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Fungal genomes tell a story of ecological adaptations

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ABSTRACT

One genome enables a fungus to have various lifestyles and strategies depending on environmental conditions and in the presence of specific counterparts. The nature of their interactions with other living and abiotic elements is a consequence of their osmotrophism. The ability to degrade complex compounds and especially plant biomass makes them a key component of the global carbon circulation cycle. Since the first fungal genomic sequence was published in 1996 mycology has benefited from the technolgical progress. The available data create an unprecedented opportunity to perform massive comparative studies with complex study design variants targeted at all cellular processes.

KEY WORDS: fungal genomics, osmotroph, pathogenic fungi, mycorrhiza, symbiotic fungi, HGT

Fungal ecology is a consequence of osmotrophy

Fungi play a pivotal role both in industry and human health (Fisher *et al.* 2012). They are involved in biomass degradation, plant and animal infections, fermentation and chemical industry etc. They can be present in the form of resting spores, motile spores, amebae (in Cryptomycota, Blastocladiomycota, Chytrydiomycota), hyphae or fruiting bodies. The same fungal species depending on environmental conditions and in the presence of specific counterparts can display various lifestyles and strategies for example entomopathogenic fungi are often encountered as leaf endosymbionts (Spatafora *et al.* 2007). Since fungi are involved in complex relationships with other organisms, their ecological repertoire is reflected in their genomes. The nature of their interactions with other organisms and environment is defined by their osmotrophic lifestyle. Nutrient acquisition and communication with symbionts and hosts are mediated by secreted molecules. Fungi possess complex repertoires of secreted molecules and membrane transporters efficiently transporting specific compounds in both directions (Richards

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& Talbot 2013). Their ability to degrade plant debris, especially lignin and cellulose makes them a key component of the global carbon circulation cycle**.** Organism adaptation is achieved by gene duplications leading to increased gene families. Expanded specific gene families result from adaptation towards a specific lifestyle, carbohydrate degrading enzymes are characteristic of plant related fungi whereas proteases of dermatophytes.

Fungal genomics and 1KF genomes

Fungi have been used as model organisms to study eukaryotic genetics for decades. Their compact genomes, well studied biology and availability of molecular tools made them ideal candidates for genome sequencing (a PubMed search results in 30932 articles related to "fungal genomes", July 2014). The genomic sequence of *Saccharomyces cerevisiae* published in 1996 was a milestone in mycology and genetics (Goffeau *et al*. 1996, Engel *et al*. 2014).

Currently the 1000 Fungal Genomes Project (1KF) is aiming at sequencing two representatives of each fungal family, everyone can nominate a sequencing candidate *via* a web service [http://genome.jgi.doe.gov/pages/fungi-1000-projects.jsf]. The available data create an unprecedented opportunity to perform massive comparative studies with complex study design variants targeted at all cellular processes.

Osmotrophic lifestyle and xenobiotic utilization

Fungal genomes are much more compact than plant and animal ones. Genes involved in a common metabolic pathway are often not only co-regulated by the same transcription factor, but also co-localized in the genome. In some cases this genomic proximity leads to gene cluster formation. Gene clusters are claimed as HGT (horizontal gene transfer) prone. Their direct acquisition leads to a straightforward evolutionary benefit and outcompeting other microbes (Richards & Talbot 2013). Since fungi are osmotrophs their adaptation to new ecological niches means developing strategies to degrade new substrates. Mechanistically this is often achieved by gene duplications with subsequent substrate specificity alterations of the resulting paralogs. As an example, the genome of *Ceriporiopsis subvermispora* shows its adaptation to lignin degradation in the expansions of laccases and manganese peroxidases (Fernandez-Fueyo *et al.* 2012)**.**

Gene organization, co-expression and co-regulation are essential when a metabolic pathway includes steps with toxic intermediates. Such pathways are sometimes conserved among distant evolutionary groups. Interestingly homologs of gene clusters with toxic intermediates which co-localize in fungi are often co-regulated in distant organisms, even in humans (McGary *et al*. 2013). It seems that clustering genes involved in a common pathway with toxic intermediates is selected for protection against the accumulation of such intermediates.

Genome size, genome richness, compactness

According to a growing body of population genetics evidence increased genome sizes result from decreased effective population size in the course of evolution of eukaryotic populations. The drop in effective population size leads to

continuously growing complexity of genomes with expanded gene families, genes fragmented by introns and surrounded by regulatory regions and mobile elements which eventually results in greater overall genome sizes. All genome components seem to expand together. However, in fungi the coding genome size is less variable than other genome components, with gene numbers between six and twenty thousand. Fungi vary in genome size and genome compactness. *Tuber melanosporum* (Pezizomycotina) an ectomycorrhizal forming fungus has a 125 Mb long genome with many transposable elements (Martin *et al*. 2010) whereas plant pathogenic *Taphrina deformans* (Taphrinomycotina) has only a 14 Mb genome (Cissé *et al*. 2013). Micorosporidia have extremely compact and reduced genomes which can be treated as a course book example of adaptation to obligate intracellular parasitism (Cuomo *et al*. 2012). The peculiarities of obligate pathogen genomes cause problems in phylogeny reconstruction. Microsporidia position within fungi has been long discussed and currently they are considered as one of basal fungal lineages together with Cryptomycota (James *et al*. 2013). Genome sizes and gene numbers can be immediately elevated as a consequence of a whole genome duplication (WGD). The ohnologs (paralogs resulting from WGD) are known to evolve in an asymmetric manner, often leading to gene loss or to neofunctionalizing. WGDs have been detected in the evolutionary history of distant fungal lineages for example in *Rhizopus delemar* (Mucorales) (Ma *et al*. 2009) and *Saccharomyces cerevisiae* (Saccharomycetales) (Byrne & Wolfe 2007).

At least some fungi are said to possess a two-speed genome i.e. a genome with housekeeping genes and the other genome with additional features, encoding specific effectors. The former, primary genome is more compact, has a few and usually inactive mobile elements and introns. The latter, accessory genome is less gene dense, encodes many species/strain specific genes, has more mobile elements, often from younger families, sometimes still active and intact. As an example rust fungi possess one of the biggest genomes among fungi, with areas abundant in mobile elements and short genes coding effector proteins.

Effector proteins

Short, secreted, cysteine rich proteins are said to mediate interactions with a host organism either a plant or an animal. Effectors play an immunomodulatory role, both in pathogenic and symbiotic fungi, they can suppress host immune response and thus facilitate tissue colonization (de Jonge *et al*. 2011, Schmidt & Panstruga 2011). One of the plant defence mechanisms is *via* jasmonic acid and mycorrhizal fungi like *Laccaria bicolor* (Agaricales) can promote mutualism by blocking the signalling (Plett *et al*. 2011). The repertoire of effector proteins evolves much faster than of housekeeping genes because it is in a constant 'arms race' with the host immune system. These effectors are often clustered with transposons which alter their expression. Host adaptation and host switching is often reflected in encoded effector protein composition. Between closely related taxa of *Nectria haematococca* and *Fusarium oxysporum* this adaptation is achieved by proteins encoded on

supernumerary chromosomes carrying, among others, host specific effectors and mobile elements (Coleman *et al*. 2009, Ma *et al*. 2010). Conditionally dispensable chromosomes and lineage specific chromosome regions are rich in repeat sequences, unique and duplicated genes and reflect the species habitat/niche.

Mobile elements in fungal genomes

Lynch (Lynch & Conery 2003) postulated that overall genome size correlated with mobile element content and decreasing effective population size. In fungi, the genome size indeed correlates well with the overall mobile element content. Transposons (TE) are DNA fragments capable of moving to new locations within a single genome in a process called transposition. Genomes and transposons have probably coevolved for all their history. TEs are usually dormant components of the genome, activated under stress conditions (Capy *et al*. 2000, Abe *et al*. 2009). Epigenetic silencing probably evolved to control transposition (Hua-Van *et al*. 2011). Transposons influence the expression of genes both in *cis* and in *trans*, alternate splicing, can lead to gene inactivation and become a source of new exons. Being a target of epigenetic machinery they change even the chromatin state of huge parts of a genome. Fungi defend their genomes by means of repeat-induced point mutation (RIP), meiotic silencing and quelling (Aramayo & Selker 2013). This richness of mechanisms reflects the significance of maintaining balance between different genome components. Studies of transposons at the kingdom level were limited to the best studied taxonomic groups and transposon types, LTR retrotransposon (Muszewska *et al*. 2011), YR retrotransposons (Muszewska *et al*. 2013), non-LTR retrotransposons (Novikova *et al*. 2009). These and other analyses have showed that plant pathogens often possess large and repeat-rich genomes (*Magnaporthe grisea*, *Mycosphaerella graminicola*, *M. fijensis*, *Blumeria graminis*) (Raffaele & Kamoun 2012). This rule is not universal and there are well studied examples such as *Ustilago maydis* with a small genome almost devoid of repeats. Transposons are involved in adaptation to new hosts and lead to altered virulence by changing genomic regions with clustered effector and avirulence genes (Kang *et al*. 2001, Van de Wouw *et al*. 2010). Transposons seem to provide an advantage in host-pathogen 'arms race' (Raffaele & Kamoun 2012). In the genome of *Pyrenophora tritici-repentis* TEs mediated adaptation towards pathogenicity by contributing to novel gene creation, effector diversification, facilitating horizontal gene transfer events and transduplication (Manning *et al*. 2013). There seems to be a link between symbiotic lifestyle in *Amanita* species and TE proliferation (Hess *et al*. 2014).

Horizontal gene transfer

HGT is a major force shaping prokaryotic genomes, in Eukaryota its impact is less pronounced. However, HGT from and to fungi has been reported in multiple studies. Unique mechanisms such as anastomosis and parasexual processes as well as close relationships, either symbiotic or pathogenic, of fungi with other organism provide additional ways for HGT within the fungal

kingdom. Fungi-like oomycetes have acquired many fungal genes enabling them to utilize compounds which are difficult to degrade (Richards & Talbot 2013). It is expected that genes coding genes used for nutrient acquisition will be transferred more successfully compared to those coding machinery involved in genetic material maintenance and processing. This bias towards niche-related genes is expected, because the housekeeping genes are involved in complex interaction networks and a newly acquired gene has to fit into the genetic environment of an acceptor. It is an open question now whether HGT between fungi is more common than from prokaryote to fungi (Richards 2011). There is a significant number of documented prokaryote to fungi transfers (Marcet-Houben & Gabaldón 2010). There are cases of entire biosynthetic pathway transfer between fungi (Richards 2011). *Fusarium* species have transferred fumonisin biosynthetic gene cluster many times in evolution (Proctor *et al*. 2013). HGT is known to occur rarely in Eukaryota, but co-transfers functionally link genes and therefore plays a role in adaptation to a certain niche.

Mobile elements and speciation

Mobile elements accumulation and proliferation leads to increasing incompatibility between taxa. The latter is one of the mechanisms underlying speciation, leading to the formation of separated taxa. The concept of species is one of the most discussed issues in theoretical biology (Taylor *et al*. 2000). The overall pattern of TEs in a species seems to be fixed with single elements being activated and widespread. The differences in TE content can be analysed not only in relation to the ratio of each group of elements in the total TE content, but also to the abundance of each major type of elements, e.g. recent separation of *Paracoccidioides brasiliensis* and *P. lutzii* sibling species upon genomic and physiological analyses (Teixeira *et al*. 2009, Desjardins *et al*. 2011). The aforementioned example of *P. brasiliensis* is an argument in favour of considering TEs as components of the definition of a species. The degree of divergence among strains of a species of interest has to be individually defined taking into account data from related taxa.

Sexual reproduction

Sexual reproduction is beneficial in many ways, it helps to get rid of deleterious mutations and fix beneficial mutations, to escape from pathogens, can be a source of genetic diversity and in consequence is widely spread among Eukaryota. However, this comes at cost of finding a mating partner, transmitting only 50% of own genetic material and breaking apart well adapted genomic configurations (Heitman *et al*. 2013). In contrast to animals, fungi seldom are obligatory sexual, usually they can reproduce asexually for many generations and sexually from time to time. Arbuscular mycorrhiza forming fungi have been considered asexual for a long time. However, genomic studies showed they have key components of the meiotic machinery (Halary *et al*. 2011). Recently mating type high mobility group (MATA-HMG) domain proteins which are sex determinants and play key roles in sexual reproduction in many fungal lineages such as Mucoromycotina, Euascomycotina, in the *Candida* clade,

have been identified in *Rhizophagus irregularis* (Glomeromycota). *R. irregularis* not only possesses MATA-HMG coding genes, but actually has an elevated number of MATA HMG copies (Riley *et al.* 2014).

Sexual reproduction models in fungi include tetrapolar, bipolar and unipolar systems (Heitman *et al*. 2013). The mating type loci can be very simple ranging from >1000 bp long loci with a single homeodomain transcription factor (TF) to 120 kb long genomic regions in *Cryptococcus neoformans*. The best studied bipolar system was described in *Sacharomyces cerevisiae.* It consists of one haploid a cell with an a locus coding a single homeodomain transcription factor, and another haploid α cell with an α locus coding two TFs, a homeodomain TF which forms an a-α heterodimer during mating and another homeodomain TF which regulates α-specific genes. A tetrapolar system has been described for *Ustilago maydis* with two different, unlinked mating type loci *a* and *b*, *a* coding for homedomain TFs, and *b* for pheromone and pheromone receptors. The *a* locus is biallelic and the *b* locus is multiallelic which results in a multitude of possible combinations. Adaptation towards pathogenicity leads to convergent changes from ancestral tetrapolar systems to bipolar systems and occurred repeatedly in Basidiomycota evolution in e.g. *Ustilago hordei*, *Malassezia restricta* and *M. globosa* and in *Filobasidiella* taxa. Moreover, unisexual reproduction seems to be widespread in pathogens, and can lead to diverse progeny even if only one parent was present (Heitman *et al*. 2013). There is a variable balance between inbreeding and outcrossing, and in animal pathogens it seems to be favourable to enhance inbreeding. According to Heitman and colleagues (2013) unisexual reproduction not only generates genetic diversity *de novo* but eliminates the costs of sexual reproduction and evolved repeatedly.

Conclusions

Fungi possess a combination of features among others osmotrophic nutrient acquisition, close interactions with other organisms, complex metabolic potential, variable reproduction models, which together have to be reflected in their genomes. Recent advancement in sequencing has revealed more and more fascinating individual stories and showed both common and specific adaptations towards particular ecological niches. More genetic mechanisms underlying fungal ecological adaptations will be elucidated with the progress of functional genomics studies.

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Streszczenie

Grzyby odgrywają zasadniczą rolę w ekosystemach jako patogeny, saprotrofy i symbionty. Ich wszechstronne zdolności metaboliczne czynią z nich kluczowe ogniwo w obiegu węgla w przyrodzie. Dla człowieka stanowią głównie źródło infekcji, ale również zyskują na znaczeniu w biotechnologii (Fisher *et al*. 2012). Grzyby są obecne

w naszym otoczeniu w formie zarodników, pełzaków, grzybni i owocników. Te same gatunki grzybów w zależności od warunków otoczenia mogą prezentować rożne formy morfologiczne i tworzyć różne relacje z otoczeniem, na przykład owadobójcze grzyby są często spotykane jako endosymbionty roślin (Spatafora *et al*. 2007). Całe to bogactwo znajduje odzwierciedlenie w genomach grzybów. Osmotroficzny tryb życia grzybów narzuca charakter interakcji grzybów z otoczeniem, która odbywa się przy pomocy wydzielanych na zewnątrz enzymów rozkładających pożywienie, białek efektorowych oraz toksyn wpływających na inne organizmy. Grzyby posiadają złożone kompozycje wydzielanych cząsteczek oraz transportery błonowe przystosowane do efektywnego przenoszenia związków chemicznych w obu kierunkach (Richards & Talbot 2013). Zdolność do rozkładania ligniny i celulozy odpowiada w dużej mierze za sukces ewolucyjny grzybów. Adaptacja organizmu do nowego ekosystemu zwykle przebiega poprzez duplikację genów z ich późniejszymi asymetrycznymi zmianami prowadzącymi do szybkiej zmiany specyficzności substratowej jednego z paralogów. Wielokrotne duplikacje jednej grupy genów prowadzą do rozrostu rodziny kodowanych przez nie białek i rozszerzenia zakresu możliwości np. rozkładanych przez nie wariantów substratów. Zwiększenie liczby genów związanych z metabolizowaniem danej grupy substratów jest jednym z podstawowych sposobów adaptacji do danej niszy ekologicznej widzianej z perspektywy genomu. Charakterystyczne więc dla grzybów związanych z roślinami będzie kodowanie licznych enzymów degradujących węglowodany, a dla dermatofitów – proteazy i lipazy. Kolejnym poziomem adaptacji patogenów/symbiontów jest zmiana profilu ekspresji genów i stały „wyścig zbrojeń" z gospodarzami. Ponadto geny te często sąsiadują z transpozonami, w obrębie szybciej ewoluującej części genomu. Geny związane z metabolizowaniem ksenobiotyków częściej ulegają też horyzontalnemu transferowi genów aniżeli geny metabolizmu podstawowego. Inna wyróżniającą grzyby cechą jest posiadanie różnorodnych modeli rozmnażania płciowego nawet pomiędzy spokrewnionymi gatunkami. Model rozmnażania jest jednym z ważniejszych sposobów dostosowania do trybu życia. Rozmnażanie jednopłciowe pojawiało się wielokrotnie w ewolucji grzybów i wydaje się być adaptacją do patogennego trybu życia.

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Aeromycology: studies of fungi in aeroplankton

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ABSTRACT

Air is a natural environment for spores of many genera and species of fungi. Despite its small size and a significant dispersion they have a great impact on human health and different areas of our activities, such as agricultural production. The study on spores of fungi that belong to aeroplankton or bioaerosole is called aeromycology. The most frequent fungi present in the air are *Cladosporium* and *Alternaria* species. Their numbers are abundant regardless of latitude and height above the sea level and above the ground. They mostly originate from agricultural environment. Other frequently listed species of fungi, whose spores are present in the air include of *Aspergillus, Penicillium, Fusarium, Sclerotinia* and *Ganoderma*. The concentration of spores in the air strongly depends on the abundance of their formation during the studied period. This in turn relates to geobotanical region, vegetation, degree of urbanization, climatic conditions, season, current weather, wind force and direction, local microclimate, and many other factors. Changes in humidity affect the concentration of different types of fungal spores. In general they are divided to 'dry' (*Alternaria, Cladosporium, Puccinia, Ustilago, Melampsora, Epicoccum, Drechslera*) and 'wet' (*Didymella, Fusarium, Ganoderma, Gliocladium, Leptosphaeria, Verticillium*). Study of the composition of species and genera are being done using different types of spore samplers, mostly volumetric instruments. Visual identification is based on colony morphology of the fungus and the shape and size of spores. The identification at the species level is possible with molecular tools. Methods based on DNA/RNA amplification are very sensitive and accurate. They allow the identification below the species level, e.g. chemotypes, mating types or isolates with genes or alleles of interest. Aerobiological monitoring is widely used in the epidemiology of human diseases (inhalant allergies) and infections of arable crops (decision support systems for the protection of cultivated plants). Aeromycology is interconnected with such diverse areas as industrial aerobiology, bioterrorism, ecology, climatology or even speleology and cultural heritage.

KEY WORDS: aerobiology, aeromycology, aeroplankton, *Alternaria, Cladosporium*

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Introduction

Air is a natural habitat of numerous fungi, mostly existing there in the form of spores, their clusters, or – on contrast – spore and mycelium fragments. Spores of some fungal genera are very frequent in air samples, often outnumbering pollen grains, on which most of research have focused for many years. Small size and great difficulties to identify the species based on single spores led to great underestimation of their presence in the atmosphere. Recent fast development of aeromycology, supported with molecular tools shows the importance of fungi in aeroplankton. Fungal spores and mycelium fragments passively float in the air and they are often dispersed by air currents (Southworth 1974). The distance of transport greatly depends on the spore size and shape, the height, thickness and location of the air layer, its physical properties such as temperature, as well as air movements. Most of fungal spores are transported in between local habitats; however, there are strong scientific evidences on spores being transported within and between geographic regions or even continents. This phenomenon is called a long-distance dispersal and it is regarded as an important survival strategy for many organisms. Moving of spores with the air currents allows fungi infect new plants of the same host, find new hosts, reach and colonize new regions or move in between summer and winter habitats (Brown & Hovmøller 2002). Modern agriculture, with greatly reduced plant biodiversity and huge monocultures increases the risk of global spread of some plant pathogens. Limited genetic variability of modern plant cultivars and their distribution in different parts of the world make it easy to establish infections by fungal spores blown to new territories. Major pathogens of crop plants such as rice, wheat, soybean, oilseed rape, potato as well as coffee or banana are similar in different parts of the globe, in spite of geographical or political barriers, resulting in no seed or seedling exchange. These obstacles can be easily overcome by spore invasions with the air currents.

Methods used in aerobiology

Fungal spores present in the air are subjected to many studies with the use of spore traps, that may be passive, when spores drop down on certain surfaces or gather in containers, due to their gravity (Koch sedimentation method) or active, when the collection of spores is connected with swirling or sucking, that allows capturing of higher spore loads (Fleischer *et al.* 2006). The most popular and advanced methods of spore trapping use volumetric samplers. They allow to re-calculate the number of observed spores to a given air unit, mostly 1 cubic meter of the air. The first volumetric sampler was used by Pierre Miguel in Parc Montsouris in Paris in 1883. It was an air pump sucking 20 liters of the air via an orifice, with the glass slide covered with glycerin. This equipment allowed the first calculations of fungal spores in the air with high precision; hence this researcher is often regarded as the first professional aerobiologist, and one of the main fathers of aerobiology and aeromycology (Comtois 1997). Since then, the development of volumetric methods has been greatly pushed forward, especially by English researchers Philip Gregory and John Hirst (Hirst 1991). Currently used volumetric spore samplers, such as

Burkard or Lanzoni apparatus, are based on models designed by Gregory and Hirst. The suction power is standardized, what allows the comparison of results obtained by different research teams worldwide.

There are also other types of apparatus, e.g. the MicroBio or Air Ideal samplers as well as the Andersen impactor – the facilities that combine volumetric methods with the culture on media. Both of them allow growing fungal spores into colonies on microbiological substrates. Moreover, the Andersen impactor allows sieving the spores into groups of different sizes. The recognition of fungi present in the air are then based on traditional visual assessments (Kasprzyk & Worek 2006, Grinn-Gofroń & Strzelczak 2011, Stępalska *et al.* 2012, Pusz *et al.* 2013), as well as on molecular tools (Kaczmarek *et al.* 2009, Karolewski *et al.* 2012, Piliponyte-Dzikiene *et al*. 2014).

Identification of fungal spores in air with molecular tools

The recognition of fungal spores based on spore shape and size is possible for such frequent 'air-flyers' as *Botrytis, Chaetomium, Coprinus, Didymella, Entomophthora, Epicoccum, Erysiphe, Ganoderma, Nigrospora, Pithomyces, Polythrincium, Stemphylium, Torula, Ustilago* and some others (Kasprzyk 2008). However, numerous spores of different species or even different fungal genera can be mixed up in studies based on microscope analysis only. Possibilities created by impactors allowing to subculture these fungi partly solve this problem, as they allow identifying fungal genera, species and variants below the species level, but they demand much time and laboratory space. Moreover, great parts of fungal spores present in the air are produced by the biotrophs, which are unculturable on conventional microbiological media. The broadcasting of spores by wind is crucial for all these species as their survival relies on quick finding the relevant host. Their detection and proper identification is possible due to molecular methods.

PCR-based methods allow precise spore identification s of many fungal species, including the most dangerous plant pathogens (West *et al.* 2008a). Moreover, recent introduction of Real-time PCR is designed to quantify the numbers of spores or gene copies (Kaczmarek *et al*. 2009, Karolewski *et al*. 2012). Molecular tools allow proper identification of fungal species with similar or identical spores and study the genetic changes in fungal populations, such as fungicide resistance (Fraaije *et al.* 2005) or AVR gene alleles (Kaczmarek *et al.* 2014b). Molecular methods allow simultaneous studies of several fungal species, allergens or genetic changes. The greatest hope is currently connected with isothermal methods, such as LAMP, allowing a simplified DNA isolation and greatly reducing the possibilities of reaction inhibition (Jędryczka *et al.* 2013). Molecular methods are sometimes criticized for no distinguishing the difference between viable or dead spores, as they both contain DNA. This may be solved by RNA-based studies, as this nucleic acid is produced by living cells only and it is rapidly broken down in dead tissues, unlike DNA. Due to difficulties with RNA stability and extraction, current RNA-based studies are confined to RNA viruses and human pathogens, rather than to plant pathogens (Schmale *et al*. 2005).

Most frequent fungal spores in air

Although a lot of scientific literature in aeromycology is devoted to fungal plant pathogens, the majority of spores belong to species that do not infect plants directly, but grow saprophytically on their surfaces. Skjøth *et al.* (2012) proved that at the time of crop harvest local load of airborne spores greatly increases and it is an agricultural area, which substantially contributes to the sudden raise of fungal spores captured by samplers located in cities. Major part of air spora is composed of *Alternaria* spp. and *Cladosporium* spp., and in general – these two genera constitute the greatest part of each spore lot in the air, at almost all geographical latitudes (Kasprzyk 2008) and heights (Pusz *et al*. 2013). Other frequently encountered spores belong to *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. Due to great similarities in their spore size and shape it is not possible to identify them based on morphological characteristics. To properly identify these spores DNA- or RNA-based detection methods are required, alternatively, the use of suction trap which impacts the spores on microbiological media.

Air contains spores of different modes of appearance. Telio- and urediniospores of rusts (e.g. *Puccinia*, *Ustilago* or *Melampsora* spp.), as well as *Alternaria*, *Cladosporium*, *Epicoccum* or *Drechslera* are present in the air of low humidity, and therefore they are called 'dry spores', whereas *Didymella*, *Fusarium*, *Ganoderma*, *Gliocladium*, *Leptosphaeria* or *Verticillium* fungi release spores after rainfall events, that puts them to the group of fungi producing 'wet spores'. In general, numerous ascospores of plant infecting fungi belong to 'wet spores', that directly relates to their biology. High humidity is a prerequisite for the successful infection of a host-plant. This is why natural mechanisms evolved fruiting bodies with layers reacting to the presence of atmospheric water that directly causes osmotic changes and allows ascospore release to air currents.

Fungal spores of different taxa are subjected to specific diurnal and seasonal cycles, which greatly depend on climate, weather conditions, circadian clock (changes of day and night) as well as the availability of fresh substrates, necessary for fungus development (Calderon *et al*. 1995). In moderate climate most of spores occur in summer or early autumn (Kasprzyk & Worek 2006), whereas in tropical areas they are more abundant in cold seasons of the year (Hasnain 1993). Microclimate greatly influences the release and appearance of spores, what relates not only to local rainfalls or the effect of ponds, lakes and water reservoirs, but it may also relate to temperature. The delayed presence of airborne spores in the season caused by lower temperatures and snow cover were found in the mountainous regions (Pusz *et al*. 2013). On contrast, in 'urban heat islands' plants usually produce more pollen and their seasonal occurrence starts earlier and lasts longer (Calderon *et al.* 1997), what partially applies also to the abundance of fungal spores. Microclimate and local turbulences make it difficult to accurately predict the occurrence of spores. The studies are easier when airborne fungal spores are transported by big air currents, which can be tracked by back-trajectories (Sadyś *et al*. 2014).

Disease forecasting systems

Forecasting of spore release, based on biology of a particular fungal taxon and the weather finds direct use in plant and human protection against infectious diseases. The systems check if a regional or local weather favored the production and release of pathogen inoculum and the infection of the host (West *et al*. 2008b). Disease forecasting systems used in modern agriculture are usually based either on mathematical models, which were built up following the experiments on pathogen biology, or they refer to stages of development of plant pathogens, controlled at time points that are crucial for plant epidemics. The effective plant control is currently a compulsory policy of the European Union (Kaczmarek *et al.* 2014a). The challenge of plant disease control is a key component of increasing food production and food security (West 2014). It was proven that the use of decision support systems brings economic rewards to farmers and better protects the environment.

Poland runs the System for Forecasting Disease Epidemics SPEC (Jędryczka *et al.* 2012) – the third world's biggest system based on aerobiological methods. The system has been constantly operating since 2004 (Jędryczka *et al*. 2006). Similar attempts have been recently undertaken in the Czech Republic (Jędryczka *et al*. 2010). The studies concentrate on *Leptosphaeria maculans* and *L. biglobosa* – the pathogens infective to oilseed rape (*Brassica napus*), which belongs to one of top cash crops. Air samples captured with volumetric spore traps are quantified with light microscope and molecular techniques (Kaczmarek *et al*. 2009, 2014b). Airborne spores that form inoculum responsible for disease epidemics allow predicting the risk of yield losses as well as its quality. Timely applications of fungicides are beneficial to agriculture. High precision of plant protection treatments, based on aerobiological data, allows combating the pathogen with the highest efficiency.

Outdoor and indoor air spora allergenic to humans

High concentration of airborne spores may lead not only to plant diseases, but often cause skin, eye or nasal irritation and diseases of human respiratory systems, resulting in shortness of breath, alveolitis and asthma. Rapiejko *et al*. (2004) elaborated threshold values for the spores of *Alternaria* and *Cladosporium* spp. necessary to evoke allergic symptoms. In case of the other fungi such values have not been published. It is also clear that these numerical values are only indicative figures, and the allergic reaction greatly depends on patients' age, health condition and numerous individual characteristics. Modern traps enable the collection of bioaerosols with allergenic proteins that can be detected with ELISA tests or other molecular diagnostic methods. Initially the detection concerned *Penicillium roqueforti*, the fungus responsible for food spoilage and serious diseases of cheese workers (Campbell *et al.* 1983). The symptoms ranged from cough, dyspnea and malaise to reduced lung volumes and hypoxemia. The studies revealed that serum and lavage fluids of the patient contained antibodies to *P. roqueforti*. This is one of numerous cases allowing understanding a serious influence of airborne fungi on human health, which started a series of studies on indoor air spora in numerous countries, including Poland (Lipiec 1997, Górny & Dutkiewicz 2002, Lipiec &

Samoliński 2002, Karwowska 2003, Filipiak *et al.* 2004, Stryjakowska-Sekulska *et al.* 2007, Dumała & Dudzińska 2013). Since then, the whole selection of different apparatus and methods has been elaborated to monitor fungal spores and allergens in the air, ranging from static samplers to minute, portable instruments. Most studies were done in public buildings such as universities, schools and hospitals. The monitoring revealed that the concentration of microorganisms only rarely exceeded its recommended limits. However, some of fungal species present in the indoor air of studied rooms could negatively affect human health.

The compositions of fungal air spora in man-made places greatly differed, even at sites located in seemingly similar conditions. Speleomycological research done in unfinished Nazi military complex "Riese" located in underground complex Osówka in the Sowie Mountains showed that *Cladosporium* spp. were most frequently isolated both from internal and external atmosphere (Pusz *et al*. 2014b). However, in shafts of copper mine located in Lubiń mining site (property of KGHM Polska Miedź SA) the most numerous fungi belonged to *Penicillium* spp. and *Aspergillus* spp., with *P. notatum* and *P. urticae* found as best adapted to grow in these specific conditions (Pusz *et al.* 2014a). Significant differences between the composition and size of fungal species between the shafts and sample collection sites supported the hypothesis on substantial influence of microclimate on fungal air spora. Fortunately, in this particular case the concentration of fungal spores also did not present a health risk to the mine workers.

Concluding remarks

The potential of fungal spores in the air is in contrast to their 'invisibility' caused by vast dispersion and small size of particular spores. However the impact on humans and their economy may be enormous, with great influence on such crucial branches as agriculture or human safety. Based on a review on primary biological aerosol particles in the atmosphere, written by Després et al. (2012), it is clear that aeromycology is connected to numerous basic and applied sciences, such as allergology, bioclimatology, biological pollution, biological warfare and terrorism, mycology, biodiversity studies, ecology, plant pathology, microbiology, indoor air quality, industrial aerobiology, speleology, cultural heritage and many other disciplines.

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Streszczenie

Powietrze jest naturalnym środowiskiem dla zarodników licznych rodzajów i gatunków grzybów. Pomimo niewielkich rozmiarów i znacznego rozproszenia mają one wielki wpływ na zdrowie ludzi i różne kierunki ich działalności, w tym w szczególności na produkcję rolniczą. Badania nad zarodnikami grzybów stanowiącymi część aeroplanktonu są przedmiotem aeromykologii. Niezależnie od szerokości geograficznej i wysokości nad poziomem morza w powietrzu szczególnie często występują grzyby z rodzajów *Cladosporium* i *Alternaria*, a ich źródłem jest najczęściej środowisko rolnicze. Innymi często notowanymi rodzajami grzybów, których zarodniki występują w powietrzu są m.in. *Aspergillus*, *Penicillium*, *Fusarium*, *Sclerotinia* i *Ganoderma*. Stężenie zarodników w powietrzu jest ściśle uzależnione od obfitości ich tworzenia w danym okresie, co jest pochodną regionu geobotanicznego, szaty roślinnej, stopnia zurbanizowania danej lokalizacji, warunków klimatycznych, pory roku, aktualnej pogody, siły i kierunku wiatru, lokalnego mikroklimatu i wielu innych czynników. Zmiany wilgotności powietrza wpływają na stężenie zarodników różnych rodzajów grzybów, określanych na tej podstawie jako „suche" (*Alternaria*, *Cladosporium, Puccinia*, *Ustilago, Melampsora*, *Epicoccum*, *Drechslera*) lub „mokre" (*Didymella*, *Fusarium*, *Ganoderma*, *Gliocladium*, *Leptosphaeria*, *Verticillium*). Badania składu rodzajowego i gatunkowego prowadzone są przy zastosowaniu różnego rodzaju chwytaczy zarodników, a identyfikacja wizualna na podstawie morfologii kolonii grzyba oraz kształtu i wymiarów zarodników uzupełniana jest obecnie przez wyjątkowo czułe metody detekcji molekularnej, specyficzne względem rodzajów, gatunków, chemotypów, a nawet składu genów i kompozycji poszczególnych alleli. Monitoring aerobiologiczny znajduje bezpośrednie wykorzystanie w epidemiologii chorób ludzi (alergologia) i roślin uprawnych (systemy wspierania decyzji w ochronie roślin uprawnych). Badania z zakresu aeromykologii znajdują zastosowanie w tak

różnych kierunkach jak aerobiologia przemysłowa, bioterroryzm, ekologia, dziedzictwo kulturowe, klimatologia lub speleologia.

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Moulds in biodeterioration of technical materials

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ABSTRACT

Moulds are microorganisms which play the key role in biodeterioration of technical materials which results from their physiological features and metabolism. Technical materials constitute the source of carbon and energy (wood, paper, textiles, fuels, leather) or the surface for fungal growth (bricks, stone, metal, glass). Moulds characterized by a high biodeterioration activity – enzymatic and acidic, belong mainly to the following genera: *Aspergillus, Penicillium, Trichoderma, Cladosporium, Paecilomyces* and *Chaetomium*. Members of some taxa (besides the aforementioned also e.g. *Stachybotrys, Alternaria, Epidermophyton, Microsporum, Scopulariopsis, Trichophyton*) growing on technical substances and producing allergens and mycotoxins cause health hazards. Therefore, basing on the knowledge about conditions for mould development and biodeterioration mechanisms, we should appropriately preserve materials against mould growth. Looking for new disinfection methods safe for technical substances in order to inhibit mould growth is also important. Protective applications of biocides should be limited only to materials most sensitive to biodeterioration (paper, textiles, fuels, paints). On the one hand we should take into consideration environmental protection, on the other production of durable, biodegradable materials ensuring the product life cycle.

KEY WORDS: moulds, biodeterioration, technical materials, biocides, disinfection

Introduction

Biodeterioration of technical materials (destruction caused by the activity of organisms) causes serious economic losses resulting from the disturbance of their properties, disasters and accidents in industry and health hazards when potential and actual pathogens grow on various surfaces. There are many described examples of microbial accidents during production, storage and usage of materials in the textile, paper, building and other industries (Zyska 2001).

It was estimated that the economic losses caused by microbial deterioration each year amounted to 2% of Gross

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National Product (Zyska & Żakowska 2005). Based on the data of GUS (Central Statistical Office) for Poland in 2013 this value was 3.2×10^{10} PLN. Moreover, it is impossible to estimate the loss of culture heritage when we take into account destruction of historical objects or the health threat to workers and users of materials.

Moulds are dominant and the most important microorganisms in biodeterioration of technical materials considering their ubiqiuty and the amount of damage and threats. However, we must remember that some technical substances are often exposed to bacteria, algae, insects, higher fungi from lichenized *Ascomycota* and *Basidiomycota*. However, development of actual damaging factors depends on the material composition and environmental conditions. In the case of wood, higher fungi and insects are responsible for its destruction; bricks and stones are destroyed mainly by algae and cyanobacteria, when they have access to light.

Significance of moulds in biodeterioration of technical materials results from their physiological features and metabolism (Fig. 1). Oligotrophic character and expansion in natural environments are the most important. A wide spectrum of produced enzymes and metabolites enables them to colonize many environments and to use almost every organic substrate. Moreover ease of spreading, different ways of propagation, creation of spores, production of secondary metabolites (antibiotics, toxins) to inhibit the growth of other organisms and high tolerance of unfavorable environmental conditions (UV, disinfection, low pH, humidity) are features which facilitate colonization of technical materials and make fungi difficult to eliminate.

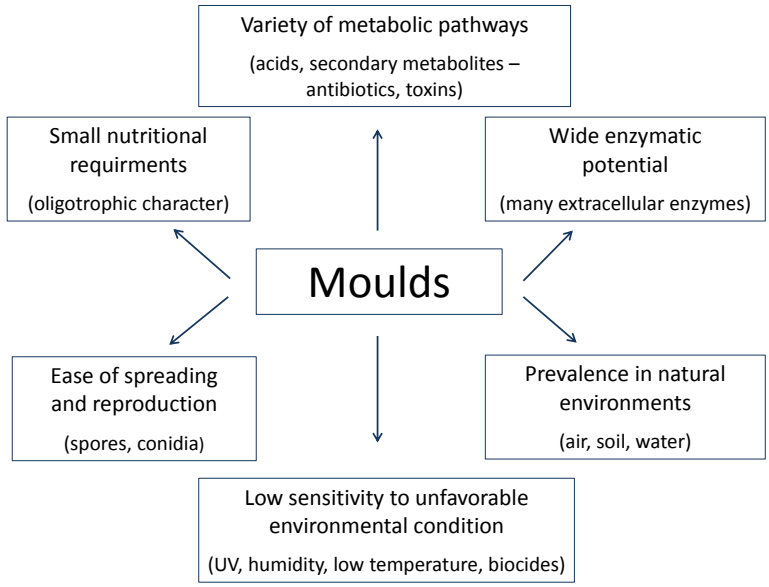

Figure 1. Mould physiological features which allow biodeterioration

High humidity of the environment (optimum $a_w > 0.9$) and access to oxygen (moulds are aerobic organisms growing on surfaces) are factors facilitating their development. However, well-known xerophilic moulds from genera *Aspergillus* and *Penicillium* are able to grow at low substrate humidity (aw = 0.8) and at low relative humidity of the air RH=60%; also production of spores in soil at low oxygen concentration was observed (numerous mould species were isolated from historical objects from tombs).

In the view of the above facts, possibility of limiting the adverse influence of moulds on technical materials should be considered. This review presents the literature addressing the following issues:

1. which technical materials are most exposed to mould development?
2. which moulds have the greatest biodeterioration potential?
3. whether and when disinfection of mouldy technical materials or antifungal materials should be applied?

Mechanisms of mould-induced biodeterioration of technical materials

There are two mechanisms of technical material biodeterioration – biological degradation and biological corrosion (Zyska & Żakowska 2005). Materials of the natural origin - wood, paper, fabrics, leather, petroleum products, oils, fuels, paints, rubber are mould-degradable (biodegradation). Moulds belonging to genera *Aspergillus, Penicillium, Alternaria, Cladosporium, Trichoderma* and others are producers of extracellular enzymes which degrade polymers into monomers using them as a source of carbon and energy.

Enzymatic profiles of moulds change according to biochemical induction of environment, in which they grow. The same species growing on carton-gypsum board or on mortar produces different enzymes (Gutarowska 2010). Total mineralization occurs very quickly on organic substances containing cellulose: cotton and paper (e.g. cotton stored in the soil for a few weeks at high humidity becomes degraded by moulds to CO_2 and H_2O). Five-week experiment concerning fuel degradation with *Cladosporium* species showed important changes in the fuel chemical composition leading to the reduction in its octane number (Zyska & Żakowska 2005). Technical materials like cotton, paper, carton-gypsum board, emulsion paints are most sensitive to mould development and biodegradation processes. Biocorrosion, resulting from chemical reactions between organic acids produced by fungi and technical material is the other mechanism of mould-triggered degradation. This process causes decay of mineral surfaces of building materials (stone, brick, mortars, concrete), metal and glass (Krumbein 1988, May *et al.* 1993). Moulds belonging to *Aspergillus* and *Penicillium* produce significant amounts of organic acids such as citric, oxalic, malic and others which react with elements (Al, Mg, Fe, Si, Mn), cause material demineralization, production of soluble salts or the deposition of insoluble salts forming a layer on surfaces e.g. calcite (Cwalina 2003). Additionally, mycelium damages the material mechanically due to hyphae 3-5 cm penetration into it. All this results in loss of cohesion and increase in porosity, furthermore organic acids buffer the materials, making them susceptible to halophilic bacteria which intensifies destruction. The type of material is crucial, because it stimulates the production of specific organic acids (Gutarowska & Czyżowska 2009).

Among inorganic surfaces stone, brick, mortar are sensitive to mould growth under high moisture conditions and presence of contaminants.

Moulds involved in the biodeterioration of technical materials

The chemical composition of technical materials determines the development of different moulds (Table 1). Moulds from genera *Aspergillus* and *Penicillium* (called moulds of warehouses) are most common. High air humidity and favorable chemical composition of the material make these fungi not only able to deteriorate many substrates, but also produce secondary metabolites (mycotoxins, volatile compounds) that are toxic to animals (Larssen & Frisvad 1994, Singh 2005). Some allergic aspergilli and penicillia found growing in damp buildings and present in indoor air were also isolated from lung specimens at autopsy (Flannigan & Miller 1994).

Technical materials containing cellulose (wood, paper, cotton, linen) or its derivatives in the form of esters, ethers (emulsion paints) are degraded by fungi belonging to the genera *Aspergillus, Penicillium, Chaetomium, Trichoderma, Cladosporium*, *Alternaria* and others.

Cellulose substrates used in buildings readily taking up water from environment (wallpaper, carton-gypsum boards) can provide a source of nutrients necessary for growth of *Stachybotrys chartarum*, which create serious mycotoxin hazard in buildings (Flannigan & Miller 1994). A number of macrocyclic trichothecenes (verrucarins, satratoxins, trichoverrins) were found in colonized materials. Paper and wood are easily degraded by the cellulase enzyme complex; the problems associated with the development of moulds during production of paper in paper mills, as well as during storage of paper in archives and libraries are described in literature (Zyska & Żakowska 2005). The development of moulds in paper factories begins already during storage of raw material (wood, paper) and production of cellulose pulps. It has been shown that unbleached mass produced from recycled substrates is more contaminated than the unbleached pulp originating from trees (in the bleaching process a biocide, titanium dioxide, is added). The mould growth was also observed on production machinery in the form of biofilms and pulp tanks (Gutarowska & Cichocka 2009). Degradation of paper in archives and libraries mainly by *Aspergillus* and *Penicillium* is a serious problem, especially under the conditions of high humidity of paper/air, such as disaster-related flooding of warehouses or during storage in uncontrolled microclimate (periodical humidity of the air RH<70%). Under such conditions, development of fungi on books is possible; there is also a hypothesis that moulds are involved in the formation of a 'Foxing' phenomenon (orange-brown small spots on paper) common in nineteenth century books (Florian & Manning 2000, Strzelczyk 2001). Numerous examples of mould-induced destruction of historical objects are also described, including paintings, monuments, paper, leather and textiles (Strzelczyk 2001, Strzelczyk *et al.* 2003, Florian 2004). The primary reasons of their biodegradation include mechanical damage, an organic nature, age and the presence of contaminants.

Materials containing proteins (wool, leather) are colonized by *Mucor, Rhizopus, Aspergillus, Penicillium, Cladosporium, Acremonium, Alternaria* fungi and other taxa with proteolytic properties. What is more, in materials

containing proteins such as keratin and collagen human pathogens such as *Epidermophyton, Microsporum, Scopulariopsis, Trichophyton* can grow. Many species of these genera cause mycoses and are classified to the class 3 of Biosafety Level (BSL) in terms of risk to human health. They pose a serious threat to people working in tannery, leather and wool product warehouses, as well as technicians, people exposed to fungi in mouldy buildings or users of these materials (Singh 2001).

Table 1. Moulds participating in biodeterioration of technical materials.

Technical material	Fungal taxon*	Reference
Wood	*Aspergillus* sp. (*A. brasiliensis*), *Chaetomium globosum, Penicillium* sp., *Trichoderma viride*	Fojutowski 2003
Paper	*Alternaria* sp., *Aspergillus* sp. (*A. brasiliensis, A.versicolor*), *Chaetomium globosum, Cladosporium* sp. (*C. herbarum*), *Geotrichum* sp., *Mucor* sp., *Paecilomyces variotii* , *Penicillium* sp., *Phoma* sp., *Stachybotrys* sp., *Talaromyces funiculosus, Trichoderma* sp.	Woźniak &Tymińska 2003, Zyska & Żakowska 2005
Textiles	*Alternaria tenuissima, Aspergillus* sp. (*A. auratus, A. brasiliensis, A. carbonarius, A. fischeri, A. flavus, A. fumigatus, A. nidulans, A. raperi, A. terreus, A. ustus, A. wentii*), *Chaetomium* sp. (*Ch. globosum, Ch. cochlioides*), *Fusarium* sp., *Penicillium* sp. (*P. aurantiogriseum, P. canescens, P. citrinum, P. gladioli, P. granulatum, P. simplicissimum, P. wortmannii*), *Talaromyces funiculosus, Trichoderma viride*	Szostak-Kot 2001, 2003, Abdel-Kareem 2009, Błyskal & Syguła-Cholewińska 2001
Leather	Acremonium sp., Alternaria sp. (A. alternata), Aspergillus sp. (A. brasiliensis, A. flavus, A. fumigatus, A. terreus, A. versicolor, A. wentii), Chaetomium globosum, Chrysosporium sp., Cladosporium sp. (C. cladosporioides, C. carrioni), Epidermophyton floccosum, Fusarium sp. (F. oxysporum), Haematonectria haematococca, Microascus brevicaulis, Microsporum canis, Mucor plumbeus, Paecilomyces variotii, Penicillium sp. (P. aurantiogriseum, P. citrinum, P. glabrum, P. ochrochloron, P. purpurogenum, P. verrucosum), Rhizopus stolonifer, Spicaria sp., Scopulariopsis sp. , Stemphylium sp., Syncephalastrum racemosum, Talaromyces ruber, Thamnidium elegans, Trichoderma sp., Trichophyton sp. (T. mentagrophytes, T. rubrum, T. tonsurans)	Orlita 2001, Strzelczyk *et al.* 2003, Perkowski & Goździecki 2003, Falkiewicz-Dulik 2003

* Fungal taxa according to Index Fungorum (www.indexfungorum.org, accessed 22.07.2014)

Technical material	Fungal taxon*	Reference
Petroleum, oil, fuels	*Alternaria* sp., *Amorphotheca resinae, Aspergillus* sp. (*A. fumigatus, A. versicolor, A. ustus*), *Fusarium* sp., *Humicola* sp., *Paecilomyces variotii, Penicillium* sp. (*P. canescens, P. spinulosum*), *Sarocladium strictum*	Kwiatkowska 2003
Paints	*Aureobasidium pullulans, Paecilomyces variotii,*	Zyska & Żakowska 2005
Rubber, insulation materials	*Aspergillus* sp., *Chaetomium* sp., *Cladosporium* sp., *Penicillium* sp., *Microascus brevicaulis, Trichoderma* sp.	Michalski *et al.* 2001
Plastics	*Aspergillus* sp. (*A. awamori, A. brasiliensis, A. flavus, A. terreus*), *Paecilomyces variotii, Penicillium* sp. (*P. expansum, P. ochrochloron*), *Talaromyces funiculosus, Trichoderma viride*	Żakowska *et al.* 2003, Nowak *et al.* 2012
Stone, concrete, brick, mortar	*Alternaria tenuissima, Aspergillus* sp. (*A. brasiliensis, A. fumigatus*), *Aureobasidium pullulans, Cladosporium* (*C. cladosporioides, C. herbarum*), *Penicillium* sp. (*P. chrysogenum, P. rubrum*), *Phaeospora* sp., *Phoma putaminum, Purpureocillium lilacinum, Trichoderma* sp., *Ulocladium consortiale*	Cwalina 2003
Glass	*Alternaria alternata, Aspergillus* sp. (*A. brasiliensis, A. versicolor*), *Talaromyces funiculosus*	Sitarz *et al.* 2003

* Fungal taxa according to Index Fungorum (www.indexfungorum.org, accessed 22.07.2014)

Biodeterioration of building and finishing materials in residential and public buildings is also common. It was estimated that 40% of people in Poland live and work in buildings with high humidity level RH>80% (European Environmental and Health Information System 2006), and in 20% of residential houses there are mould-related problems. They not only reduce the aesthetics of the buildings, but also pose a threat to health of residents and workers. Moulds are capable of growing on most construction, finishing and insulation materials used in buildings (Gutarowska 2010). Moulds from genus *Aspergillus* (*A. brasiliensis, A. flavus, A. versicolor*), *Penicillium* (*P. chrysogenum, P. expansum*), *Alternaria* and *Cladosporium cladosporioides* are frequently isolated from buildings. It has been shown that the type of building material significantly modifies mould allergenicity and toxicity (Gutarowska 2013).

Growth of moulds with lipolytic properties such as *Cladosporium*, *Acremonium* and *Paecilomyces* in metalworking fluids or oils constitutes a significant threat in industrial factories. Moulds are able to change the chemical composition of oils and to contribute to biocorrosion of metal surfaces, moreover together with bacteria they produce bioaerosols which are harmful for

workers (Cyprowski *et al.* 2007). Some species of *Cladosporium* and *Paecilomyces* are able to grow on polymers, rubber and also in the fuels. The processes of degradation of these materials take several months to several years, however, they are the cause of major economic losses, especially due to the destruction of insulations and fuel tanks (Berryman 1987). Studies conducted in Poland showed the presence of moulds in 50% of samples taken from fuel tanks (Kwiatkowska 2003). Assessment of strength change of external rubber covers of telecommunication cables revealed 80% of mass loss caused by growth of actinomycetes (filamentous bacteria) and moulds (Zyska & Żakowska 2005).

The frequency and scale of the devastation of technical substances and health risks caused by moulds make it necessary to look for new methods of disinfection and protection of materials against biodeterioration.

Protection against mould growth and methods of their elimination from technical materials

Similarly as in pharmaceutical, cosmetic and food industries also in plants producing technical materials systems of high quality production have been introduced, including high quality of technological water and raw substrates (paper, paints, building materials), as well as cleanliness of equipment, tanks and warehouses for storing both raw materials and products. The microclimate parameters, which should not exceed RH<60%, 10-15% humidity of materials (depending on the material) and air temperature in warehouses 18-21°C are also monitored.

In the case of textiles, paper, plastics also access to light should be minimized (Abe 2010). However, susceptibility of paper, paints, building materials, wood to harmful impact of microorganisms makes biocide usage during production necessary.

Some examples of biocides and preparations applied to inhibit moulds growth and to produce materials with antifungal properties are presented below (Tab. 2).

Market of chemical substances and preparations (complexes containing biocides and excipients, extending their action) that can be used to protect industrial materials is regulated by 98/8/EC Directive of the European Parliament and of the Polish Council Act of biocidal products (Dz.U.2002 No. 175, poz. 1433) and the Ordinance of the Minister of Health on the categories and types of biocidal products according to their purpose (Dz.U. 2003 No 16 poz 150) (Brycki 2012).

Selection of a biocide suitable to conserve a given technical material is a big challenge for manufacturers. The main feature of a good biocide is its effectiveness in eliminating different forms of microorganisms (in the case of moulds it is essential to eliminate both mycelium and spores). However, we must remember that biocides added to technical materials must meet a number of additional requirements: they must be non-toxic, non-allergic to animals and humans, they cannot influence properties of the material or permanently adhere to it, must be insensitive to the process parameters, cheap and stable during product life cycle. Taking into account these conditions the choice of a biocide requires cooperation of experts, production technicians, chemists and microbiologists. Also it should be noted that this substance must be biodegradable

after usage. Common usage of some biocides (such as nanosilver, Triclosan) to produce antimicrobial materials can cause their accumulation in the environment and in organisms, and subsequently elimination of certain organisms which play a crucial ecological role, as well as contribute to the appearance of resistant microorganisms. It is therefore prudent to decide whether addition of a biocide to a technical material is indispensable and use it only in justified circumstances.

Table 2. Selected biocides and preparations with antifungal activity used to protect technical materials against biodeterioration.

Technical material	**Biocide / preparation**	**References**
Wood	quaternary ammonium salts, triazols, boric acid, propiconazol, ionic liquids, essential oils	Krajewski 2001, Fojutowski 2003, Zabielska-Matejuk *et al.* 2012
Paper	ethylene oxide, essential oils (thymol), ozone, phenylacetic acid, quaternary ammonium salts, isopropanol, ionic liquids phenylphenol/Irgasan, Nipagin, Rotanox	Woźniak & Tymińska 2003, Karbowska-Berent *et al.* 2009, Koziróg et al. 2012
Textiles	quaternary ammonium salts, , ethylene oxide, propylene oxide, salicylanilide, o-phenylphenol, 5-chlorophenol, nanosilver and nanocopper / Preventol, Irgasan	Montegut *et al.* 1991, Szostak-Kot 2003, Abdel-Kareem 2009, Foksowicz-Flaczyk *et al.* 2012, Gutarowska & Michalski 2012
Leather	4-chloro-3 methyl phenol, chlorocresol, 2-phenylphenol, phenoxyethanol, 2-thio-thiocyano- methyl-benzothiazole, isothiazoline compounds, diiodomethyltolyl-sulfone, nanosilver, nanozinc and nanocopper / Sanitized	Didato & Yanek 1999, Orlita 2001, Falkiewicz-Dulik 2003; 2012
Petroleum, oil, fuels	Preventol P109 (2-bromo 2-nitro-1,3 propanediol and isothiazolones), MAR 71 (N, N'- methylene-bis-5-methyloxazolidine), Kathon FP1.5 (5-chloro-2-methyl-4 isothiazolin-2-one and 2-methyl-4 isothiazolin-3 –one)	Kwiatkowska 2003

Technical material	Biocide / preparation	References
Paints	Copper alkylbenzenesulphonate, quaternary ammonium salts, titanium dioxide, ionic liquids	Shirakawa *et al.* 2002, Michalczyk & Cieniecka-Rosłonkiewicz 2009, Markowska-Szczupak *et al.* 2012
Plastics	nanosilver and nanocopper	Jeziórska *et al.* 2012
Stone, concrete, brick, mortar	quaternary ammonium salts/ Boramon, Mycetox	Cieniecka-Rosłonkiewicz & Komorowska-Kulik 2003, Piontek and Lechów 2012

Disinfection of technical materials which are already biodeteriorated to stop growth of moulds is another important issue. There is a range of methods used to address this problem: laser used for wood (Piotrowska *et al.* 2003); fumigation with gases (ethylene oxide, propylene oxide), essential oils and nanosilver particles used for paper and textiles (Tymińska 2001, Woźniak & Tymińska 2003, Gutarowska *et al.* 2012); gamma radiation used for textiles, paper and leather (Machnowski *et al.* 2012, Perkowski & Goździecki 2003, Woźniak & Tymińska 2003); microwaves for building and finishing materials (Górny *et al.* 2012); photocatalitic ionization (Pietrzak & Gutarowska 2013) and biocides (Brycki 2012).

Choosing a method of disinfection one should consider not only the inhibition of fungi growth, but also safety for the technical material. This is particularly important for historical objects. Many of the disinfection methods have a negative impact on the treated materials, they change their strength and optical parameters and may also contribute to their faster biodegradation in the future (Machnowski *et al.* 2012, Gutarowska *et al.* 2014). Therefore, it is important to know the effects of disinfection for both microorganisms and for materials to select appropriate process parameters.

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Streszczenie

Pleśnie są mikroorganizmami, które odgrywają kluczową rolę w biodeterioracji materiałów technicznych, co wynika z ich cech fizjologicznych i metabolizmu. Materiał techniczny stanowi dla nich albo źródło węgla i energii (drewno, papier, tekstylia, paliwa, skóra) albo jest podłożem do ich wzrostu (cegły, kamień, metal, szkło). Grzyby charakteryzujące się wysoką aktywnością biodeterioracyjną - enzymatyczną i kwasotwórczą należą głównie do *Aspergillus, Penicillium, Trichoderma, Cladosporium, Paecilomyces* i *Chaetomium*. Przedstawiciele niektórych rodzajów grzybów (oprócz wymienionych powyżej, również m.in. *Stachybotrys, Alternaria, Cladosporium, Epidermophyton, Microsporum, Scopulariopsis, Trichophyton)* rosnąc na substancji technicznej oraz wytwarzając alergeny i mykotoksyny stwarzają zagrożenia zdrowotne. Dlatego na podstawie wiedzy na temat warunków rozwoju pleśni i mechanizmów biodeterioracji należy odpowiednio zabezpieczać materiały przed rozwojem grzybów. Istotne jest również poszukiwanie nowych, bezpiecznych dla materiałów technicznych metod dezynfekcji w celu zahamowania rozwoju grzybów. Zastosowanie biocydów w celach ochronnych należy

ograniczyć jedynie do materiałów najbardziej narażonych na biodeteriorację (papier, tekstylia, paliwa, farby), mając na uwadze aspekty ochrony środowiska, a także produkcję materiałów biodegradowalnych, zapewniając cykl życia produktu.

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The effect of particular active substances of hallucinogenic mushrooms

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ABSTRACT

Magic mushrooms have accompanied man for thousands of years. Formerly they were used for religious and culture purposes. Those fungi belong mainly to the genera *Conocybe, Copelandia, Panaeolus, Psilocybe* and *Stropharia.* A number of these fungal species produce substances, like psilocybin, psilocin, ibotenic acid or muscimol. Because of their chemical similarity to naturally occurring neurotransmitters like serotonin and GABA these substances, after ingestion, affect brain neurochemistry and by this induce hallucinations.

This mini review presents the influence of psilocybin, psilocin, ibotenic acid and muscimol on the nervous system. Also the effects of the above mentioned substances on emotion and mental health of people are discussed.

KEY WORDS: magic mushrooms, psilocin, psilocybin, ibotenic acid, muscimol

Introduction

Hallucinogenic mushrooms have accompanied man for thousands of years. Rock paintings, dated back to 3.500 years BC, which were found in one of the caves in present Algeria, depict dancing figures, holding mushrooms in their hands. This thesis is also supported by similar findings from the region of Latin America. Numerous data suggest that hallucinogenic mushrooms were being used in Indian cultures of Mexico and Central America; the Aztecs' and the Maya's religious rituals were based on the consumption of mushrooms containing psilocybin. Although, the colonization, together with Christianization of those areas of America, led to the imposition of the official ban on their usage, the custom has survived in secret within the indigenous population, as it was proved later. Gordon Wasson in 1957 published in Life (Meyer & Quenzer 2005) a description of the original Indian ritual, during which they used hallucinogenic mushrooms. That publication led to the popularization of those fungi within Western culture, especially among

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contemporary youth subcultures. Then hallucinogens, psilocin and psilocybin, produced by fungi were identified and the pharmaceutical company Sandoz has synthetized psilocybin. At the time, it was found that also in Europe hallucinogenic mushrooms can be commonly found (Meyer & Quenzer 2005). In Poland, we meet that group of fungi, among which prevails *Psilocybe semilanceata* (Fr.) P. Kumm. (Sebelska 2013).

A number of fungal species produce alkaloids with hallucinogenic properties. Those fungi, which were often called *magic mushrooms,* belong mainly to the genera: *Conocybe*, *Panaeolus* (incl. = *Copelandia*), *Psilocybe* and *Stropharia*. Their effects, depending on the species of fungus, typically occur relatively fast after ingestion of a few grams of dry substance. Their influence appears about 30 minutes after ingestion and may maintain for several hours. Those fungi can be consumed in several forms: as dried material, fungus' brew or as a supplement to other food. In most fungi, hallucinogenic substance are psilocybin and its' active derivative, psilocin. After ingestion, psilocybin is enzymatically converted to psilocin, which in fact manifests psychoactive properties (Meyer & Quenzer 2005).

Mushrooms containing psilocybin and psilocin are not the only that can affect the activity of the CNS. Other psychoactive compounds, which are present for instance in fly agaric (*Amanita muscaria*), should be mentioned. Psychoactive substances of that mushroom are muscimol, ibotenic acid and mukazon. Ingestion of fly agaric, in various forms, leads to a state resembling alcoholic intoxication (Meyer & Quenzer 2005).

The influence of psilocybin / psilocin on the nervous system

Psilocybin, such as psilocin are metabolic products of a number fungi belonging mainly to the following genera: *Conocybe, Inocybe* or *Psilocybe*. The consumption even of a small amount of fungus, typically from 1 to 5 grams, leads to the appearance of a number somatic and psychological changes. Substances contained in them can cause heart disorders or neurodegenerative changes, especially if taken chronically. On the other hand, psilocybin, being a precursor of psilocin, in certain circumstances may cause mystical reactions. Altered states of consciousness, caused by religious beliefs are often being amplified by taking psychedelic substances. Intention behind that custom is to facilitate contact with ancestors or creation of prophecy. Those symptoms obviously are not free from side effects. In the state of impaired consciousness caused by the action of both psilocybin and psilocin, negative emotional experience, such as fear, undefined anxiety, shame and a feel of guilty may occur. Therefore, religious practices combined with the usage of psychedelic drugs, often lead to the disorder of psychosocial identification (Stebelska 2013). Such mental syndrome, caused by various reasons, is often dangerous and may be trigger the development of severe pathologies of mental nature, including states similar to some forms of schizophrenia (Hyde *et al*. 1978, McDonald 1980). Due to reasons mentioned above, psilocybin and psilocin are being currently used in animal models investigating substrates of schizophrenia (Stebelska 2013).

About 50% of psilocybin is absorbed after oral administration and, as shown by experimental data obtained with

carbon C^{14}, its' distribution in the body is rather uniform (Hopf & Eckert 1974, Passie *et al*. 2002). In the body psilocybin under the influence of alkaline phosphatase, is the subject to dephosphorylation and is converted to psilocin, which is the main and perhaps the only substance showing psychopharmacological activity. About 8 hours after ingestion, the concentration of this substance in the tissues is very low, albeit in the liver and adrenal glands it remains even to about 48 hours (Hopf & Eckert 1974, Passie *et al*. 2002, Stebelska 2013).

After oral administration of 3 to 5 mg of psilocin, which depends on the individual characteristics of each organism, the first characteristic symptoms of excitation the sympathetic autonomic nervous system appear, while the hallucinogenic effects are not being observed, yet. All effects characteristic to psilocybin appears after about 70 - 90 minutes after oral administration of 8 to 25 mg of the substance. Research also indicates that about 30 minutes after taking psilocybin, psilocin appears in the plasma. Psilocin is a substance with lipophilic nature, and therefore may relatively easy pass blood - brain barrier (Passie *et al*. 2002).

Both psilocybin and psilocin possess the indole ring and belong to the same group of indolamines as serotonin, the endogenous CNS neurotransmitter. For this reason, similarly to the other hallucinogenic tryptamines, both; psilocybin and psilocin show resemblance to serotonergic system membrane receptors. The central effects of psilocybin and psilocin run mainly with the participation of 5-HT_{2A} receptor. That was confirmed by using an antagonist of these receptors, ketanserin, which prevented the development of the most psychotic symptoms caused by psilocybin or psilocin administration. However, as Stebelska (2013) reported in her review article, some of the effects of these tryptamines shall not be removed, and would even undergo intensification. This is particularly the weakening of cognitive response, expressed as difficulty in maintaining attention and reduced vigilance (Carter *et al*. 2005, Delgado & Moreno 1998, Stebelska 2013). The same author, summarizing the cited results, states that the serotonergic system is responsible for the psychotic effects of these substances, by the excitation of 5-HT_{2A} receptors within areas of the cerebral cortex, but also by the combination with the presynaptic 5-HT1A receptors, which are found in serotonergic cells within the dorsal raphe nucleus (DRN). Psilocin leads to deactivation of nerve cells within the DRN, which resembles that observed during REM sleep. Consequently, it leads to a reduction in serotonin release from terminals of ascending serotonergic projections to the cortex (Stebelska 2013, Carter *et al*. 2005). This type of reduction in the concentration of serotonin in serotonergic projection target area, beginning in the DRN, may lead to the occurrence of hallucinations similar to those that occur among people suffering from schizophrenia (Aghajanian & Marek 1999, Gonzalez-Maeso & Sealfon 2009).

Because psilocin is a derivative of tryptamine, therefore its inactivation in the body takes place with the participation of the same enzymatic processes as serotonin. This mainly concerns the action of monoamine oxidase, an enzyme naturally occurring within the synapses in the central nervous system and involved in the deactivation of monoaminergic neurotransmitters, which are released into the synaptic cleft. Therefore, the usage of monoamine

oxidase inhibitors can intensify the psilocin activity; its hallucinogenic effects may be stronger and last longer (Halpern 2004).

Psychotic states and hallucinations that occur after the ingestion of psilocin can often return, what is known as the effect of "flashback". This phenomenon occurs especially when psilocin is combined with other substances, such as alcohol or marijuana (Ikeda *et al*. 2005, Stebelska 2013). Although psilocybin and psilocin are not strongly addictive substances, in some cases can become extremely dangerous for people who are taking them. This applies especially to young people, often emotionally instable due to their individual development. There have been noted some fatal cases, in which, after taking magic mushrooms, the person receiving them jumped out of the window. This may indicate a strong emotional destabilization or, what is characteristic to hallucinations caused by these substances, the phenomenon of waking dream, often accompanied by a feeling of floating or even possessing the ability to fly (Muller *et al*. 2013, Stebelska 2013).

The influence of ibotenic acid and muscimol on the nervous system

Ibotenic acid and muscimol, are substances produced by fungi of the genus *Amanita*, in case of which, they repel insects and larvae feeding on their fruit bodies (Halpern 2004, Tsujikawa *et al*. 2006). These fungi are widely present throughout the world, they are also common in Poland.

Due to the chemical structure of its' molecule, ibotenic acid shows resemblance to receptors of naturally occurring excitatory amino acid in the nervous system, the glutamic acid. While, muscimol is a $GABA_A$ receptor agonist, whose natural ligand in the nervous system is γ-aminobutyric acid. Unlike muscimol, ibotenic acid, due to its cytotoxic property is much more dangerous. It is being used in animal test models, in order to damage specific brain regions, which are characteristic, inter alia, for the changes seen in Alzheimer's disease (Halpern 2004, Stebelska 2013, Tsujikawa *et al*. 2006).

In the body, ibotenic acid is metabolized to muscimol, which has the ability to pass across the blood-brain barrier. This compound connects to mentioned $GABA_A$ receptors mainly in the areas of the forebrain, including the caudate nucleus and putamen, the thalamus and the hippocampal formation. This leads to opening of the receptor associated with the chloride ion channel, which in turn leads to inhibition of neuronal activity, where these receptors are located (Stebelska 2013).

According to Stebelska (2013), psychedelic effects occur after taking approximately 6 mg of muscimol. However, in case of ibotenic acid that dose varies from 30 to 60 mg, which fulfils intake of one fly agaric fruit body with the mass of about 70 grams (Satora *et al*. 2005, Stebelska 2013). The first symptoms of ingestion of fungus occur relatively quickly, just after 15 to 30 minutes. They are usually nausea, vomiting, diarrhea, vasodilatation, sweating and salivation. Interesting effect of hallucinogenic that occurs after eating of fly agaric is the phenomenon called macroscopia. It consists of a vision of surrounding objects as much bigger than they are in reality. In case of humans, ingestion of relatively large dose of muscimol leads to hyperthermia, mydriasis, mood improval, concentration difficulties, anorexia, ataxia, catalepsy and hallucination. A number of these

changes, resembling drunkenness, is also characteristic to the effects caused by the classical hallucinogen such as LSD (Meyer & Quenzer 2005). Fly agaric poisoning symptoms persist relatively long and disappear after about 8 hours of ingestion of mushrooms (Satora *et al*. 2005).

Conclusions

Hallucinogenic mushrooms have being eaten for thousands of years for ritual and religious purposes. Active substances, like psilocybin, psilocin and muscimol characterizes with relatively low toxicity and usually do not cause addiction typical to cocaine or opiates. However, due to the nature of the induced hallucinations and individual organism features such as age or emotional state, they may become dangerous to health. In turn, their resemblance to the serotoninergic system, and consequent ability to interact with other neurotransmitter systems of the brain are danger, because can lead to psychosis, often specific to those occurring among patients suffering from schizophrenia. Regardless to the dangers associated with the consumption of hallucinogenic mushrooms, as well as active substances produced by them, it should be noted that the latterly mentioned are being commonly used in clinical neurophysiology. Being agonists or antagonists of membrane receptors for certain neurotransmitters, they help in understanding their physiological functions and point out possible pathologies caused by abnormalities within the particular neurotransmitter systems.

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Streszczenie

Działanie niektórych substancji aktywnych grzybów halucynogennych

Grzyby halucynogenne towarzyszą człowiekowi od tysięcy lat. Malowidła naskalne datowane na 3500 lat przed naszą erą, które odnaleziono w jednej z jaskiń na terenie dzisiejszej Algerii, przedstawiają tańczące postacie, trzymające w rękach grzyby. Potwierdzają to również znaleziska archeologiczne z rejonów Ameryki Łacińskiej. Liczne dane wskazują, że grzyby halucynogenne stosowano w kulturach Indian Meksyku i Centralnej Ameryki, Azteków i Majów w rytuałach religijnych, związanych z ich życiem, a opartych na spożywaniu grzybów zawierających psylocybinę. Co prawda, kolonizacja i chrystianizacja tych rejonów Ameryki doprowadziła do wprowadzenia oficjalnego zakazu ich stosowania jednak, jak później dowiedziono, zwyczaj przetrwał w tajemnicy wśród rdzennej ludności. Gordon Wasson w 1957 roku opublikował w Life opis oryginalnego indiańskiego rytuału, podczas którego wykorzystano grzyby halucynogenne (Meyer & Quenzer 2005). Wspomniana publikacja doprowadziła do spopularyzowania tych grzybów w kulturze Zachodu, szczególnie w ówczesnych subkulturach młodzieżowych. Następnie zidentyfikowano związki halucynogenne, psylocynę i psylocybinę, produkowane przez grzyby, a firma farmaceutyczna Sandoz wprowadziła na rynek psylocybinę (Meyer & Quenzer 2005). W tym samym czasie stwierdzono, że także w Europie powszechnie występują grzyby o działaniu halucynogennym. W Polsce spotykamy tę grupę grzybów, wśród których zdecydowanie dominuje łysiczka lancetowata (*Psilocybe semilanceata*) (Stebelska 2013).

Szereg gatunków grzybów wytwarza alkaloidy o właściwościach halucynogennych. Grzyby te, często określane jako *grzyby magiczne*, należą głównie do rodzajów *Conocybe, Copelandia, Panaeolus, Psilocybe oraz Stropharia*. Efekty ich działania, zależnie od gatunku grzyba, pojawiają się zwykle po zażyciu już kilku gramów suchej substancji stosunkowo szybko, po upływie około 30 minut po spożyciu i utrzymują do kilku godzin. Zróżnicowana może być także postać, w jakiej grzyb jest spożywany; może to być materiał wysuszony, przygotowany napar lub grzyb przygotowany jako dodatek do innego pokarmu. W przypadku większości grzybów halucynogennych substancją aktywną jest psylocybina oraz jej pochodna, psylocyna. Po spożyciu, psylocybina podlega enzymatycznemu przekształceniu do psylocyny, która to w rzeczywistości przejawia własności psychoaktywne (Meyer & Quenzer 2005).

Grzyby zawierające psylocynę i psylocybinę nie są jedynymi, które mogą wpływać na aktywność OUN. Należy zwrócić uwagę także na inne związki psychoaktywne, które występują choćby w muchomorze czerwonym (*Amanita muscaria*). Substancje psychoaktywne tego grzyba to muscimol, kwas ibotenowy i mukazon. Spożycie muchomora czerwonego, w różnej postaci, prowadzi do stanu przypominającego upojenie alkoholowe (Meyer & Quenzer 2005).

Wpływ psylocybiny/psylocyny na układ nerwowy

Zarówno psylocybina, jak też psylocyny są produktami metabolizmu szeregu grzybów należących między innymi do rodzajów *Conocybe, Inocybe* lub *Psilocybe*. Spożycie niewielkiej ilości grzybów, zwykle od 1 do 5 gramów, prowadzi do pojawienia się szeregu zmian, tak somatycznych jak i psychicznych. Substancje w nich zawarte, mogą wywoływać zaburzenia pracy serca czy zmiany neurodegeneracyjne

szczególnie, jeśli są przyjmowane chronicznie. Z drugiej strony psylocybina, będąca prekursorem psylocyny, w określonych warunkach może wywoływać reakcje mistyczne. Stany zmienionej/odmiennej świadomości, powodowane wierzeniami religijnymi, często są „wzmacniane" przyjmowaniem substancji psychodelicznych, co w zamierzeniach ma powodować ułatwienie kontaktu ze światem przodków lub kreowaniu przepowiedni. Tego rodzaju symptomy nie są jednak wolne od efektów ubocznych stosowania wspomnianych substancji. W stanie zaburzeń świadomości powodowanej działaniem psylocybiny/psylocyny, mogą pojawiać się negatywne doznania emocyjne, jak choćby strach, nieokreślony lęk, wstyd oraz poczucie winy. Tym samym, wszelkie praktyki o charakterze kultu religijnego, w połączeniu z zastosowaniem środków psychodelicznych, prowadzą często do zaburzeń identyfikacji psychosocjalnych (Stebelska 2013). Tego rodzaju syndrom psychiczny, wywoływany z różnych powodów, często jest niebezpieczny i może stanowić początek rozwoju poważnych patologii o charakterze psychicznym, w tym podobnych w przebiegu do niektórych postaci schizofrenii (Hyde *et al*. 1978, McDonald 1980). Z tego powodu psylocybina lub psylocyna są obecnie stosowane w modelach zwierzęcych badających podłoże schizofrenii (Stebelska 2013).

Około 50% psylocybiny podlega absorpcji po podaniu doustnym, a jak wskazują dane doświadczalne uzyskane z zastosowaniem związku znakowanego izotopem węgla C^{14}, jej dystrybucja w organizmie jest mniej więcej równomierna (Hopf & Eckert 1974, Passie *et al*. 2002). W organizmie, psylocybina, pod wpływem zasadowej fosfatazy, podlega procesowi defosforylacji i przekształcana jest do psylocyny, która jest główną, prawdopodobnie jedyną, substancją aktywną psychofarmakologicznie. Po upływie około 8 godzin od zażycia, stężenie substancji w tkankach jest bardzo niskie, jednak w wątrobie i nadnerczach utrzymuje się do około 48 godzin (Hopf & Eckert 1974, Passie *et al*. 2002, Stebelska 2013).

Po doustnym przyjęciu od 3 do 5 mg psylocyny, co uzależnione jest od indywidualnych cech każdego organizmu, pojawiają się pierwsze objawy charakterystyczne dla wzbudzenia części współczulnej autonomicznego układu nerwowego, natomiast brak jeszcze efektów halucynogennych. Wszystkie efekty, charakterystyczne dla psylocybiny, zaznaczają się po upływie około 70–90 minut po doustnym przyjęciu od 8 do 25 mg tej substancji. Badania wskazują również, że około 30 minut po przyjęciu psylocybiny, w osoczu krwi pojawia się psylocyna. Psylocyna jest substancją o charakterze lipofilnym, dlatego stosunkowo łatwo przechodzi barierę krew – mózg (Passie *et al*. 2002).

Zarówno psylocybina, jak i psylocyna posiadają pierścień indolowy i należą do tej samej grupy indoloamin, w której znajduje się serotonina, endogenny neurotransmiter ośrodkowego układu nerwowego. Z tego powodu, podobnie do innych tryptamin o charakterze halucynogennym, wykazują powinowactwo do receptorów błonowych układu serotoninergicznego. Ośrodkowe efekty działania psylocybiny i psylocyny przebiegają głównie przy udziale receptora $5\text{-}HT_{2A}$. Potwierdzono to stosując antagonistę wspomnianych receptorów, ketanserynę, która zapobiegała rozwojowi większości objawów psychotycznych powodowanych podaniem psylocybiny lub psylocyny. Jednakże, jak podaje w swojej pracy przeglądowej Stebelska (2013), niektóre efekty działania tych tryptamin nie zostają zniesione, a nawet ulegają nasileniu. Dotyczy to szczególnie osłabienia reakcji kognitywnych, wyrażanych trudnością w utrzymaniu uwagi oraz obniżeniem czujności (Carter *et al*. 2005, Delgado

& Moreno 1998, Stebelska 2013). Ta sama Autorka, podsumowując cytowane wyniki badań stwierdza, że system serotoninergiczny odpowiada za psychotyczne efekty działania tych substancji, zarówno przez wzbudzenie receptorów 5-HT_{2A} w obrębie obszarów kory mózgowej, ale także przez połączenie się z presynaptycznymi receptorami 5-HT_{1A}, które znajdują się w obrębie komórek serotoninergicznych grzbietowego jądra szwu. Psylocyna prowadzi do wyłączenia aktywności komórek nerwowych w obrębie grzbietowego jądra szwu (DRN), które przypomina to, obserwowane podczas snu REM. W konsekwencji doprowadza to do obniżenia uwalniania serotoniny z zakończeń wstępującej, dokorowej projekcji serotoninergicznej (Stebelska 2013, Carter *et al*. 2005). Tego typu obniżenie stężenia serotoniny w docelowych obszarach projekcji serotoninergicznej, rozpoczynającej się w obrębie grzbietowego jądra szwu, może doprowadzić do występowania halucynacji podobnych do tych, które występują u osób cierpiących na schizofrenię (Aghajanian & Marek 1999, Gonzalez-Maeso & Sealfon 2009).

Ponieważ psylocyna jest pochodną tryptamin, dlatego jej dezaktywacja w organizmie przebiega przy udziale tych samych procesów enzymatycznych, którym podlega serotonina. Dotyczy to przede wszystkim działania monoaminooksydazy, enzymu naturalnie występującego w obrębie synaps w ośrodkowym układzie nerwowym i uczestniczącego w dezaktywacji neurotransmiterów monoaminergicznych, uwalnianych do przestrzeni synaptycznej. Dlatego zastosowanie inhibitorów monoaminooksydazy nasila działanie psylocyny, efekty halucynogenne mogą być silniejsze i utrzymują się dłużej (Halpern 2004).

Stany psychotyczne i halucynacje, które występują po przyjęciu psylocyny mogą często nawracać, co znane jest jako efekt „flashback". Zjawisko to pojawia się szczególnie wtedy, gdy psylocyna łączona jest z innymi substancjami, jak choćby alkohol lub marihuana (Ikeda *et al*. 2005, Stebelska 2013). Mimo, że psylocybina i psylocyna nie są substancjami silnie uzależniającymi, to jednak w niektórych przypadkach mogą być wyjątkowo niebezpieczne dla osób, które je zażywają. Dotyczy to szczególnie ludzi młodych, często niezrównoważonych emocjonalnie w związku z rozwojem osobniczym. Zanotowano kilka przypadków śmiertelnych, w których po zażyciu *magicznych grzybów*, osoby je przyjmujące wyskakiwały przez okno. Może to wskazywać na silną destabilizację emocjonalną albo, co jest charakterystyczne dla halucynacji wywoływanych prze te substancje, zjawiska snu na jawie, któremu często towarzyszy uczucie unoszenia się lub nawet umiejętności fruwania (Muller *et al*. 2013, Stebelska 2013).

Działanie kwasu ibotenowego i muscimolu w układzie nerwowym

Kwas ibotenowy oraz muscimol, to substancje, które są wytwarzane przez grzyby z rodzaju *Amanita*, w przypadku których odstraszają owady oraz larwy, żerujące na ich owocnikach (Halpern 2004, Tsujikawa *et al*. 2006). Grzyby te są szeroko rozpowszechnione w całym świecie, występują również często w Polsce.

Ze względu na chemiczną budowę cząsteczki, kwas ibotenowy wykazuje powinowactwo do receptorów naturalnie występującego w układzie nerwowym aminokwasu pobudzającego, kwasu glutaminowego. Z kolei muscimol jest agonistą receptora $GABA_A$, którego naturalnym ligandem w układzie nerwowym jest kwas γ-aminomasłowy. W odróżnieniu od muscimolu, kwas ibotenowy, ze względu na swoje własności cytotoksyczne jest znacznie bardziej niebezpieczny. Jest on stosowany w

modelach badań na zwierzętach, w celu uszkodzeń wybranych obszarów mózgu, które są charakterystyczne między innymi dla zmian obserwowanych w chorobie Alzheimera (Halpern 2004, Stebelska 2013, Tsujikawa *et al*. 2006).

W organizmie, kwas ibotenowy jest metabolizowany do muscimolu, który ma zdolność do przechodzenia przez barierę krew-mózg. Związek ten łączy się ze wspomnianymi receptorami $GABA_A$, głównie w obszarach przodomózgowia, między innymi w obrębie jądra ogoniastego i przegrody, wzgórza oraz formacji hipokampa. Powoduje to otwarcie związanego z tym receptorem kanału dla jonu chlorkowego, co w efekcie prowadzi do zahamowania aktywności neuronów, na których znajdują się wspomniane receptory (Stebelska 2013).

Jak podaje Stebelska (2013), efekty psychodeliczne pojawiają się po zażyciu około 6 mg muscimolu. Natomiast w odniesieniu do kwasu ibotenowego dawka ta mieści się w przedziale 30 do 60 mg, co odpowiada mniej więcej spożyciu jednego owocnika *Amanita muscaria* o masie około 70 gramów (Satora *et al*. 2005, Stebelska 2013). Pierwsze objawy po spożyciu muchomora występują stosunkowo szybko, ponieważ już po około 15 do 30 minutach. Zwykle są to nudności, wymioty, biegunka, rozszerzenie naczyń krwionośnych, pocenie się i ślinotok. Interesującym efektem halucynogennym, który występuje po zjedzeniu muchomora jest zjawisko makroskopii. Polega ono na widzeniu otaczających przedmiotów jako znacznie większych, niż są one w rzeczywistości. W przypadku ludzi, doustne przyjęcie stosunkowo dużych dawek muscimolu prowadzi do hipertermii, rozszerzenia źrenic, poprawy nastroju, trudności z koncentracją, anoreksji, ataksji, katalepsji oraz halucynacji. Szereg tych zmian przypomina stan upojenia alkoholowego, jest także charakterystycznych do efektów powodowanych przez klasyczne związki halucynogenne, jak choćby LSD (Meyer & Quenzer 2005). Objawy zatrucia muchomorem utrzymują się stosunkowo długo i ustępują po upływie około 8 godzin od spożycia grzybów (Satora *et al*. 2005).

Podsumowanie

Grzyby halucynogenne są spożywane od tysiącleci w celach rytualnych i religijnych. Substancje aktywne, psylocybina, psylocyna i muscimol są stosunkowo mało toksyczne i z reguły nie powodują uzależnień, charakterystycznych choćby kokainy lub opiatów. Niemniej jednak, ze względu na charakter wywoływanych halucynacji oraz indywidualne cechy organizmu, jak wiek, stan emocjonalny mogą być groźne dla zdrowia. Z kolei na ich powinowactwo do receptorów układu serotoninergicznego, a przez to na możliwość interakcji z innymi systemami neurotransmisyjnymi mózgu, stanowią niebezpieczeństwo rozwoju psychoz, często charakterystycznych dla tych, występujących u pacjentów ze schizofrenią. Niezależnie od niebezpieczeństw związanych ze spożywaniem grzybów halucynogennych, jak też produkowanych przez nie substancji aktywnych, należy wskazać, że te ostatnie znajdują powszechne zastosowania w badaniach neurofizjologicznych. Będąc agonistami lub antagonistami receptorów błonowych dla niektórych neurotransmiterów, pozwoliły zrozumieć ich funkcje fizjologiczna oraz wskazały na możliwe patologie powodowane zaburzeniami w obrębie poszczególnych systemów neurotransmisyjnych.

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The biotechnology of higher fungi - current state and perspectives

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ABSTRACT

This review article concisely describes methodology of biotechnological processes with the use of cultures of higher fungi, their application in bioremediation and to obtain biologically active preparations. Advantages and disadvantages of biotechnological methods used to cultivate mushrooms are analyzed. This paper contains overview of higher fungi species most commonly used in biotechnological processes, of cultivation methods applied to produce fungal biomass, of enzymes and bioactive metabolites and of the strategies for submerged cultivation of the mycelial cultures. The problems of optimization of strains and biotechnological processes are briefly discussed.

KEY WORDS: medicinal mushrooms, mycoremediation, submerged cultivation, process optimization

Introduction

Since the beginnings of biotechnology, more or less formally recognized scientific discipline, fungi – the organisms used in biosynthesis and biotransformations of various types of substances have been in the focus of interest. However, for a long time researchers concentrated mainly on species informally classified as lower fungi. In pharmaceutical biotechnology, for example, filamentous fungi of the genera *Penicillium, Cephalosporium, Aspergillus* or *Fusidium* are employed in the production of antibiotics, vitamins, enzymes and organic acids (citric acid, itaconic acid, fusaric acid and gluconic acid). In recombinant DNA techniques (e.g. in insulin production), *Saccharomyces cerevisiae* Meyen ex E.C. Hansen yeast is used as a recipient of DNA. Among higher fungi, a systematically heterogeneous group of fungi belonging to *Ascomycotina, Basidiomycotina,* and former *Deuteromycotina*, characterized by the ability to form fruiting bodies, for a long time the only process that could be classified as biotech (a process which comprises cultivating of the inoculum, preparation and sterilization of the substrate, inoculation and culturing the strain under defined conditions) was

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cultivation of edible mushrooms under semicontrolled conditions.

Research conducted by Gregory can be considered as the first attempt to use the submerged cultures of higher fungi in classical biotechnological processes. In 1966, Gregory published the results of the search for new antitumor substances in the fruiting bodies of more than 200 species of fungi belonging to *Basidiomycetes* class. He searched for pharmacologically active substances also in approximately 7 000 post-culture liquid media used for submerged cultivation of different species of higher fungi. The isolated substances (mainly polysaccharides) demonstrated an inhibitory effect on tumor cells, including cancers such as Kaposi S-180, adenocarcinoma 755 and leukemia L-1210. Recently submerged culture of mycelium, conducted in bioreactors of different structures, in liquid or solid media has been the most typical biotechnological process with the use of higher fungi. Development and optimization of such processes focus mainly on:

- isolation of biologically active metabolites, often pharmacologically active substances (drugs, vitamins), synthesized by fungal cells (from mycelium or a culture medium);
- production of biomass rich in nutrients to be used as food, functional foods and food supplements;
- production of biomass rich in biologically active substances (mainly antioxidants) to be used in cosmetology;
- isolation of enzymes (mainly peroxidases), synthesized by cultured fungi, which are subsequently used in the processes of biotransformation and bioremediation or in chemical syntheses;
- the use of cultured mycelium in bioremediation processes (mycoremediation).

The advantages and disadvantages of biotechnological methods of mushroom cultivation

Biotechnological methods of cultivation of higher fungi in many respects surpass the methods used for cropping:

- the major advantage consists in short cultivation time in bioreactors, especially in liquid media. In comparison with the duration of mushroom cropping, this significantly reduces the time necessary to obtain a comparable biomass;
- the mycelial cultures in bioreactors are carried out under repeatable conditions, resulting in a stable composition of the biomass grown. This facilitates the standardization of the preparations derived from fungi, for example for pharmaceutical use;
- optimization of the composition of the culture media and the physico-chemical factors of the culture allows to a regulate metabolism of the cultivated mycelia, thus significantly increasing in efficiency of the biosynthesis of biologically active compounds (e.g. secondary metabolites);
- technology of the biotechnological processes ensures monitoring and maintaining of biochemical and genetic identity of mycelia grown in a fermenter.

There are also serious difficulties in the use of modern biotechnological methods in cultures of higher fungi:

- not all strains of the higher fungi are able to grow efficiently as mycelial cultures in bioreactors;

- in the case of certain species of fungi there are significant differences in the chemical composition of fruiting bodies and mycelium cultivated biotechnologically. These differences are not always advantageous when mycelial cultures are used to prepare biologically active preparations;

- the metabolic pathways of biosynthesis of biologically active substances by fungi are still not well characterized and described, as compared to plants or filamentous fungi. This makes it difficult to design and to optimize a biotechnological process, for example by the selection of precursors of biosynthesis or strain growth promoters;

- it is difficult to use genetic engineering methods in higher fungi, due to the lack of complete knowledge on the genes encoding the biosynthesis pathways for the whole or part thereof.

Higher fungi species used in biotechnological processes

Biotechnological processes with the use of saprotrophic mushrooms belonging to white rot fungi are among the most studied, well-developed and in practice easiest to conduct. In particular the white rot fungi regarding as medicinal mushrooms are often used in biotechnological processes.

Higher fungi of the class *Basidiomycetes* represent about 30% of all fungal species (Kirk *et al*. 2008). About 700 of them are able to synthesize metabolites with pharmacological, very often (651 species) anticancer and immunostimulating activity. This group of mushrooms is reffered to as 'medicinal mushrooms' (Wasser and Weiss 1999). Among the most studied medicinal mushrooms there are about 30 species of fungi. They are able to synthesize compounds of diversified pharmacological activity: antitumor, immunomodulatory, antiviral (including anti-HIV), antibacterial, anti-inflammatory, antifungal, anti-diabetic, hepatoprotective, nerve tonic, hypotensive, as well as the activity lowering blood levels of cholesterol and triglycerides (Sumiyoshi *et al*. 2010, Elisashvili 2012, Lo *et al*. 2012, Patel & Goyal 2012, Cheng *et al*. 2013, Croccia *et al*. 2013, Kylyc *et al*. 2013, Lei *et al*. 2013, Lin *et al*. 2013, Mendez-Espinoza *et al*. 2013, Mizuno & Nishitani 2013, Park *et al*. 2002, Rony *et al*. 2013, Rouhana-Toubi *et al*. 2013, Wu X. *et al*. 2013, Yamanaka *et al*. 2013, Yu *et al*. 2013, Zhu *et al*. 2013, Hsu *et al*. 2014).

Overview of the most interesting species of medicinal mushrooms and their pharmacological activity is presented in Table 1, showing a modified version of the data published by Wasser and Weiss (1999). The most valuable species, from the pharmacological point of view as well as their use in production of pharmaceutical formulations (drugs, food supplements, functional foods), include *Lentinula edodes, Ganoderma lucidum, Trametes versicolor, Schizophyllum commune, Hericium erinaceus,* and *Grifola frondosa.* There are four formulations (registered in several countries as drugs) used in cancer therapy, isolated from the fruiting bodies or mycelia of basidiomycetes (Mizuno 1999):

- *Lentinan* – a polysaccharide fraction isolated from *Lentinula edodes,*
- *Schizophyllan* (SPG, sonifilan, sizofilan) – a polysaccharide fraction isolated from *Schizophyllum commune,*
- *Grifolan* – a polysaccharide fraction isolated from *Grifola frondosa*,
- *Krestin* – a polysaccharide PSK and PSP-complex polysaccharide-protein isolated from *Trametes versicolor.*

Table 1. Cross index of the most interesting species of medicinal mushrooms and their pharmacological activity (Wasser & Weiss 1999, modified). x = commercially developed mushroom product (drug or dietary supplement); + = non commercially developed mushroom product; * = the most widely used species of fungi and their most important activities from pharmacological point of view are in bold.

	PHARMACOLOGICAL ACTIVITY														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Antifungal	Antiinflammatory	**Anticancer ***	Antiviral (e.g. anti-HIV)	Antibacterial	Blood pressure regulation	Cardiotonic	Cholesterol lowering	Antidiabetic	**Immunomodulatory**	Kidney tonic	Hepatoprotective	Nerve tonic	Sexual potenting	Antiasthmatic
Auricularia auricula-judae (Bull.) Quél.			+			+	x	x							x
Tremella fuciformis Berk.		+	+					+	+	+		+			x
***Schizophyllum commune* Fr.**		x	x		x					x	x	x			
Dendropolyporus umbellatus (Pers.) Jülich			x							x		x			x
***Grifola frondosa* (Dicks.) Gray**	+		x	x	x	x			x	x		+			+
Fomes fomentarius (L.) Fr.				+		+						+			
Fomitopsis pinicola (Sw.) P. Karst.		+	+		+										
***Trametes versicolor* (L.) Lloyd**			x	x	x						x	x			
Piptoporus betulinus (Bull.) P. Karst.	+		+			+									
***Hericium erinaceus* (Bull.) Pers.**			x							x			x		x
Inonotus obliquus (Ach. ex Pers.) Pilát		x	x							x		x			
Lenzites betulina (L.) Fr.			+				+								
Laetiporus sulphureus (Bull.) Murrill	+		+												
Ganoderma lucidum (Curtis) P. Karst.		x	x	x	x	x	x			x	x	x	x	x	x
Ganoderma applanatum (Pers.) Pat.			+	+	+					+					

	PHARMACOLOGICAL ACTIVITY														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Antifungal	Antiinflammatory	**Anticancer ***	Antiviral (e.g. anti-HIV)	Antibacterial	Blood pressure regulation	Cardiotonic	Cholesterol lowering	Antidiabetic	**Immunomodulatory**	Kidney tonic	Hepatoprotective	Nerve tonic	Sexual potenting	Antiasthmatic
***Lentinula edodes* (Berk.) Pegler**		x	x	x	x	x		x	x	x	x	x		x	
Pleurotus ostreatus (Jacq.) P. Kumm.			x	+	+			x		x			+		
Flammulina velutipes (Curtis) Singer	+	x	x	+						x					
Oudemansiella mucida (Schrad.) Höhn.	x														
Armillariella mellea (Vahl) P. Karst.	+					x	x							x	
Hypsizygus marmoreus (Peck) H.E. Bigelow			x										x		
Marasmius androsaceus (L.) Fr.		x													
Agaricus blazei Murrill			x												
Agaricus bisporus (J.E. Lange) Imbach			x							x	x				
Volvariella volvacea (Bull.) Singer			+	+	+			+							
Agrocybe aegerita (V. Brig.) Singer	+		+					+							

Methods for the submerged cultivation in the mycelial cultures of practically all of above mentioned mushroom species have been developed and published (Kim *et al*. 2002, Chang *et al.* 2006, Cui *et al*. 2006, Liu & Wang 2007, Malinowska *et al*. 2009, Kim *et al*. 2010, Turło *et al*. 2010c, Elisashvili 2012, Lo *et al*. 2012, Patel & Goyal 2012, Atli *et al*. 2013, Habijanic *et al*. 2013, Hsu *et al*. 2014, Khan *et al*. 2013, Liang *et al*. 2013, Jeong *et al*. 2013, Yue *et al*. 2013, Wu F.C *et al*. 2013).

Production of the mushroom-derived products is a rapidly expanding industry. As mentioned above, the mushroom-derived immunomodulating and anticancer compounds are used in clinical applications as adjuvant to standard chemotherapy (Lindequist *et al*. 2005, Arora *et al*. 2013, Durgo *et al*. 2013). There are also several types of

dietary supplements derived from the medicinal mushrooms: dried and pulverized fruiting bodies, hot water and alcohol extracts of fruiting bodies, biomass or extracts of mycelia, or broth harvested from submerged liquid cultures. Commercial preparations are available as tablets, capsules or elixirs in most Asian countries and their presence in the USA, New Zealand, Australia, and Europe increases. In 1999 worldwide sales of mushroom dietary supplement products (nutriceuticals) had the value of $5-6 billion US (Chang and Buswell 1999, Wong and Cheung 2008) and have been increasing by between 10-20% annually. The current market value has been estimated to exceed of $14 billion per year (U.S. Food and Drug Administration). According to Wong and Cheung (2008), nearly 80% of medicinal mushroom products are derived from fruit bodies (e.g. *Lentinan* from *L. edodes, Grifon D* from *G. frondosa,* and practically all preparations from *Ganoderma lucidum*), 15% are based on extracts from mycelium (e.g. *Krestin* and *PSP* from *T. versicolor, LEM* and *LAP* from *L. edodes*), the smallest part from a culture medium (e. g. *Sonifilan* from *S. commune, PSPC* from *Tricholoma lobayense* R. Heim). However, due to increasing demands for quality and standardization of products, the share of biotech-derived preparations in the market is constantly growing.

The white rot fungi, including species unformally classified as higher fungi, are unique in their ability to completely degrade lignin in biotechnological processes (Tien and Kirk 1988). This process is mediated by fungal redox enzymes: lignin peroxidases (LiP), Mn-dependent peroxidases (MnP), versatile peroxidases (VP), other peroxidases, laccases, and tyrosinases (Bucke 1998, Mester *et al*. 1997, Singh 2006, Turło & Turło 2013). The fungal redox enzymes are non-specific for substrate and non-stereoselective, therefore able to transform a broad spectrum of organic pollutants, such as polycyclic aromatic hydrocarbons, pesticides, dyes, plastics and explosives (Bumpus *et al*. 1985, Aust & Benson 1993, Field *et al*. 1992, Wong 2009, Hammel & Cullen 2008, Shukla & Varma 2011). The lignolytic enzymes of the white rot fungi are active extracellulary, therefore these organisms are better candidates for the biotransformation of apolar pollutants than non-lignolytic microorganisms (Field *et al*. 1992). The fungal redox enzymes are produced under nutrient-limiting conditions (Moreira *et al*. 2000, Couto *et al*. 2002). Their synthesis is not induced by the presence of pollutants (Barr & Aust 1994). All the above mentioned features make the white rot fungi applicable in bioremediation processes, implemented *in situ* or *ex situ*. *Ex situ* processes are performed as typical submerged cultures carried out in bioreactors. Submerged cultures of the white rot fungi are also used for biosynthesis of lygnolytic enzymes that, after the isolation from the post-cultivation medium, are used, native or immobilized, in the biotransformation of xenobiotics. The list of fungal strains used in mycoremediation (a form of bioremediation that uses conditioned native fungi or fungal mycelium to remove and degrade contaminants; Singh 2006) is very long and includes also the white rot fungi described above as medicinal mushrooms. Besides widely examined *Phanerochaete chrysosporium* Burds. (Kubatova *et al*. 1998, Takada *et al*. 1996), several other white rot fungi, e.g. *Pleurotus ostreatus* (Kubatova *et al*. 1998, Beaudette *et al*. 2000), *Coriolopsis polyzona* (Pers.) Ryvarden (Vyas *et al* 1994, Novotny *et al*. 1997), *Trametes*

(*Coriolus*) *versicolor* (Berry *et al*. 1993, Sasek *et al*. 1993, Cloete & Celliers 1999, Beaudette *et al*. 2000, Koller *et al*. 2000, Ruiz-Aguilar *et al*. 2002), *Bjerkandera adusta* (Willd.) P. Karst. (Beaudette *et al*. 2000), *Trametes trogii* Berk. (Levin *et al*. 2003), *Phlebia lindtneri* (Pilát) Parmasto (Singh 2006, Kamei & Kondo 2005), *Trametes* (*Coriolus*) *hirsuta* (Wulfen) Lloyd (Orihara *et al*. 2005), *Phanerochaete sordida* (P. Karst.) J. Erikss. & Ryvarden (Valli *et al*. 1992), *Pleurotus pulmonarius* (Fr.) Quél. (Masaphy *et al*. 1996), *Hypholoma fasciculare* (Huds.) P. Kumm., *Stereum hirsutum* (Willd.) Pers. (Bending *et al*. 2002), are also known to metabolize organopollutants. Numerous processes using higher fungi for degradation of environmental pollutants have been patented; however, a significant part of them is still at the stage of preliminary experiments. Only a few companies (e.g. Earth Fax Development Corporation in United States, Gebruder Huber Bodenrecycling in Germany) employ fungal cultures for soil bioremediation, but a broader use probably will take place in the future.

Submerged cultures of mycorrhizal fungi raise more problems, especially when optimizing the culture media, but are also possible to conduct. In the experiments performed in our laboratory we have successfully conducted the bioreactor cultures of such mushroom species as *Lactarius deliciosus* (L.) Gray, *Boletus edulis* Bull., *Tuber aestivum* Vittad., *Tuber brumale* Vittad. The purpose of these experiments was to obtain biomass of preferred nutritional composition, including typical flavor and aroma volatiles of fungi.

The cultivation methods for the production of fungal biomass, enzymes and bioactive metabolites

In fungal biotechnology there are used several different techniques and substrates. In general regarding the substrates used, the methods are divided into:

- solid-state fermentation (SSF) defined as a process occurring in the absence or near absence of free liquid, employing an inert or natural substrate as a solid support. The method is used for bioconversion of plant waste materials into foods (mushroom fruit bodies), fodder, enzymes, secondary metabolites (e.g. drugs, food supplements). The advantages of SSF: small energy consumption, cheap substrates (natural lignocellulosic materials, food-industry residues), concentrated media resulting in smaller bioreactor dimensions (Pandey *et al.* 2000, Couto & Toca-Herrera 2007, Petre & Teodorescu 2012). The disadvantages: problems with isolation and purification of the products, difficult or impossible control of the process parameters (pH, temperature, aeration), inhomogeneous culture conditions (e.g. difficulties in oxygen transport, agitation);

- submerged liquid cultures working as homogenous systems under the full process control (pH, agitation, concentration of medium components, oxygenation, medium density). This method permits fully standardized production of the fungal biomass with high nutritional value or biosynthesis of mushroom metabolites with predictable composition. The downstream processing after the submerged cultivation is easier as compared with SSF. However, submerged cultivation induces high energy cost required for agitation, oxygen supply, stabilization of the temperature of the medium. This method

has significant industrial potential also due to the possibility of the process upscalling and operation of the large scale bioreactors.

The choice of the technique for the submerged cultivation of higher fungi mycelial cultures depends on the desired effect (the product), and on the fungi physiological and morphological peculiarities.

Strategies for submerged cultivation of mycelial cultures

The most frequently used technique for the submerged mushroom cultivation is *batch culture.* In the batch cultures no fresh nutrients are introduced into a substrate and no end products of metabolism are discharged during the process. Shake flask cultures are the simplest form of this technique. They are commonly used in cultivation of the inoculum prepared for inoculation of the bioreactor culture, and in experiments on the optimization of the culture medium (Asatiani *et al*. 2007, Turło *et al*. 2008, Malinowska *et al*. 2009a, Porras-Arboleda *et al*. 2009, Lin 2010, Xu *et al*. 2011, García *et al*. 2014, Homolka 2014). On a larger scale the mushroom cultures are grown in bioreactors of different construction, most commonly in air-lift type (stirred by the air stream) or in stirred-tank type (stirred with a mechanical stirrer) ones (Lee *et al*. 2004, Kim *et al*. 2007, Elisashvili *et al*. 2009, Turło *et al*. 2010a, b). In a fermenter it is possible to control the culture conditions, such as temperature, agitation, dissolved oxygen, temperature, substrate and metabolite concentrations and pH of the medium (Elisashvili 2012). Cultivation of higher fungi in the bioreactor submerged cultures is loaded, however, with greater difficulties than the cultures of single-celled organisms. In the submerged cultures morphological form of pellets is characteristic of higher fungi. The pellet size determines the oxygen and nutrient transport into its center. In the core region of a large pellet cells death resulting from lack of oxygen and nutrients occurs, therefore reduction of the pellet diameter is advantageous. Pellet size is influenced by different variables, such as agitation regime, density of the inoculum or sugar concentration in the medium (Petre *et al*. 2010). According to our unpublished experiments, addition of polysorbate detergents (Tween) at a low concentration to a culture medium significantly reduces the diameter of pellets and does not inhibit growth of the strain (in *L. edodes* cultures). The mushroom mycelia and pellets are shear sensitive, therefore in the air-lift bioreactor the mycelial growth is better than in stirred tanks, due to lower shear forces. The culture viscosity significantly increases during cultivation, additionally fungal mycelia wrap around impellers, spread into sampling and nutrient feed lines and cause blockages. These drawbacks limit the time of operation in bioreactors.

The other strategy used for mushroom submerged culture is *fed-batch cultivation*. The fed-batch cultures are carried out with a batch or continuous dispensing of sterile medium to the fermenter, which results in reducing the inhibitory effect of metabolic products of microbial growth and increased biomass growth (Shih *et al*. 2008). In *repeated-fed batch fermentation* process, in turn, periodically a portion of broth with accumulated mushroom biomass is taken from the fermenter and supplemented with fresh medium, while maintaining its constant volume.

Successful commercial implementation of the submerged cultivation of the mushrooms to the technical scale involves, irrespective of the purpose, the development of three phases:

- inoculum preparation techniques and their improvements,
- clear technical protocols for the final design and associated engineering processes,
- protocols for monitoring, adjustment, continuity and maintenance of the engineering system.

Optimization of the strains and biotechnological processes

Currently, there are two known methods of enhancing the productivity of a strain used in the biotechnological processes: (i) modification of the strain itself, by the use of mutagenesis, fusion of protoplasts or DNA transformation methods or (ii) optimization of the process by finding the optimum composition of a cultivation medium and conditions. At present, in the cultures of higher fungi the latter method is predominantly used. However, there are described and patented several methods for genome manipulations in higher fungi e.g. *Flammulina velutipes* (Cho *et al*. 2006), *Pleurotus nebrodensis* (Inzenga) Quél. (Lin *et al*. 2008), *Pleurotus ostreatus* (Irie *et al*. 2001), *Lentinula edodes* (Terashima *et al*. 2002, Terashima *et al*. 2006, Kwan *et al*. 2012, Au *et al*. 2013, Tang *et al*. 2013) and others (Zhang *et al*. 2002, Romaine 2011). Particularly intensive studies concern edible mushrooms. *Agaricus bisporus* is one of the most intensively studied species. Despite more than 60 years of scientific investigation, advances in the genetic enhancement of this mushroom species has been impeded by its difficult genetics (Summerbell *et al*. 1989, Van Griensven 1991, Romaine 2011). Modifications of the genetic characteristics of homobasidiomycetes such as *Agaricus bisporus via* treatment with donor DNA, fusions using protoplasts and *via* matings between strains are patented (Huizing *et al*. 1995, Mikosh *et al*. 2001). These methods may be used in order to improve commercial characteristics of edible mushrooms and to commercially produce enzymes and metabolites in modified strains The use of transgenic basidiomycetes as a recombinant expression system for the production of a mucosal vaccine was also described (Florack & Rouwendal 2007). The first description of long-distance movement of a fully functional protein in a mushroom was given by Woolston *et al*. (2011). In 2006 Agarigen Inc. was founded, a Penn State spin-off company dedicated to harnessing transgenic *A. bisporus* for the biosynthesis of commercialized proteins. The correct selection of medium composition (carbon, nitrogen, phosphorus and microelement sources and concentrations, growth promoters, precursors for biosynthesis, other special supplements) and parameters of mushroom cultivation (duration of the process, temperature, pH, agitation, air supply) is crucial for the optimal mycelial growth and metabolite production. The optimization is essential for the development of an industrial-scale process. It should be taken into account that the physical and chemical factors are interconnected and affect the efficacy of the process. One-variable at a-time method for optimizing the culture medium and physical culture parameters involves changing one independent parameter (physical or chemical) while keeping the others constant. This method allows to determine the optimal

parameter (e.g. carbon source) but does not provide information on interactions and correlations between parameters. This may be reached by statistical optimization techniques that permit simultaneous optimization of many factors, thereby obtaining much quantitative information by only a few experimental trials. For example response surface methodology (RSM) enables the evaluation of the effects of many factors and their ractions to response variables. There are numerous reports on the use of this method in the optimization of the culture medium for simultaneous optimal strain growth and biosynthesis of secondary metabolite or exopolysaccharide (Feng *et al*. 2010, Luo *et al*. 2009). Similar experiments were also successfully conducted in our Department (Malinowska et al. 2009b). Our experience has shown, however, that the statistical methods for planning the experiment are not always effective in practice. We observed that optimal compositions of the substrate calculated by two different methods: based on central composite rotatable designs (CCRD) and using neural network were significantly different, moreover, none of the calculated maxima was confirmed experimentally.

Perspectives

The significant part of biotechnological processes described in this work is still at the stage of preliminary experiments. However, a large number of processes using cultures of higher fungi for biosynthesis of biologically active preparations and nutrients or for degradation of environmental pollutants have been patented. Presently, only a few companies use submerged cultures for the production of the commercially available products. Practical application of the biotechnological processes using mycelial cultures depends not only on their unique production potential, but also on development of industrial technologies for large-scale cultivation of fungal cultures and downstream processing which will ensure commercial success.

The fact is that biotechnology, as an applied science, needs for its development knowledge in many fields. Elucidation of the physiological and biochemical mechanisms regulating biosynthesis and secretion of biologically active substances will enable scientiststo design and to optimize new biotechnological processes. Gaining knowledge concerning molecular biology of fungi will help to use genetic engineering methods e.g. recombinant DNA techniques in higher fungi. The production potential and adaptability of fungal cultures is enormous. Search for new, previously undescribed fungal metabolites gives a chance to discover a number of highly interesting substances with potential use in medicine.

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Streszczenie

Od początku istnienia biotechnologii, jako mniej lub bardziej formalnie uznawanej dziedziny nauki, dużym zainteresowaniem badaczy cieszyły się grzyby, jako organizmy stosowane w biosyntezie i biotransformacjach różnego rodzaju substancji. Początkowo zainteresowanie dotyczyło jednak głównie gatunków grzybów zaliczanych nieformalnie do tzw. grzybów niższych. W biotechnologii farmaceutycznej przedstawiciele tej grupy, przykładowo, rodzajów *Penicillium*, *Cephalosporium*, *Aspergillus* lub *Fusidium,* są od dawna stosowani w produkcji antybiotyków, witamin, enzymów lub kwasów organicznych (cytrynowego, itakonowego, fusarowego, glukonowego). Drożdże z kolei są stosowane w technikach rekombinowanego DNA (produkcja insuliny) jako biorca transformowanego DNA. W przypadku tzw. grzybów wyższych, niejednorodnej pod względem systematycznym grupy grzybów, które tworzą owocniki, przez długi czas jedynym stosowanym procesem biotechnologicznym (obejmującym namnożenie inokulum, przygotowanie i sterylizację podłoża, inokulację, oraz hodowlę szczepu w określonych warunkach) była intensywna uprawa w podłożach stałych, dotycząca gatunków grzybów jadalnych.

Za pierwsze próby stosowania grzybów wyższych w innego typu procesach biotechnologicznych można uznać opublikowane w 1966 przez Gregory'ego wyniki poszukiwań substancji o działaniu przeciwnowotworowym w pohodowlanych pożywkach płynnych, stosowanych do fermentacji wgłębnej różnych gatunków grzybów z klasy *Basidiomycetes*. Współcześnie coraz większe zainteresowanie biotechnologów budzi prowadzona w podłożach płynnych, w bioreaktorach o różnej konstrukcji, hodowla wgłębna mycelium wielu gatunków grzybów wyższych, należących głównie do *Basidiomycetes*. Celem opracowania (optymalizacji) tego typu procesów jest:

- izolacja z mycelium lub podłoża pohodowlanego substancji farmakologicznie czynnych (leków, witamin) biosyntezowanych przez grzyba;
- uzyskanie biomasy o wysokiej zawartości substancji odżywczych, do wykorzystania jako żywności funkcjonalnej i do produkcji suplementów diety;
- uzyskanie biomasy o wysokiej zawartości substancji biologicznie czynnych (głównie antyoksydantów), do wykorzystania w kosmetologii;
- izolacja z hodowli biosyntezowanych przez grzyby enzymów (głównie oksydoreduktaz), stosowanych następnie w procesach biotransformacji lub bioremediacji;
- wykorzystanie hodowanego mycelium w procesach bioremediacji (tzw. mykoremediacja).

Najlepiej opracowane i najłatwiejsze do przeprowadzenia są procesy biotechnologiczne z wykorzystaniem wielu gatunków grzybów saprofitycznych, najczęściej tzw. grzybów białej zgnilizny. Wiele spośród nich należy do nieformalnej grupy grzybów leczniczych. Hodowle wgłębne grzybów mykoryzowych przedstawiają nieco więcej problemów przy optymalizacji podłoża hodowlanego, niemniej również są prowadzone.

Powodów zainteresowania biotechnologicznymi metodami hodowli grzybów jest kilka:

- ogromną zaletą jest krótki czas hodowli czystych kultur mycelialnych w fermentorach, zarówno na podłożach płynnych, jak i stałych. W porównaniu z czasem hodowli owocników grzybów daje to znaczne skrócenie czasu uzyskiwania porównywalnej biomasy;
- hodowle mycelialne w bioreaktorach mogą być prowadzone w wysoce powtarzalnych warunkach, co skutkuje stałym składem uzyskiwanej biomasy. Ułatwia to standaryzację np. preparatów leczniczych uzyskiwanych z grzybów;
- optymalizacja składu podłoży hodowlanych i warunków fizyko-chemicznych hodowli wpływa na regulację metabolizmu hodowanej grzybni. W efekcie pozwala to na znaczne podwyższenie wydajności biosyntezy związków biologicznie czynnych (np. metabolitów wtórnych);
- możliwa jest kontrola i zachowanie biochemicznej i genetycznej identyczności hodowanej w fermentorze grzybni.

Istnieją też poważne trudności związane ze stosowaniem nowoczesnych metod biotechnologicznych w przypadku grzybów wyższych:
- nie wszystkie gatunki grzybów wyższych mają zdolność efektywnego wzrostu w postaci kultur mycelialnych w bioreaktorze;
- w przypadku niektórych gatunków grzybów istnieją znaczące różnice w składzie chemicznym owocników grzyba i mycelium hodowanego metodami biotechnologicznymi. Nie zawsze różnice te są korzystne w przypadku stosowania hodowli mycelialnych do otrzymywania farmakologicznie czynnych związków;
- szlaki metaboliczne biosyntezy wielu biologicznie czynnych substancji przez grzyby wyższe są ciągle jeszcze – w porównaniu z roślinami, lub grzybami strzępkowymi – słabo poznane i opisane. Znacząco utrudnia to projektowanie i optymalizację warunków procesu biotechnologicznego, dobór prekursorów biosyntezy lub promotorów wzrostu szczepu;
- utrudnione jest stosowanie metod inżynierii genetycznej na skutek braku pełnej wiedzy o genach biosyntezy całego szlaku lub jego części.

Niemniej pomimo trudności, producenci substancji leczniczych pochodzenia grzybowego (Lentinan, LEM, Grifon-D, PSK, PSP), suplementów diety oraz enzymów grzybowych, wprowadzają metody biotechnologiczne do produkcji. Zgodnie ze stosowanym od dawna w biotechnologii przemysłowej (np. przez producentów antybiotyków) zwyczajem, warunki procesu rzadko są opisywane w publikacjach, a czasami nie są nawet patentowane – co ułatwia zachowanie ich w tajemnicy. W latach 90-tych XX wieku pojawiły się pierwsze informacje o możliwości stosowania metod rekombinowanego DNA dla grzybów wyższych. Współcześnie, liczne publikacje donoszą o opracowaniu metod transformacji oraz o uzyskaniu modyfikowanych genetycznie grzybów jadalnych.

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Analysis of indole derivatives in methanolic extracts from mycelium of *Agaricus bisporus* cultured *in vitro* on liquid Oddoux medium

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ABSTRACT

Methanolic extracts obtained from biomass of *Agaricus bisporus* (J.E. Lange) Imbach cultured *in vitro* were analyzed for qualitative and quantitative composition of non-hallucinogenic indole compounds in order to compare their amount with fruiting bodies of these species. Extracts demonstrated to contain six indole compounds. Contents of individual compounds ranged from 0.01 to 21.33 mg/100 g d.w. in biomass from *in vitro* cultures. The quantitatively dominating compounds included: 5-hydroxytryptophan (12.50 mg/100 g d.w.), L-tryptophan (14.00 mg/100 g d.w.) and serotonin (7.00 mg/100 g d.w.). Total content of the remaining indole compounds under analysis in the study was 55.32 mg/100 g d.w.

KEY WORDS: *Agaricus bisporus*, *in vitro* culture, L-tryptophan, serotonin

Introduction

The project consisted of experiments utilizing edible mushroom: *Agaricus bisporus* – White bottom mushroom (Basidiomycota), mainly because this species is widely used for commercial purposes in Poland and Europe, and among all mushroom species, it is the most frequently consumed mushroom in Polish and European society due to its taste and nutritional qualities. In addition, choice of mushroom was influenced by practical aspects – a possibility for mass production. Currently, fruiting bodies of *A. bisporus* are irradiated by UV light during process of production to increase vitamin D content (Roberts 2008, Koyalamudi *et al*. 2008). Fruiting body of *A. bisporus* also contains ergothioneine compound. This substance plays an important antioxidative and anti-mutagenic role, as well as chemo- and radioprotective (Ey *et al*. 2007). *A. bisporus* is also a highly valued source of laccase, vitamins (especially riboflavin, vitamin D3) and bioelements such as selenium, magnesium, copper, iron, calcium, zinc and potassium (Baross *et al*. 2008, Roberts 2008, Reczyński *et al.* 2013).

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The group of indole compounds that are not yet fully researched belongs to non-hallucinogenic indole type. Taking into consideration the significance of such indole derivatives as L-tryptophan, 5-hydroxytryptophan, 5-methyltryptamine, serotonin, melatonin, tryptamine – which are known as neurotransmitters or their precursors, it makes sense to examine the presence of them in edible mushrooms (Muszyńska *et al*. 2007, 2009, 2011 a, b, c, 2012a, b). A notable aspect regarding mycelium of higher mushrooms is its ability to accumulate easily absorbed substances but there is a lack of information in respects to types and degree of accumulation of such compounds introduced to culture media. Due to this, these mushrooms can be used for a research of indolic compounds accumulated in the biomass from *in vitro* cultures. The difficulty in obtaining research material (due to temporary and unpredicted occurrence of fruiting bodies from natural sites) were the reason to use biomass from *in vitro* cultures for further experiments (Muszyńska *et al.* 2012a, b). Morever, in enclosed laboratory conditions, it is easier to control accumulation of chosen metabolites.

Material and methods

Fungal material

The studies were conducted with young fruiting bodies of *Agaricus bisporus* (White button mushroom) from commercial origin. After taxonomic identification based on Knudsen and Vesterholt (2008) (representative samples of mushrooms were deposited in the Department of Pharmaceutical Botany, Jagiellonian University Collegium Medicum, Kraków, Poland), some of young sporocarps were used to derive *in vitro* cultures, from which obtained mycelium formed material for further analysis.

In vitro culture

The pieces of fruiting bodies were defatted with 70% ethanol for 15 s then sterilized in 15% hypochlorite solution for 5 min (manufactured by Unilever, Hungary). After several rinses with sterile redistilled water, mycelium fragments were transferred to Petri dishes containing agar-solidified medium with composition according to Oddoux (1957).

Experimental *in vitro* culture

After growing on solid medium, the pieces of mycelium were placed into an Erlenmeyer flask (500 mL) containing 250 mL of liquid modified Oddoux medium at initial biomass of 0.1 g. The cultures were incubated at the temperature 25 ± 2° C under 16 h light (900 lx/8 dark) and shaken at 140 rpm (shaker ALTEL, Łódź). The agitated liquid cultures of *A. bisporus* were maintained for two weeks and were subcultured afterwards. The obtained biomass was separated from the liquid medium using Büchner funnel with a filter paper, rinsed with redistilled water and immediately dried by lyophilization (lyophilizer Freezone 4.5, Labconco; temperature: - 40° C).

Dry, lyophilized materials (5 g of each species) were placed in a glass percolator and extracted with petroleum ether under dark conditions to remove oil fractions. Oil fractions were discarded and the remaining biomass was dried and

extracted again with methanol in a percolator for 24 h. The extracts were concentrated by distillation in a vacuum evaporator under reduced (200 mBa) pressure at 40° C. To remove the remaining lipids, concentrated extracts were frozen, while polysaccharides were removed using Chihara method. The residues were quantitatively dissolved in methanol, filtered through Whatman No. 3 paper and purified by TLC. For the purification of the extracts, we used TLC aluminium-backed silica gel 60 plates (Merck, Art. No 1.055540001), onto which the methanol extracts were loaded. Chromatograms were developed in mobile phase found to be optimal for separation of indole compounds: n-propanol/ethyl acetate/water (7:1:2 v/v/v). Spots containing indole compounds were identified under a UV lamp at $\lambda = 280$ nm. TLC chromatogram of extract from mycelium of *A. bisporus* is presentend in Fig. 1. The obtained fractions were extracted from chromatograms with methanol, filtered through syringe driven filter unit (Millex, Milipore Corporation, USA) than concentrated by distillation in a vacuum evaporator under reduced pressure at 40° C. The extracts, quantitatively dissolved in 1.5 mL of methanol, were subjected to HPLC analysis.

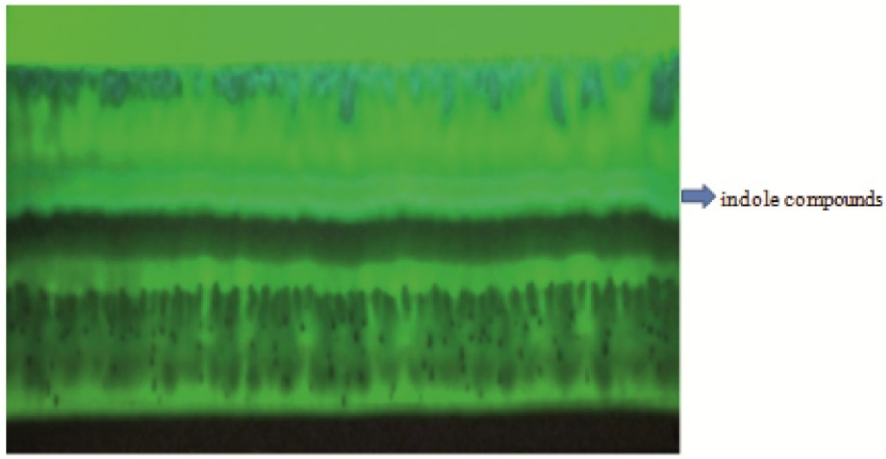

Figure 1. TLC chromatogram of extract from mycelium of *Agaricus bisporus* identified under a UV lamp at $\lambda = 280$ nm.

High performance liquid chromatography analysis (HPLC)

The obtained extracts were analyzed for contents of L-tryptophan, 5-hydroxytryptophan, 5-methyltryptamine, serotonin, melatonin, tryptamine, kynurenine sulfate, indoleacetic acid, indoleacetonitrile, indole and indoleacetamide. The analysis was performed according to the procedure by Kysilka and Wurst (1985) with our modifications (Muszyńska *et al*. 2009). HPLC analyses were performed with Hitachi apparatus equipped with L-7100 pump, reversed phase column: Purospher® RP-18 (4 x 200 mm, 5 µm) at 25° C. The solvent system used for analysis was composed of:

methanol/water/ammonium acetate (15:14:1 v/v/v) at flow rate of 1 ml/min. Detection was carried out with a UV detector, using λ=280 nm. The identification of indole compounds was made by comparing the retention times of sample peaks with those of the standards. The presence of tested metabolites in the sample was evident as an increase in peak height for the appropriate retention time. Quantitative analysis was carried out using the calibration curve method. The results are expressed in mg/100g of dry weight, calculated by internal normalization of the chromatographic peak area. A representative chromatogram is presented in Figure 2.

For each mushroom, three samples were used for the determination of the quality attribute and all the analyses were carried out in triplicate. The results were expressed as the mean values and standard deviation (SD). The experimental data were submitted for analysis of variance for completely random design to determine the least significant difference at the level of 0.05.

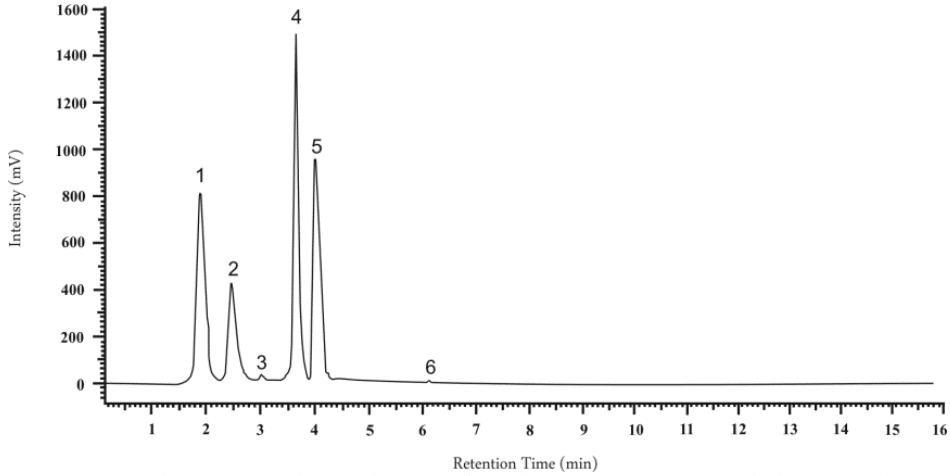

Figure 2. HPLC chromatogram of extract from mycelium of *Agaricus bisporus*: (1) -5-hydroxytryptophan, (2) - serotonin, (3) - tryptamine, (4) -5-methyltryptamine, (5) - L-tryptophan, (6) - melatonin.

Results

After several attempts to establish an optimal sterilization method, we were successful in the initiation of *A. bisporus* mycelia *in vitro* culture from hymenial part of fresh, young fruiting bodies. The best biomass growth was obtained during 3-week growth cycles in shaking liquid cultures using modified Oddoux medium. The biomass growth in the initiated cultures averaged at 8.1 g d.w./L of medium. Maximum mycelium biomass growth of *A. bisporus* was observed at initial medium pH of 6.2 and at temperature of 25º C. *In vitro* cultures maintained under laboratory conditions and optimized for maximum growth, can provide a uniform mycelium which may be a reproducible and efficient source of metabolites. The obtained biomass increments and dynamics of mycelium growth did not differ from the results that we obtained for *Boletus badius* (Fr.) Kuhn. ex Gilb, *Cantharellus cibarius* Fr. and *Tricholoma equestre* (L.) Kumm. and for *Calocera viscosa* (Pers.) Fr. cultures studied earlier (Muszyńska *et al*.

2009, 2011c, 2012b). The HPLC procedure applied to determine qualitative and quantitative content of non-hallucynogenic indole compounds offered an optimum conditions for most effective separation of the analyzed metabolites. Methanolic extracts obtained from biomass of *A. bisporus* cultured *in vitro* were analyzed for qualitative and quantitative composition of non-hallucinogenic indole compounds and their amount was compared with ones obtained from fruiting bodies of these species. The extracts were shown to contain six indole compounds: L-tryptophan, 5-hydroxytryptophan, serotonin, melatonin, tryptamine and 5-methyltryptamine. Contents of individual compounds varied ranging from 0.01 to 21.33 mg/100 g d.w. in biomass from *in vitro* cultures. The quantitatively dominating compounds included: 5-methyltryptamine (21.33 mg/100 g d.w.), L-tryptophan (14.00 mg/100 g d.w.), 5-hydroxytryptophan (12.50 mg/100 g d.w.) and serotonin (7.00 mg/100 g d.w.). The total content of the remaining indole compounds was 55.32 mg/100 g d.w. The contents of the remaining indoles: melatonin and tryptamine in mycelium from *in vitro* cultures were low, below 1.00 mg/100 g d.w. The contents of indole compounds in the methanolic extracts of mycelium of *A. bisporus* and in fruiting bodies are presented in Table 1.

Table 1. Contents of indole compounds under study (mg/100 g d. w.) in extracts from mycelium and fruiting bodies of *Agaricus bisporus*. Data are presented as the mean ± SE of 3 series.

Indole compounds	*Agaricus bisporus* mycelium from cultures *in vitro* (mg/100 g d.w.)	*Agaricus bisporus* fruiting bodies (mg/100 g d.w.) (Muszyńska *et al.* 2011 a)
L-tryptophan	14.00 +/- 0.300	0.39 +/- 0.003
5-Hydroxytryptophan	12.50 +/- 0.671	a
Serotonin	7.00 +/- 0.070	5.21 +/- 0.055
Melatonin	0.01 +/- 0.006	0.11 +/- 0.006
Tryptamine	0.48+/- 0.050	0.02 +/- 0.002
Indole-3-acetic acid	a	0.19 +/- 0.017
5-Methyltryptamine	21.33 +/- 0.755	a

a - Content lower than 0.001 mg/100 g d. w.

Discussion

The fruiting bodies of *A. bisporus* indicated presence of five indolic compounds: melatonin, tryptamine, L-tryptophan, serotonin, indolo-3-acetic acid (contents from 0.06 to 5.21 mg/100 g d.w.). Serotonin was quantitatively dominant compounds in extracts from fruiting bodies of this species (5.21 mg/100 g d.w.) (Muszyńska *et al.* 2011a). However, the mycelium from *in vitro* cultures showed a greater content of these indolic compounds. Serotonin contents were of the same order of magnitude but were slightly greater in the extracts from *in vitro* culture (7.00 mg/100 g d.w.). On the other hand, L-tryptophan contents were almost 30 times greater in the material from *in vitro* cultures compared with the fruiting bodies (14.00 and 0.39 mg/100 g d.w.,

respectively). In addition, extracts from *in vitro* cultures were characterized by the presence of 5-hydroxytryptophan and 5-methyltryptamine but the absence of indole-3-acetic acid evidenced in the fruiting bodies. To the best of our knowledge, this is the first time to identify and quantify indole compounds from *in vitro* culture of *A. bisporus*, the most popular edible mushroom. The mycelial culture seems to be a valid model for investigation of indole compounds accumulation and to study their metabolism in mushrooms. High content of serotonin and its precursors L-tryptophan and 5-hydroxytryptophan in the fruiting bodies and in the mycelium cultured *in vitro* of *A. bisporus,* demonstrate also a potential for the use of this material as a source of this physiologically important compound for humans. Serotonin is a long known compound playing the role of a regulator of sleep, body temperature, mood, maturation and regeneration and an inhibitor of cell aging, thereby contributing to general strengthening of the immune system and is used also as an antidepressant. Further optimization of conditions for *in vitro* cultures may allowan alternative method for commercial cultivation of this species. This is desirable since it may be expected that mycelium cultured *in vitro* may also be a source of other important metabolites, possessing both culinary and medicinal values, characteristic of fruiting bodies.

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Streszczenie

Lecznicze i przeciwutleniające właściwości grzybów są doskonałym połączeniem, które stanowi o ich wartości dietetycznej i umożliwia korzystanie z nich zarówno, jako żywności jak i dodatku żywieniowego. Celem niniejszej pracy była analiza zawartości fizjologicznie aktywnych związków indolowych w mycelium z kultur *in vitro Agaricus bisporus* (pieczarka dwuzarodnikowa). L-tryptofan, egzogenny aminokwas i jego pochodne, takie jak np. 5-hydroksytryptofan, muszą być dostarczane z pokarmem w codziennej diecie. Związki te mają działanie przeciwdepresyjne, są bezpośrednimi prekursorami serotoniny, a w przeciwieństwie do niej przekraczają barierę krew – mózg. Są też biogenetycznymi prekursorami innych związków indolowych, które pełnią funkcję neuroprzekaźników, co uzasadnia oznaczanie ich zawartości w grzybach jadalnych. Materiał do badań stanowiły owocniki *A. bisporus* pochodzenia komercyjnego. Z owocników *A. bisporus* wyprowadzono kultury *in vitro* na podłożu stałym Oddoux (1957). Eksperymentalne kultury *in vitro* prowadzono na płynnym, wytrząsanym podłożu Oddoux. Co dwa tygodnie prowadzenia kultur pasażowano je na świeżą pożywkę. Biomasę mrożono i suszono metodą liofilizacji. Otrzymaną biomasę z kultur *in vitro* analizowano jakościowo i ilościowo metodą HPLC na obecność niehalucynogennych związków indolowych.

Po raz pierwszy zidentyfikowane i ilościowo oznaczone zostały związki indolowe w kulturach *in vitro Agaricus bisporus* na płynnym podłożu wg Oddoux. Analiza wykazała, że ekstrakty metanolowe otrzymane z grzybni zawierają sześć związków indolowych: L -tryptofan, 5 - hydroksytryptofan, serotoninę, melatoninę, tryptaminę i 5-metylotryptamię. Zawartości poszczególnych składników w biomasie z kultur *in vitro* były zróżnicowane w zakresie od 0,01 do 21,33 mg/100 g s. m. Dominującymi ilościowo związkami były: 5-hydroksytryptofan (12,50 mg/100 g s. m.), L-tryptofan (14,00 mg/100 g) i serotonina (7,00 mg/100 g). Całkowita zawartość związków indolowych w badanym materiale wynosiła 55,32 mg/100 g s. m. Biomasa z kultur *in vitro* badanego gatunku jest dobrym źródłem 5-hydroksytryptofanu i L- tryptofanu. Kultury *in vitro A. bisporus* mogą być wykorzystane jako model do badań nad akumulacją i metabolizmem związków indolowych.

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Biologically active compounds from selected aphyllophorales mycelial cultures

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ABSTRACT

For a long time fungi belonging to *Basidiomycota* phylum have been in the center of attention because of the presence in their fruiting bodies of compounds with known therapeutic activity. Mycelial cultures of two aphyllophorales species occurring in Poland, *Hydnum repandum* L., and *Sparassis crispa* (Wulf.) Fr., were analyzed in our study. The main aim of the study was qualitative and quantitative analysis of extracts obtained from the mycelial cultures for the presence of known biologically active compounds, including phenolic acids, non-hallucinogenic indole compounds and sterols.

For analyses a reversed-phase chromatography (RP-HPLC) method was used. The presence of eight phenolic acids including gallic, gentisic, *p*-hydroxybenzoic, caffeic, *p*-coumaric protocatechuic, syringic, vanillic and cinnamic acids was confirmed in the extracts obtained from the biomass. The quantitatively predominant metabolites in biomass from *in vitro* cultures of *H. repandum* and *S. crispa* were protocatechuic acid (6.23 μg/g DW) and p-hydroxybenzoic acid (4.52 μg/g DW). Derivatives of indole such as indole, serotonin, tryptamine and tryptophan were measured quantitatively. Their total content was estimated as 1.28 μg/g DW and 3.07 μg/g DW in *H. repandum* and *S. crispa* extracts, respectively. The major metabolite found was tryptophan. In addition, ergosterol, one of the sterols present in the biomass of *in vitro* cultures of *S. crispa* was analyzed (700.87 μg/g DW).

The obtained results confirm the hypothesis that mycelial cultures of domestic species of aphyllophorales are able to accumulate biologically active metabolites.

KEY WORDS: Basidiomycota, indole compounds, phenolic acids, sterols

Introduction

Representatives of *Basidiomycota* are an important source of active compounds of the multidirectional therapeutic activity; inter alia, antiviral, bacterio- and

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fungistatic, antiparasitic, as well as antitumor, anti-inflammatory, vessel protective or hypoglycemic (Zjawiony 2004, Sułkowska-Ziaja *et al.* 2005). More and more research has been undertaken to identify the chemical composition of not only fruiting bodies, but also the mycelium extracted from *in vitro* cultures. Studies in this area have demonstrated the existence of qualitative and quantitative differences in the production of certain groups of chemical compounds between fruiting bodies and mycelium from *in vitro* cultures. This is important for both cognitive and application reasons.

The present study involved two wild edible mushrooms commonly growing in Polish forests: *Hydnum repandum* L. (Hydnaceae) and *Sparassis crispa* (Wulf.) Fr. (Sparassidaceae). These species belong to an artificial systematic group known as *Aphyllophorales*.

The aim of the study was to determine the levels of biologically-active compounds in extracts from the biomass of *in vitro* cultures. It is a continuation of an extensive analysis of the chemical composition of biomass from mycelial cultures to confirm their usefulness as potential sources of compounds with biological properties. As far as we know, our study is the first one where the above-mentioned groups of compounds in the biomass from *in vitro* cultures of the examined fungi species have been described.

Material and methods

Fungal material

Samples of mushrooms were collected in 2012 in mixed forests of northern Poland. Taxonomic identification was conducted according to Gumińska and Wojewoda (1988). Representative voucher samples were deposited in the Department of Pharmaceutical Botany UJCM, Kraków, Poland. Mycelial cultures were derived from explants originating from the hymenial part of fruiting bodies. Fruiting body fragments were sterilized, placed on Petri dishes with modified Oddoux medium (Oddoux 1957), incubated at a temperature of ±22°C and subcultured every three weeks. Experimental *in vitro* cultures were maintained in Erlenmayer flasks, containing 250 mL of medium and shaken at a rate of 140 rpm. The cultures were maintained for three weeks. Then, the biomass was separated from the medium, frozen and lyophilized.

Determination of phenolic acids

The amount of 5 g of powdered material was hydrolysed with 2 M hydrochloric acid for 2 h at 100° C. Hydrolysates were extracted with 50 mL of ethyl acetate and concentrated to dryness. HPLC analyses were conducted using an HPLC VWR Hitachi-Merck apparatus: L-2200 autosampler, L-2130 pump, LiChrospher RP-18e column (250mm×4mm, 5µm) at 25° C, L-2350 column oven, and L-2455 diode array detector at UV range 200-400nm. The mobile phase consisted of solvent A: methanol/0.5% acetic acid 1:4 (v/v), and solvent B: methanol. The gradient was as follows: 100:0 for 0–25 min; 70:30 for 35 min; 50:50 for 45 min; 0:100 for 50-55 min; 100:0 for 57–67 min. (Ellnain-Wojtaszek & Zgórka 1999). Phenolic acid standards were purchased from Fluka (Chemie AG) and Sigma (St. Louis, USA).

Determination of indole derivatives

Another 5 g of powdered material was extracted with 100 mL of methanol for 2 hand mixed extracts, which were then concentrated to dryness. The HPLC study was performed according to the procedure described by Muszyńska (Muszyńska *et al.* 2009). Briefly, the conditions were as follows: Hitachi HPLC apparatus; L-7100 pump; Purospher RP-18 column (250 mm × 4 mm, 5 μm); solvent system – methanol : water : ammonium acetate 15:14:1 (v/v/v); flow rate - 1ml/min; and UV detector (λ = 280nm). Indole standards were purchased from Sigma (St. Louis, USA).

Determination of sterols

The third 5 g portion of powdered material was mixed with 100 mL of a 75:25 (v/v) mixture of methanol and dichloromethane, followed by sonification for 10 min and centrifugation at 10 000 rpm for 5 min. Combined extracts were concentrated to dryness. The HPLC method was performed according to the procedure developed by Yuan (Yuan *et al.* 2008). The mobile phase consisted of solvent A: methanol : water 20:80 (v/v), and solvent B: methanol : dichloromethane 75:25 (v/v). A gradient procedure was used as follows: starting at sample injection, 60% of B for 5 min; a linear gradient from 60 to 100% of B for 10 min; and 100% of B for 10 min. The flow rate was 1.0 mL/min. Sterol standards, ergosterol, and ergocalciferol were purchased from Fluka (Chemie AG).

Results

The results of the analyses are shown in Table 1. Amongst the fifteen studied phenolic acids, five were detected in the extracts from the biomass from an *in vitro* culture of *H. repandum*; additionally, cinnamic acid was identified. In turn, seven phenolic acids were found in similar extracts of *S. crispa*. The total amounts of phenolic acids and cinnamic acid were 14.44 μg/g and 12.65 μg/g DW in biomass from *H. repandum* and *S. crispa*, respectively (Tab. 1). A sample chromatogram shows the separation of phenolic acids in the extract from of *H. repandum* biomass (Fig. 1).

Among the 12 indole compounds analyzed, three were detected in the extracts from *H. repandum* as well as *S. crispa*; i.e. indole, tryptamine and L-tryptophan. In addition, trace amounts of serotonin were detected in both extracts. The total amounts of indole compounds were similar (0.98 μg/g and 1.19 μg/g DW in *H. repandum* and *S. crispa* extracts, respectively) (Tab. 1). Ergosterol was the only representative of the analysed compounds with a sterol structure detected at level 700.87 μg/g DW, in *S. crispa* extracts.

Discussion

Earlier studies of the chemical composition of the fruiting bodies of the investigated species showed very similar qualitative compositions for all investigated metabolites; however, in the biomass of the *in vitro* cultures, the level

of determined compounds was slightly lower (Sułkowska-Ziaja *et al.* 2014). Phenolic acids determined in the current studies are characterized by a wide spectrum of biological activity. The strongest antioxidant activity is exhibited by vanillic acid and in a cinnamic acid derivative – caffeic acid. Gallic, *p*-hydroxybenzoic and protocatechuic acids detected in *H. repandum* and also *S. crispa* biomass are characterized by documented, multidirectional activity: antioxidant, antibacterial, antiviral, anti-inflammatory or antifungal. Furthermore, the protocatechuic acid found in the largest amounts in the conducted studies is characterized by immunomodulatory, spasmolytic, cardioprotective and antithrombotic activity (Ferreira *et al.* 2009). A previously conducted chemical analysis of fruiting bodies also showed the quantitative predominance of this metabolite.

Table 1. Contents of biologically active compounds in mycelial cultures (μg/g DW).

Metabolites	*Hydnum repandum*	*Sparassis crispa*
Indole compounds		
Indole	0.018±0.05	0.07±0.02
Serotonin	*	*
Tryptamine	0.02±1.05	0.05±0.11
L-Tryptophan	0.94±0.09	1.07±0.17
Phenolic acids		
Gallic acid	4.16±0.12	2.27±0.34
Gentisic acid	2.03±0.11	0.10±0.01
p-Hydroxybenzoic acid	nd	4.52±0.4
Caffeic acid	nd	0.10±0.16
o-Coumaric acid	nd	1.45±0.21
Protocatechuic acid	6.23±0.02	3.92±0.3
Syringic acid	0.41±0.21	0.29±0.02
Vanillic acid	0.60±0.56	nd
Cinnamic acid	1.01±0.01	nd
Sterols		
Ergosterol	nd	700.87±0.3
Ergocalciferol	nd	nd

Values are means of three experiments ± SD, nd - not detected, *- traces.

Indole-derived non-hallucinogenic compounds are also an interesting group of secondary metabolites found in fungi. Estimating the content of this group of metabolites in the investigated species seems to be important due to their biological activity as well as from a toxicological viewpoint (Muszyńska *et al.* 2011a, b). Within the scope of the study, the determined compounds have important biological functions as natural neurotransmitters and neuromodulators (serotonin, tryptamine), and are involved in the regulation of circadian rhythms, body temperature, mood, blood pressure, and appetite (5-hydroxy-tryptophan,

serotonin), as well as blood clotting (serotonin, melatonin). L-tryptophan and 5-hydroxytryptophan exhibit a hypnotic, antidepressant activity. In turn, melatonin and hydroxyindole derivatives have antioxidant potential.

The data concerning the appearance of non-hallucinogenic indole compounds in the representatives of Basidiomycota mainly refer to tryptophan, a biogenetic progenitor of all other indole compounds. Tryptophan is one of the diet supplements used as antidepressants. It can cross the brain-blood barrier and in the central nervous system can be converted to serotonin and melatonin, important neurotransmitters and neuromodulators controlling the circardian rhythm. A rich source of these metabolites are edible mushrooms e.g. *Suillus bovinus* where tryptophan content is 25.9 mg/100g d.w.

The last study demonstrates the occurence of a biogenic amine, tryptamine in many basidiomycetes. Tryptamine, synthesized from tryptophan is a direct precursor of different active compounds, including serotonin. Considerable quantities of tryptamine were detected in *Suillus luteus* (34/100d d.w.) and *Leccinum rufum* (31.71 mg/100g d.w.) (Muszyńska *et al.* 2011a, b). It has multi directional pharmacological activity i.a. it takes part in body temperature regulation, moodiness, organism maturation but also tissue regeneration and cell senescence.

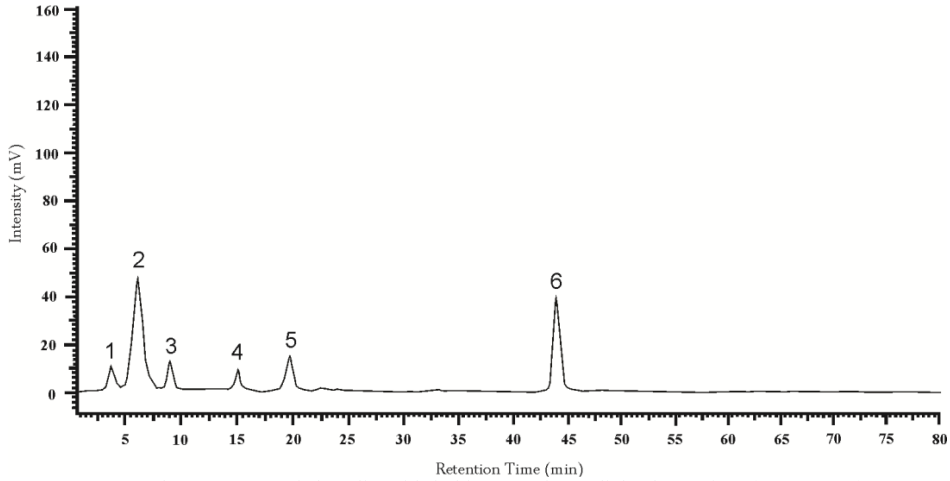

Figure 1. HPLC chromatogram of phenolic acids in biomass of mycelial culture of *Hydnum repandum* (1-gallic acid, 2-protocatechuic acid, 3-gentisic acid, 4-vanillic acid, 5-syryngic acid, 6-cinnamic acid).

One of the main components of fungal cell membranes – ergosterol – is converted into vitamin D in the presence of sunlight or another ultraviolet light source (Mattila *et al.* 2002). This vitamin plays an essential role in the prevention of cancer, through increasing tumor cells phagocytosis and facilitating other immunomodulatory functions. According to the latest studies, vitamin D also blocks the angiogenesis in tumors and impairs their growth progress (Kopczyński 2012, Mraz *et al.* 2010). The highest levels of ergosterol have

been noted in saprotrophic fungi and may constitute up to 83–89% of the entire amount of sterols. In addition, it has promising anti-allergic and immunostimulatory properties. Therefore, the search for new sources of this compound is becoming more and more popular (Yuan *et al.* 2006). Our research has shown that *S. crispa* is one of the fungi with significant ergosterol levels in mycelial cultures. The data presented in this paper confirm the significant potential of chemical components with recognized antioxidant activity. The species can be considered an alternative source of phenolic acids and ergosterol.

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Streszczenie

Grzyby z gromady *Basidiomycota* od dawna budzą zainteresowanie ze względu na występowanie w ich owocnikach szeregu związków o uznanych właściwościach leczniczych. Obiektem przeprowadzonych badań były kultury mycelialne dwóch gatunków grzybów afylloforoidalnych występujących na terenie Polski *Hydnum repandum* L. oraz *Sparassis crispa* (Wulf.) Fr. Celem podjętych badań była jakościowa i ilościowa analiza ekstraktów otrzymanych z kultur mycelialnych pod kątem

występowania związków o udokumentowanej aktywności biologicznej: kwasów fenolowych, niehalucynogennych związków indolowych oraz steroli.

Do oznaczeń wykorzystano wysokosprawną chromatografię cieczową faz odwróconych (RP-HPLC). Na podstawie analizy stwierdzono w ekstraktach z otrzymanej biomasy obecność ośmiu kwasów fenolowych: galusowego, gentyzynowego, *p*-hydroksybenzoesowego, kawowego, kumarowego, protokatechowego, syryngowego, wanilinowego oraz kwasu cynamonowego. Ilościowo dominującym związkiem był kwas protokatechowy w ilości 6,23 µg/g s.m. (*H. repandum*) oraz kwas hydroksybenzoesowy w ilości 4,52 µg/g s.m. (*S. crispa*). Spośród związków pochodnych indolu ilościowo oznaczono: indol, serotoninę, tryptaminę i tryptofan. Całkowita ich zawartość wynosiła 1,28 µg/g s. m. (ekstrakty z *H. repandum*) oraz 3,07 µg/g s.m. (ekstrakty z *S. crispa*). Ilościowo dominującym metabolitem był tryptofan. Spośród steroli oznaczono ergosterol w biomasie z kultur *in vitro S. crispa* (700,87 µg/g s.m).

Uzyskane wyniki wskazują, że przebadane kultury mycelialne krajowych gatunków grzybów afylloforoidalnych są zdolne do akumulacji metabolitów aktywnych biologicznie.

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Mushroom flavour

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ABSTRACT

Mushrooms and fungi not only present a fascinating world of shapes, both macro- and microscopic, but they are also an interesting source of flavours, fragrances and odours, e.g. garlic, coconut, flour-like, cucumber or fruit-like, as well as the most characteristic for this kingdom of living organisms mushroom-like flavour and aroma. Fungi can possess many different and interesting flavours and fragrances – starting from nice anise-like, fruit-like, cucumber, garlic, to cheese-garlic, and ending with potato or flour-like smells. Some mushrooms emit carbide or distinctly faecal-like odour. The taste of mushrooms is frequently correlated with their aroma. What components does the core of a mushroom flavour consist of? Chemical analysis of specimens reveals compounds responsible for characteristic flavour and odour. It was found that the most characteristic flavour compound is defined mainly by C_8 volatiles. Between all C_8 compounds the most important for mushroom flavour are oct-1-en-3-ol, octan-3-ol, octan-3-on and oct-1-en-3-on. Fungi and mushrooms can enable biotechnological production of some flavour components, for instance the *Nidula niveotomentosa* produces a characteristic raspberries compound – raspberry ketone in submerged cultures; the biotechnological production can also provide rare and tasty forest mushroom biomass e.g. edible boletus.

KEY WORDS: odour, fungi, aroma

Introduction

Flavours and special odours are nonconventional tool in mycology. Which of mycologists does not know a garlic like odour of *Mortierella* (Domsch *et al.* 1980) or a coconut-like fragrance of *Trichoderma* (Rifai 1969)? The same odour characterises *Hypocrea caerulescens* Jaklitsh & Voglmayr (Jaklitsh *et al.* 2012). Those who work with basidiomycete fungi as well as ordinary fans of pizza or more sophisticated dishes with mushrooms

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easily recognise the mushroom flavour. But what is the essence of the mushroom flavour? What components does it consist of? The aim of the manuscript is to show the diversity of both fungal and mushroom flavours and aromas as well as their chemical origin and importance.

Overview of aromatic fungi and compounds responsible for their flavours

Flavour industry has been developing for over 160 years. It uses the flowers and whole plants or herbal roots, spices, vegetables and fruits processing as well as essential oils for distillation (flavours and fragrances). As W. A. Poucher (1991) defines in 'The raw materials of perfumery', mushrooms may have an unpleasant odour. However, some odours can be nice and interesting and have been mentioned as a potential source of fragrances, e.g. *Cortinarius suaveolens* Bataille & Joachim has a strong orange blossom scent, which remains after drying (Poucher 1991), *Hygrophora agathosmus* – cherry laurel (Poucher 1991) or rather *Hygrophorus agathosmus* (Fr.) Fr. – an almond/marzipan odour (California Fungi 2014), *Tricholoma aurantium* (Schaeff.) Ricken – cucumber aroma (Poucher 1991); *Lacatry glyciosmos* – bergamot (probably *Lactarius glyciosmus* (Fr.) Fr.); *Lactory camphoratus* – melilot (possibly *Lactarius camphoratus* (Bull.) Fr.) or *Hygrophora hyacenthinus* – jasmine (probably *Hygrophorus hyacinthinus* Quél), *Clitocybe geotrope* – lavender (probably *Clitocybe geotropa* (Bull.) Quél), *Psatella arvensis* (probably *Pratella arvensis* (Schaeff.) Gillet = *Agaricus arvensis* Schaeff.) – aniseed. Most of the mushrooms mentioned by Poucher (1991) cannot be found in the Index Fungorum database, probably due to the errors he made in the spelling of the names. My conjectures of the proper names of those fungi might be incorrect. Although the above-mentioned fungi have not been well recognized by Poucher, it is worth pointing out that they were for the first time treated as useful sources of fragrances in cosmetic and perfumery industries.

Basidiomycetes show a broad profile of smells: from a bitter almond-like one in *Clitocybe gibba* (Pers.) P. Kumm., *C. odora* (Bull.) P. Kumm., an almond-like scent combined with a nut flavour in *Agaricus bitorquis* (Quél.) Sacc., a flour-like one in *Tricholoma equestre* (L.) P. Kumm., *Calocybe gambosa* (Fr.) Singer, *Catathelasma imperiale* (Quél.) Singer, *Entoloma sinuatum* (Bull.) P. Kumm., a carbide-like one – *Tricholoma sulphureum* (Bull.) P. Kumm., a fruit-like one in *Lepista nuda* (Bull.) Cooke, *Inocybe erubescens* A. Blytt, *Russula emetica* (Schaeff.) Pers. and *Lactarius deliciosus* (L.) Gray*, L. deterrimus* Gröger, *Fistulina hepatica* (Schaeff.) With., a cucumber combined with flour-like one in *Lyophyllum connatum* (Schumach.) Singer and in *Mycena galericulata* (Scop.) Gray, a cucumber- or herring-like one in *Macrocystidia cucumis* (Pers.) Joss., a garlic-like one in *Marasmius alliaceus* (Jacq.) Fr., *Micromphale perforans* (Hoffm.) Gray, a radish-like one in *Mycena pura* (Pers.) P. Kumm., *Volvariella speciosa* (Fr.) Singer, *V. bombycina* (Schaeff.) Singer and in *Pluteus cervinus* (Schaeff.) P. Kumm., a mushroom odour combined with an anise-like one in *Leucoagaricus leucothites* (Vittad.) Wasser and more distinct anise-like one in *Agaricus sylvicola* (Vittad.) Peck or anise odour in *Gloeophyllum odoratum* (Wulfen) Imazeki and *Trametes suaveolens* (L.) Fr., an odour of a raw potato in *Amanita citrina* Pers., a dill-like one in *Polyporus*

umbellatus (Pers.) Fr. (Snowarski 2005, 2010). Chemical analyses reveal many fungal substances which can be sources of different fragrances and flavours (Table 1). *Nidula niveotomentosa* (Henn.) Lloyd (a bird's nest fungus) is an example of a species applied in biotechnology as it is used to produce a characteristic raspberries compound – raspberry ketone [4-(4-hydroxyphenyl)butan-2-one] and betuligenol in submerged cultures (Taupp *et al*. 2008). In *Clitopilus prunulus* (Scop.) P. Kumm., *Catathelasma ventricosum* (Peck.) Singer as well as *Tricholoma virgatum* (Fr.) P. Kumm. for the cucumber odour (*E*)-non-2-enal is responsible. In Greece a high-quality mushroom *Hygrophorus russocoriaceus* Berk. & T.K. Mill. grows, whose unique cedar aroma is attributed to sesquiterpenes found only in this species of the genus *Hygrophorus*. *Lactarius helvus* (Fr.) Fr. possesses a characteristic chicory and fenugreek smell; therefore it is used as a spice mushroom, although raw it is mildly toxic. It is known as a maggi-pilz (maggi-mushroom) (Fraatz & Zorn 2010). *Ceratocystis* species have been studied because of their fruit-like aromas, *C. variospora* (R.W. Davidson) C. Moreau as a source of geraniol (Reineccius 1994). Some fungi show a significant anise odour, in *Clitocybe odora* *p*-anisaldehyde masks other flavour compounds. Anisaldehyde is also responsible for an odour in other *Clitocybe* spp., but in *Agaricus essettei* Bon and *Gyrophragmium dunalii* (Fr.) Zeller a mixture of benzaldehyde and benzyl alcohol makes a profile of its odour (Rapior *et al.* 2002, Fraatz & Zorn 2010). *Lentinellus cochleatus* (Pers.) P. Karst. as well as *C. odora* and *A. essettei* are known for their anise-like scent, but *L. cochleatus* has a different compound profile: *p*-anisaldehyde, methyl *p*-anisate, methyl(*Z*)-*p*-methoxycinnamate and methyl (*E*)-*p*-methoxycinnamate are responsible for an anise-like aroma (Rapior *et al*. 2002).

Although fungi as large-fruited mushrooms are edible for humans, they are not of vital importance for the diet. Boletus King Bolete (*Boletus edulis* Bull.) is appreciated for its special mild taste and nice mushroom scent (tab. 1), and is one of the most popular edible mushrooms in Europe. The other one is a white button mushroom (*Agaricus bisporus* (J.E. Lange) Imbach) - the most commonly cultivated and consumed species in the world (Fraatz &Zorn 2010). Another important but not so famous species is an oyster mushroom (*Pleurotus ostreatus* (Jacq.) P. Kumm.). Finally, truffles (*Tuber aestivum* (Wulfen) Spreng. – summer truffle, *T. melanosporum* Vittad. – black truffle) are famous for their special aroma and for prices they obtain on the market. Truffles differ strongly in aroma depending on the geographical origin. In order to 'reveal' their distinct mushroom aroma for which oct-1-en-3-ol, octan-3-one, octan-3-ol and oct-1-en-one are responsible, the fruiting bodies with a characteristic sulphurous odour must be left open to the air. The aroma profile of mushrooms changes not only because of the geographical origin but also due to the environmental differences, processing, storing and the age of mushrooms as well as part of fruiting body (caps or gills or hyphae) (Fraatz & Zorn 2010). Some small truffles: *Tuber beyerlei* Trappe, Bonito & Guevara, *T. castilloi* Guevara, Bonito & Trappe, *T. guevarai* Bonito & Trappe, *T. lauryi* Trappe, Bonito & Guevara, *T. mexiusanum* Guevara, Bonito & Cázares, *T. miquihuanense* Guevara, Bonito & Cázares and *T. walker* Healy, Bonito & Guevara

grown in Mexico and the USA have a garlic odour (Guevara *et al.* 2013).

Volatile compounds of *A. bisporus* were broadly studied and it was found that the most characteristic flavour compound is defined mainly by C_8 volatiles. Among all volatile compounds, the participation of C_8 volatiles can vary in the case of white button mushroom (from 44% to 98%) and in other mushrooms. It depends on the differences between the species type, conditions of production, time of growth, nitrogen and carbon sources, conditions of post-harvest processing. Among all C_8 compounds, the most important is oct-1-en-3-ol (1-octene-3-ol), but finally over 150 different volatiles have been recovered in the white button mushroom. Besides C_8 volatiles, other aromatic compounds were identified: benzyl alcohol, benzaldehyde, *p*-anisaldehyde (4-methoxybenzaldehyde), benzyl acetate, phenyl ethanol (Dijkstra 1976, Dijkstra & Wikén 1976, Fraatz & Zorn 2010).

The oct-1-en-3-ol occurs in two optically active forms, a predominant in nature (R) – (-)-oct-1-en-3-ol has a fruity mushroom-like odour identified as more intense than (S) – (+)- isomer (note of mould and grass). Additionally the oct-1-en-3-ol was found in black currants, strawberries and potatoes (Dijkstra 1976, Dijkstra & Wikén 1976, Fraatz & Zorn 2010). The other odour is due to oct-1-en-3-one and is described as an odour of boiled mushrooms. With the increase of its concentrations a metallic note appears. In the miscellaneous fungi the predominant components are: oct-1-en-3-ol, oct-1-en-3-one, oct-2-en-1-ol, octan-1-ol, octan-3-ol, octan-3-one, benzaldehyde, limonene, N(2phenyethyl)acetamide, geranyl acetone, farnesyl acetone, (*E,E*)-farnesol (Fraatz & Zorn 2010).

The oct-1-en-3-ol (1-octene-3-ol) was isolated for the first time by Freytag and Ney in 1968 (Dijkstra & Wikén 1976), its typically mushroom aroma is produced by *Aspergillus oryzae* (Ahlb.) Cohn and blue cheese fungus (*Penicillium* spp., *P. roqueforti* Thom). During ripening *P. roqueforti* produces ketones: 2-pentanone, 2-heptanoneand 2-nonanone probably of the prime importance for the typical flavour of Roquefort, Cheddar and other cheeses (Reineccius 1994). Garlic-like and cheese-like aromas with distinct note of mature Camembert were found in *Kalpuya brunnea* M. Trappe, Trappe, & Bonito, a new truffle (Morchellaceae) species described in Oregon, where it is known as an Oregon truffle and is locally collected for commercial use (Trappe *et al.* 2010). Dijkstra and Wikén (1976) found that nucleotides, amino-acids and carbohydrates also contributed significantly to the mushroom flavour of *Agaricus bisporus*, whereas a less significant influence was observed in the case of (the less contribution showed) low-boiling volatiles: benzaldehyde, benzyl alcohol, 1-octen-3-one, *n*-butyric acid and isovaleric acid. No synergistic influence of those components on the flavour was observed by Dijkstra (1976) and Dijkstra and Wikén (1976).

The intensity of flavour differs due to the loss of volatiles and some decomposition of flavours. This is important in the production of commercial mushroom concentrates as Hansen and Klingenberg (1983) showed analysing the differences between distillates of twelve mushrooms concentrates obtained from European manufactures. They recognized 1-octen-3-ol, 1-octen-3-one, L-glutamic acid, guanosine-5'-monophosphate as main

flavour components. GLC/MS analysis revealed components of mushroom flavour related to 1-octen-3-ol (1-octanol, 3-octanol), aromatic compounds (benzaldehyde, phenylethanol, phenyl acetaldehyde), terpenes (terpinene-4-ol), N-containing components (e.g. pyrazine derivatives). Some of these components were not genuine, e.g. phenylethanol, phenylacetaldehyde, terpinene-4-ol, some of them were recovered only in trace amounts. In some cases C_8 volatiles were not present, although they were described as 'similar to mushroom and yeast'. In the processing as well as cultivation of mushrooms for a natural flavour, it is important that conditions of these processes are shaped with special care, according to the principles suggested for shiitake mushrooms by Yoshii (1980).

The concentration of oct-1-en-3-ol and benzylic acid increases with the maturation of fruiting bodies of mushrooms. These compounds, and broadly C_8 volatiles, are emitted by mushrooms to attract insects to distribute their spores; in some cases oct-1-en-3-ol plays a role as an aggregation hormone for certain beetles. The phenomenon is also discussed with reference to *Fomitopsis pinicola* (Sw.) P. Karst., *Fomes fomentarius* (L.) Fr. as well as in genera *Aspergillus* and *Penicillium* (Fraatz & Zorn 2010). The concentration of oct-1-en-3-ol decreases with an increasing age of the fruiting bodies of shiitake (*Lentinula edodes*), while the amount of octan-3-one increases (Fraatz & Zorn 2010).

Growing fungi in artificial media should be preceded with careful investigation on media composition and conditions of culture (e.g. irradiation). A periodically illuminated culture of *Nidula niveotomentosa* synthesises raspberry compounds from L-phenylalanine. Typically mushroom compounds, oct-1-en-3-ol and octan-3-one, can be produced in submerged cultures by *Penicillium vulpinum* (Cooke & Massee) Seifert & Samson as lipid degradation products. *Pleurotus ostreatus* shows a varied flavour composition in a liquid medium depending on the age and culture conditions, although the predominant compounds oct-1-en-3-ol and octan-3-one were detected. *Pleurotus florida* (=*P. ostreatus* f. *florida* Cetto) obtained in laboratory cultures differed from fruiting bodies and showed a sweet anise and almond scent resulting from the synthesis of p-anisaldehyde and benzaldehyde, whereas the natural compound of *P. florida* oct-1-en-3-ol occurred only in minute amounts (Taupp *et al.* 2008, Fraatz & Zorn 2010).

Woźniak (2007) described a procedure for producing the biomass of mycelium of three varieties of King Bolete designed for consumption. She recommended the procedure for the production of mycelium for dried biomass as a supplement of dried mushrooms, although the concentration of C_8 compounds as well as oct-1-en-3-ol was significantly lower in the mycelium obtained from a submerged liquid culture than in fresh fruiting bodies. The investigations will enable the biotechnological production of mushroom compounds important as flavours and odours. These volatile compounds are commonly known and they are recognized by smell and taste receptors as typical of mushroom or fungal origin. On the other hand, fungi and mushrooms can serve as a source of other compounds and in this way give a possibility of cheaper production than isolation from a natural source.

Table 1. The most popular fungal flavour and smell compounds (according to Nyegue *et al.* 2003, and Fraatz & Zorn 2010).

Fungal species	Number of detected compounds	Predominant compound of a flavour	Additional flavour compounds
Agaricus bisporus (J.E. Lange) Imbach (white button mushroom)	>150 (also 70 or 22 depending on the method)	C_8: oct-1-en-3-ol, octan-3-ol, octan-3-one, oct-2-en-1-ol	Benzyl alcohol, benzaldehyde, *p*-anisaldehyde, benzyl acetate, phenylethanol
Boletus edulis Bull. (King Bolete)	70 or ~50 depending on the method	C_8: in canned oct-1-en-3-ol, octan-1-ol, octan-3-ol, in boiled octan-3-one; in dried oct-1-en-3-one, γ-octalacton, octa-2,4-dien-1-ol, octanal	--
Calocybe indica Purkay. & A. Chandra (milky mushroom)	--	C_8 predominant	--
Cantharellus cibarius Fr. (chantarelle)	--	α-humulene, α-copaene, β-caryophyllene	--
Hygrophorus spp. (waxy caps)	45	3-methylbutanal, hexanal, p-cymene, octan-3-one, oct-1-en-3-one, octan-3-ol, methyl benzoate,	sesquiterpenes in *H. russocoriaceus*
Lactarius helvus (Fr.) Fr. (Maggi-pilz)	38	capric acid, sotolon, 2-methylbutanoic acid	--
Lentinula edodes (Berk.) Pegler (shiitake)	130; 18 sulphur containing compounds	oct-1-en-3-ol, octan-3-ol, octan-3-one, oct-4-en-3-one, lenthionine (a cyclic sulphur-containing compound)	dimethyl disulphide, dimethyl trisulphide, 1,24-trithiolane
Marasmius alliaceus (Jacq.) Fr. (garlic mushroom)	16 and 27 depending of the method	1,3-dithietane, benzaldehyde, 2,3,5-trithiahexane, 2,3,4,6- tetrathiaheptane, dimethyl disulphide, dimethyl, dimethyl trisulphide, tetrasulphide, 2,4,5,7-tetrathiaoctane,	--
Phallus impudicus L. (common stinkhorn)	--	dimethyl disulphide, dimethyl trisulphide, linalool, (E)-ocimene, phenyl acetetylaldehyde	--
Pleurotus florida (= *P. ostreatus* f. *florida* Cetto) (paddy straw and cotton mushroom; Florida oyster mushroom)	--	C_8: oct-1-en-3-ol	--

Fungal species	Number of detected compounds	Predominant compound of a flavour	Additional flavour compounds
Pleurotus ostreatus (Jacq.) P. Kumm. (oyster mushroom)	28	C_8: oct-1-en-3-ol, octan-3-ol, octan-3-one, octanal, oct-1-en-3-one, (*E*)-oct-2-enal,octan-1-ol octan-3-one (80%), octan-3-ol (14%)	Benzaldehyde (almond odour) Benzyl alcohol (sweet-spicy)2-phenylethanol (rose scent)
Polyporus sulphureus (Bull.) Fr. [*Laetiporus sulphureus* (Bull.) Murrill] (sulphur polypore)	40	in young fruiting bodies: oct-1-en-3-one, oct-1-en-3-ol, 3-methylbutanoic acid, 2-phenylethanol, 2-phenylacetic acid	in aged: 2-methylpropanoic acid, butanoic, acid, 2-phenlylacetic acid,
Termitomyces shimperi Heim. (a mushroom cultivated by termites)	24	oct-1-en-3-ol, 2-phenylethanol, hexanal	--
Tricholoma matsutake (S. Ito & S. Imai) Singer (pine-mushroom)	23	oct-1-en-3-one, oct-1-en-3-ol, octan-3-ol, octan-3-one, (E)-dec-2-enal, α-terpineol, phenylethyl alcohol, ethyl 2-methylbutanoate	2-methylbutyrate, linalool, methional,
Tuber aestivum (Wulfen) Spreng. (summer truffle), *T. melanosporum* Vittad. (black truffle)	72-89 17	dimethyl sulphide, dimethyl trisulphide, oct-1-en-3-ol, octan-3-one, octan-3-ol, oct-1-en-3-one	butan-2-on, butan-2-ol
Volvariella volvacea (Bull.) Singer (padi straw mushroom)	--	oct-1-en-3-ol, oct-2-en-1-ol, limonene, oct-1,5-dien-3-ol, octan-3-ol, octan-1-ol,	--

Mushrooms and fungi can also emit unpleasant odours (Pildain *et al.* 2010). *Hygrophorus paupertinus* A.H. Sm. & Hesler, is the one whose odour is defined as ‘’exceedingly strong, penetrating, and disagreeably and distinctly faecal-like’. Volatile compounds responsible for the odour are highly odoriferous 3-chloroindole, indole and 1-octen-3-ol, although the last is known as a compound responsible for a nice mushroom aroma. 3-Chloroindole is known for its faecal-like odour when is concentrated, but simultaneously the same compound becomes pleasant in highly diluted solutions (Poucher 1991). Indole and 3-methylindole were identified in other species with unpleasant odours: *Tricholoma bufonium* (Pers.) Gillet, *T. inamoenum* (Fr.) Gillet, *T. lascivum* (Fr.) Gillet, *T. sulphureum, Lepiota bucknallii* (Berk. & Broome)

Sacc., *Morchella conica* Pers., *Coprinus picaceus* (Bull.) Gray, *Boletus calopus* Pers., *Gyrophragmium dunalii* (Wood *et al.* 2003). Other indole compounds were also found in the fruiting bodies of some mushroom species - *Auricularia polytricha* (Mont.) Sacc., *Suillus bovinus* (L.) Roussel, *Macrolepiota procera* (Scop.) Singer, *Lentinula edodes* (Berk.) Pegler, *Leccinum scabrum* (Bull.) Gray, both before and after thermal processing (Muszyńska *et al.* 2013).

Alcohol (ethanol) is another flavour of fungal origin. Yeasts and *Rhizopus* spp. are responsible for its smell in the case of beer, wine, vodka and exotic fermented beverages such as parakari (fermented cassava) (Henkel 2005). May this popular flavour close the door of fungal world of flavour, odours and aromas as well as fragrances.

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Streszczenie

Grzyby w naszym otoczeniu stanowią nie tylko ciekawy świat kształtów, ale także są nośnikami ciekawych zapachów. Mykolodzy procujący z grzybami mikroskopowymi doskonale wiedzą, że niektóre z nich wydają charakterystyczną woń, jak np. woń czosnku typową dla rodzaju *Mortierella* lub kokosową w przypadku *Trichoderma* spp. W świecie grzybów spotkać można także takie zapachy, którym towarzyszy specyficzny smak. Któż nie zna typowego określenia „smak/aromat grzybowy”? Każdy, nawet osoba niezwiązana zawodowo z grzybami wie, co kryje się pod tym określeniem. Ale co stanowi, że możemy zdefiniować to określenie od strony chemicznej? Kluczową grupą substancji odpowiedzialnych za typowy smak i aromat grzybowy są lotne pochodne związków z grupy C_8, a wśród nich najbardziej istotne okazują się być oct-1-en-3-ol, octan-3-ol, octan-3-on, oct-1-en-3-on.

Grzyby dostarczają rozmaitych wrażeń zapachowych, począwszy od miłych anyżkowych, owocowych, poprzez ogórkowe, czosnkowe, serowo-czosnkowe, następnie przypominające ziemniaki lub mąkę, aż do zapachów nieprzyjemnych, jak zapach karbidu lub fekaliów. Smak niektórych z nich jest skorelowany z zapachem, w wielu przypadkach smaków nie znamy. Za każdym z tych aromatów ukrywają się związki chemiczne pozwalające czasem na uzyskanie wstępnej informacji taksonomicznej.

Bogactwo aromatów w świecie grzybów pozwala na biotechnologiczne wykorzystanie ich do otrzymywania bądź to czystych związków, jak np. w przypadku ketonu malinowego pozyskiwanego z *Nidula niveotomentosa*, bądź np. aromatycznej grzybni mogącej zastąpić rzadkie i pożądane gatunki grzybów leśnych, np. borowików.

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Small mammals feeding on hypogeous fungi

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ABSTRACT

Fungi serve as a food source for a wide variety of animals. Among mammals, most species feed on fungi occasionally or accidentally while foraging for other type of food, but some species are frequent mycophags and fungi can be a dominant component of their diet. Examples of mycophags can be found among marsupials: wallabies and bettongs; and rodents: squirrels, chipmunks, voles and mice.

Hypogeous fungi produce closed, underground sporocarps without opening mechanisms, and thus are unable to release their spores into the air. In case of those fungi, animals feeding on sporocarps and spreading spores in their faeces are considered to be the main vector of spore dispersal. Animals that frequently feed on fungi and other heavy digestible food have developed morphological adaptations such as longer gut retention and a spiral construction of the proximal colon, to digest more fungal material which is rich in nitrogen.

The spores stay viable after passing through the animal gut, and in some cases their ability to germinate and form mycorrhiza is enhanced after leaving the intestine. Hypogeous fungi are mycorrhizal partners for plants and it is therefore possible that the interactions between mycorrhizal fungi and animals spreading their spores also play an important role in ecosystem functioning.

KEY WORDS: mycophagy, fungivory, spore dispersal rodents

Introduction

Mycophagy, or fungivory is a feeding habit of consuming fungi. Depending on the degree to which animals feed on fungi, mycophagy can be: obligatory – the diet consists entirely or mostly of fungi; preferential – fungi are preferred to other food types but the animal feeds regularly on different food sources; opportunistic – fungi are eaten occasionally and accidental when fungi are eaten while foraging for a different kind of food (Trappe *et al*. 2009).

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Fungivory is very common and is mostly associated with snails and insect larvae feeding on "grubby" fruit bodies (Trappe *et al*. 2009), but many groups of vertebrates, like mammals, also make use of this food source. Examples of mammal mycophags can be found in the families of Sciuridae (squirrels and chipmunks), Cricetidae (voles), Muridae (mice), Macropodidae (kangaroos and wallabies), Potoroidae (rat-kangaroos and bettongs), and bigger animals, like Suidae (pigs) and Cervidae (deer) (Fogel & Trappe 1978). Insectivorous mammals, such as shrews (Soricidae), are examples of accidental or opportunistic mycophags that feed on hypogeous fungi while foraging for invertebrates (Kataržyte & Kutorga 2011). Recently primates are becoming a new and interesting group in studies on mycophagy. Mushrooms are not a common food source for those mammals and they mostly enrich the animals' diet, when available. Some examples of primate mycophagy can be observed among macaques, marmosets and lemurs (Hanson *et al*. 2003, Hilario & Ferrari 2011).

As animals can eat the whole fruit body, traces of animal foraging may be difficult to observe and track with the naked eye. Therefore, the prime method for determining whether mammals feed on fungi is microscopic and DNA analysis of faecal samples and intestine contents, for presence of spores. The fungal material can even be found in samples from stomachs and faeces of predatory mammals, since they feed on mycophagous animals (Fogel & Trappe 1978, Lehmkuhl *et al*. 2004).

The aim of this paper is to show some aspects of mammalian mycophagy regarding feeding on a particular food source that are hypogeous fungi. The case studies presented here will consider two mammal groups: rodents (Rodentia) and marsupials (Marsupialia), having well known records of mycophagy and hereafter referred as small mammals.

Hypogeous fungi as a food source

Macroscopic fungi produce fruit bodies on the ground to enable spore dispersal which is additionally enhanced by releasing mechanisms. This, however, does not occur in hypogeous fungi. These fungi produce closed, underground sporocarps with no opening mechanisms. As the spores cannot be released into the air, the main way for their dispersion is through animal activity (Johnson 1996). Animals take part in spore dispersion in a couple of ways: by digging up, and thus opening the sporocarps and releasing spores into the air, by eating the sporocarps and spreading spores in faeces or by carrying spores on their bodies after walking through an already decayed sporocarp (Cork & Kenagy 1989, Johnson 1996, Trappe *et al*. 2009). Some hypogenous fungi produce sporocarps in more than one season of the year. For example, *Elaphomyces,* which is the most common genus of truffle-like fungi in Poland, produces fruit bodies in the spring, summer and autumn, and usually more than one generation of fruit bodies can be found (immature, mature and overriped). In humid periods, old fruit bodies break up, producing an intense smell (Ławrynowicz *et al*. 2006). The mature fruit bodies produce characteristic aromas resembling hormones attracting animals. The chemistry of those odours and animal reaction to them differ among species (Fogel & Trappe 1978, Johnson 1996, Trappe & Claridge 2005). Chemical analyses suggest that the major compound responsible for the characteristic smell of truffles is dimethyl

sulphide. An earlier hypothesis that those aromas resemble pheromones was rejected experimentally using dogs and pigs (Johnson 1996).

Fungal cell walls are built of carbohydrates, primarily of chitin, which can be digested only by some animals (Cork & Kenagy 1989, Claridge *et al*. 1999). For those who can digest them, hypogeous fungi are a source of phosphorus, potassium, calcium, magnesium, and most important – nitrogen (Johnson 1996, Claridge *et al*. 1999, Trappe *et al*. 2009). 80% of the nitrogen is contained in the indigestible spores, and from the remaining 20%, only a half is in the form of proteins, and the other half is built into complex and mostly indigestible structures of cell walls (Cork & Kenagy 1989, Johnson 1996, Claridge *et al*. 1999, D'Alva 2007, Trappe *et al*. 2009).

Fungi are also a source of water, which constitutes 80-90% of their mass (Claridge *et al*. 1999, Trappe *et al*. 2009). It is possible that the high concentration of water in hypogeous fungi, and their relatively low dry mass makes them nutritious, when eaten in large numbers. Therefore, in the autumn, when hypogeous fungi appear in abundance, the cost of foraging for this type of food is lower than for other food sources. Moreover, animals can easily find intensively smelling matured fruit bodies, and along with them, a concentration of more fruit bodies than they can consume in one intake (Cork & Kenagy 1989, Johnson 1996). In the case of small mammals, the balance of costs and benefits from foraging for fungi is little above zero. As a result, although this is enough for them, it is not enough for larger mammals, like deer, which in turn eat fungi less frequently (Fogel & Trappe 1978, Cork & Kenagy 1989, Trappe *et al*. 2009).

The spores of hypogeous fungi pass through an animal's digestive system with no changes in their structure and stay viable after leaving it (Cork & Kenagy 1989, Claridge & Lindenmayer 1998, Claridge *et al*. 1999, Trappe *et al*. 2009). While inside the alimentary canal, spores are subject to heat and chemical treatment, of which both can stimulate spore germination. However, the mechanism of these factors' influence on the spores remains unclear and the evidence is mixed. The laboratory studies by Colgan and Claridge (2002) support the hypothesis that the passing of spores through an animal's digestive system can enhance the spores' ability to germinate, but it differs depending on the mycophagous animal species. This is due to the differences in mycophags gut retention, body temperature and digestive system structure (Colgan & Claridge 2002). Another factor are the conditions required for germination, which also differ among fungal species (Trappe & Claridge 2005).

Examples of small mammal mycophagy on hypogeous fungi

Small mammals usually eat fungi as a part of a diverse diet that includes fruit, seeds, herbs, invertebrates and other food sources but they may, in some cases, prefer fungi to other food items (Fogel & Trappe 1978, Johnson 1996, D'Alva 2007). Examples of mycophagous species along with the dietary volume of consumed fungal material are shown in table 1. The volume for *Bettongia gaimardi* is cited after Johnson (1996), and the other species are cited after Fogel and Trappe (1978) with data taken from works of Trevis (1953), Mckeever (1964), Steinecker and Browning (1970), and Drożdż (1966).

Table 1. The annual dietary volume of consumed fungal material.

	Species	**Volume (%)**
Marsupialia, Potoroidae	*Bettongia gaimardi*, Tasmanian bettong	90
Rodentia, Sciurudae	*Sciurus griseus,* Western gray squirrel	52
	Spermophilus lateralis, Golden mantled ground squirrel	61
	Tamias amoenus, Yellow-pine chipmunk	37
	Tamias quadrimaculatus, Long-eared chipmunk	66
	Tamias speciosus, Lodgepole chipmunk	32
	Tamias townsendii, Townsend's chipmunk	72
	Tamiasciurus douglasii, Douglas's squirrel	56
Rodentia, Cricetidae	*Myodes glareolus,* Bank vole	7
Rodentia, Muridae	*Apodemus flavicollis,* Yellow-necked mouse	1

Hypogeous fungi serve as a food source for various species of small mammals characterised by different foraging behaviour (Fogel & Trappe 1978, Trappe *et al*. 2009). Australian wallabies, for example, find fruit bodies a couple of centimetres below soil surface, whereas bettongs, which are equipped with longer claws, can dig to the lower parts of the ground profile, thus making their diet more diverse (Verns & Lebel 2011). Australian mammals that feed on fungi are mostly small, eat less plants and their digestive system is adapted for longer gut retention times and fermentation to assimilate more nutrients from heavy digestible fungi (Danks 2012). Some rodents, like voles, have similar adaptations. They are able to digest complex polysaccharides, like chitin, which indicates a complicated fermentation process in the digestive system. Voles are also very effective in reducing losses of nitrogen in faeces, due to the colonic separation mechanism, and a characteristic spiral construction of the proximal colon. This enables them to digest fungal material sufficiently. Some species of voles, like bank vole *Myodes glareolus* and field vole *Microtus agrarius*, practice coprophagy (consumption of faeces) which is also an adaptation for digesting heavy food, particularly cellulose (Cork & Kenagy 1989, Lee & Houston 1993, Claridge *et al*. 1999).

Many squirrels feed frequently on hypogeous fungi, among them the American red squirrel *Tamiasciurus hudsonicus*, Townsend's chipmunk *Tamias townsendii* and northern flying squirrel *Glaucomys sabrinus* (Colgan & Claridge 2002, Bertolino *et al*. 2004). Studies on *G. sabrinus* show that it

prefers hypogeous fungi in its diet and consumes them when available. Flying squirrels actively search for fungi on the ground, despite the higher risk of predation from lynxes and coyotes (Trappe *et al*. 2009).

Kataržyte and Kutorga (2011) observed that the *Apodemus* mice, and the bank vole *Myodes glareolus* feed on fungi for most of the year with the number of faecal samples containing spores increasing from 50% in the spring to 83% in the autumn. The number of fungal species found also increased. The most frequently observed genus was *Elaphomyces*. Studying the faecal samples from small mammals in search for spores can be helpful in evaluating the biodiversity of hypogeous fungi on given terrain. Kataržyte and Kutorga (2011) found 9 species of hypogeous fungi in samples from mice *Apodemus sp.*, bank vole *Myodes glareolus*, common shrew *Sorex araneus* and pygmy shrew *S. minutus*, while only 5 species were found during the search for fruit bodies. Moreover, the presence of *Chamonixia caespitosa*, and fungi of the genus *Genea* in Lithuania are documented only from faecal samples from small mammals (Kataržyte & Kutorga 2011).

Relationships in ecosystems – mycophagy and mycorhiza

In comparison with anemochoric spores, zoochoric spores have a significantly larger range of dispersion because foraging areas of small mammals can range from 1 to even 100 ha (Johnson 1996). Studies on population structure of hypogeous fungi show little genetic diversity between neighbouring sites, which means that long distance spore spreading prevents losses in the genetic pool of the population (Johnson 1996, Bertolino *et al*. 2004). Animals carry spores into early successional habitats, like glacier forefronts and burnt down forest patches, where the fungi have fewer competitors (Cazares & Trappe 1994). Additionally, less frequent species of fungi are prevented from competitive exclusion by more widespread species because animals feed on a variety of species and spread spores equally (Johnson 1996).

Hypogeous fungi occupy a very specific niche, being mycorrhizal partners for roots of vascular plants (Fogel & Trappe 1978, D'Alva *et al*. 2007, Trappe *et al*. 2009). They have a positive effect on their host plants, and may also influence the plant community structure in the given area as well as the overall condition of the ecosystem. The interactions between mycorrhizal fungi, their tree hosts and spore dispersing mycophags are the topic of multiple studies conducted in various regions in Europe, North and South America and Australia (Claridge *et al*. 1999). Experimental works have shown that some fungi that originated from spores that passed through animals' digestive systems form mycorrhiza with seedlings more rapidly than fungi from spores that were deposited into soil directly from the fruit body (Johnson 1996, Claridge *et al*. 1999, Colgan & Claridge 2002).

Many animals, both mycophages and predators, depend on trees for shelter, food and breeding places. In turn, the growth of trees is aided by mycorrhizal fungi. Therefore, mycorrhiza and mycophagy may be inseparable phenomena influencing the structure, functioning and stability of the forest ecosystem (Johnson 1996). Any disturbance in this complex net of relations can influence all its other parts. It is vital to expand the knowledge of the forest ecosystem and the interactions

between organisms composing it, as it would give us a wider perspective regarding the forest management (Colgan *et al*. 1999).

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Streszczenie

Grzyby stanowią pokarm dla wielu gatunków zwierząt. Spośród ssaków, większość gatunków żywi się grzybami w niewielkiej ilości oraz natrafiając na nie w czasie poszukiwania innego pokarmu, jednak dla niektórych gatunków, grzyby mogą stanowić dominujący element diety. Najwięcej przykładów mykofagicznych ssaków można znaleźć wśród małych zwierząt: torbaczy (walabie i kanguroszczury) oraz gryzoni (wiewiórki, myszy i nornice) (Trappe *et al*. 2009).

Grzyby podziemne zajmują bardzo specyficzną niszę ekologiczną, jako partnerzy mykoryzowi drzew, tworzący zamknięte owocniki, nie przystosowane do dyspersji zarodników z prądami powietrza. Z tego powodu głównymi wektorami rozpraszania tych grzybów są zwierzęta odżywiające się podziemnymi owocnikami i roznoszące zarodniki w odchodach. Zwierzęta które regularnie żywią się grzybami posiadają fizjologiczne i morfologiczne adaptacje do trawienia tego typu pokarmu i uzyskania z niego jak największej ilości przyswajalnej materii. Zarodniki pozostają zdolne do dalszego rozwoju po wydaleniu na zewnątrz organizmu zwierzęcego. Badania laboratoryjne wskazują, że w przypadku niektórych gatunków wpływa to wręcz korzystnie na tempo dalszego rozwoju zarodników, oraz na ich zdolność do zawiązywania mykoryzy.

Zwierzęta w ekosystemie leśnym zależą od drzew jako od miejsc schronienia, żerowania i rozmnażania. Tyczy się to zarówno gatunków mykofagicznych, roznoszących zarodniki grzybów podziemnych, jak i zwierząt drapieżnych. Z kolei drzewa zależą od grzybów mykoryzowych wpływających na ich rozwój i kondycję. Ważnym jest zatem, aby poszerzać wiedzę o powiązaniach między organizmami tworzącymi ekosystem leśny, gdyż jakiekolwiek zaburzenie w tej sieci zależności (jak na przykład selektywna wycinka drzew, lub ograniczanie populacji gryzoni uznanych za szkodniki), może wpłynąć na pozostałe elementy ekosystemu (Colgan *et al*. 1999).

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Species diversity of macrofungi on fallows in the buffer zones of the landscape parks in Łódzkie province

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ABSTRACT

This study presents the species structure of macrofungi in different plant communities formed on fallows as a result of secondary succession. The mycological observations were carried out in 2012 and 2013 in the buffer zones of all landscape parks in the Łódzkie province, i.e. Bolimów LP, Spała LP, Sulejów LP, Warta-Widawka LP, Łódź Hills LP, Przedbórz LP and Załęcze LP. The botanical research identified fallows representing 7 types of plant communities. In total 46 macromycetes species were found on the fallows. The diversity of macrofungi depended on the type of plant community. The highest number of fungi species was found in the communities with an admixture of trees (*Pinus sylvestris, Betula pendula*), while the lowest was collected on fallows almost completely covered by *Cirsium arvense* and *Solidago canadensis*. Considering the trophic classification of macrofungi found on fallows, most species were saprotrophic and mycorrhizal. Wood inhabiting saprotrophs were represented by only two species.

KEY WORDS: fallows, secondary succession, macromycetes, landscape parks, Łódzkie province

Introduction

The acreage of fallows in Poland continues to increase (Harkot *et al.* 2011) due to the low profitability of farming on low fertility soils and changes in the demographic structure of rural populations. No uniform definition of a fallow exists. In general, a fallow is defined as former agricultural land where human interference has been abandoned for many years (Flis 1985). Traditionally, 'fallow' refers only to an arable land that is no longer used. However, in case of abandoned meadows and pastures the relevant definitions do not exist. The prolonged cessation of farming on all above listed land leads to the similar consequences, i.e. the secondary succession progression. Therefore, it seems reasonable to extend the definition of fallows to overgrown meadows and pastures as well (Krysiak 2011). In this paper the term fallow was adopted to all

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agricultural land (arable fields, meadows, pastures) where farming has been abandoned.

The information about macrofungi on former agricultural land can be found in a limited number of mycological reports (e.g. Kujawa & Kujawa 2008, Kałucka 2009). The purpose of this study was to define the species structure of macrofungi in various plant communities formed on fallows as a result of secondary succession.

Material and methods

The mycological observations were carried out in 2012 and 2013. Fallows selected for the study are located in the buffer zones of all landscape parks in the Łódzkie province, i.e. Bolimów LP, Spała LP, Sulejów LP, Warta-Widawka LP, Łódź Hills LP, Przedbórz LP and Załęcze LP. Seven belt transects were established on the fallows. Each transect was divided into 5-6 study plots (100 m x 100 m). In total, 39 study plots were established. Fallows were initially identified based on orthophotomaps scaled 1: 10 000 (the flight was done in 2009), then verified and finally plotted on the land. Each plot was surveyed in the current growing vegetation. The search for macrofungi on the study plots was made three times each year.

The applied nomenclature of macrofungi follows Index Fungorum (2014).

Results

The botanical survey revealed fallows representing 7 types of plant communities:

1) the community without vegetation or with very limited vegetation, with growing lichens and bryophytes,

2) the community dominated by grasses, with one grass species being dominant, e.g. *Agrostis capillaris* L. or *Elymus repens* (L.) Goul., and the low number of other plants,

3) the community dominated by *Hieracium pilosella* L. and with the low share of other plant species,

4) the community dominated by 2-3 grass species and with the high number of other plants,

5) the community dominated by *Cirsium arvense* (L.) Scop. and *Solidago canadensis* L., with the low share of other species,

6) the community dominated by *Calamagrostis epigeios* (L.) Roth, with the low share of other plants,

7) the community with highly diversified species composition and a significant share of bryophytes.

Trees and shrubs constituted another element of fallows vegetation that is important for the development of macrofungi. In the studied communities they were represented by *Pinus sylvestris* L., *Quercus robur* L., *Betula pendula* Roth, *Padus serotina* (Ehrh.) Borkh. and *Pyrus communis* L. em. Gaertner.

So far, in a two-year observation, 46 macromycetes species were found on the seven types of fallows. The diversity of fungi depended on the type of plant community developing on a particular fallow type. In the communities formed only by lichens, a limited number of fruit bodies of gasteroid basidiomycetes from *Bovista, Lycoperdon* and *Calvatia* genera were recorded. Fungi in the communities dominated by one grass species or *Hieracium pilosella* were presented by gasteroid species as well as *Crinipellis scabella* (Alb.& Schwein) Murrill, *Marasmius oreades* (Pers.) Gillet,

Conocybe tenera (Schaeff.) Fayod, *Panaeolus fimicola* (Pers.) Gillet and *Psilocybe coronilla* (Bull. Ex DC.) Noordel.

The lowest number of fungi species was found on the fallows almost completely covered by *Cirsium arvense* and *Solidago canadensis* (tab. 1). In these communities only single fruit bodies of *Conocybe tenera* and, scarcely, *Macrolepiota procera* (Scop.) Singer were found. The highest number of macrofungi species was found in the communities with an admixture of trees, regardless of the type of vegetation that was dominating there. On study plots with young pines, large numbers of mycorrhizal macrofungi from the genera *Amanita, Boletus, Chalciporus, Inocybe, Laccaria, Paxillus, Suillus,* and *Xerocomus* were found. Some of these species, e.g. *Laccaria laccata* (Scop.) Cooke and *Suillus luteus* (L.) Roussel produced fruit bodies in abundance. The fruit bodies of the mycorrhizal macrofungi (mainly *Amanita muscaria* (L.) Lam. and *Suillus luteus*) were also found on fallows without trees; all were located near tree stands among fields. In the communities where the cover rate of bryophytes was high, species growing on bryophytes, e.g. *Rickenella fibula* (Bull.) Rathelh. and *Arrhenia lobata* (Pers.) Kühner & Lamoure ex Redhead were found.

Table 1. Quantitative participation of macrofungal species in plant communities on surveyed fallow types.

Type of plant community	Number of species
Community with lichens and bryophytes	12
Community dominated by grasseswith a single grass species dominant	16
Community dominated by *Hieracium pilosella* with trees	31
Community dominated by 2-3 grass species	18
Community dominated by *Cirsium arvense* and *Solidago canadensis*	7
Community dominated by *Calamagrostis epigeios*	10
Community with a significant share of bryophytes	29

Considering the trophic classification of macrofungi found on fallows, most species were saprotrophic and grew on the soil, plant parts or fruits. Mycorrhizal macrofungi were also numerous and were found on more than ten study plots in the second year of observation. Of these, the highest number of fruit bodies was formed by *Amanita muscaria, Boletus edulis* Bull., *Laccaria laccata*, *Suillus bovinus* (L.) Roussel and *S. luteus*. Saprotrophic fungi growing on wood were represented by only two species *Trichaptum abietinum* (Dicks.) Ryvarden and *Schizophyllum commune* Fr., both forming fruit bodies on fallen pine branches.

Discussion

Fallows are specific habitats with special soil structure formed under the strong influence of long-term cultivation. Plant communities of fallows are unstable, and their species diversity may increase over time (Kurus & Podstawka-

Chmielewska 2006). It seems that the pattern of succession for the vegetation emerging spontaneously on fallows may be associated with the soil properties and the vegetation on the adjacent areas as well. A two-year mycological study offered a chance to observe only some selected trends in the macrofungal colonization of abandoned land with different types of vegetation developed. The previous studies demonstrated that the succession of plant cover is always linked with the succession of fungi (Arnolds 1992, Keizer and Arnolds 1994, Kałucka 2009). Therefore, the diversity of fungi species on the studied fallows will change in time. Afforestation of former agricultural lands plays an important role in establishing conditions favourable to various macrofungi species (Kujawa & Kujawa 2008). Very few of the fallows studied are spontaneously colonised by trees (mainly pines). On some fallows the young individuals of invasive species *Padus serotina,* can be found and they can have negative effects on the potential growth of native trees. We can deduct that changes in the fungi populations on the studied fallows will be driven by many factors. The mycological observations of the same study plots should to continue in order to identify these factors.

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Streszczenie

Przemiany użytkowania ziemi w ostatnich 20 latach przyczyniły się do powstania w naszym kraju znacznych powierzchni odłogów, które stwarzają nowe siedliska dla rozwoju spontanicznej roślinności i grzybów. Dotychczasowe dane o grzybach wielkoowocnikowych gruntów porolnych są znikome. W prezentowanej pracy przedstawiono wyniki dwuletnich obserwacji macromycetes na powierzchniach odłogów, usytułowanych w strefach otaczających wszystkie parki krajobrazowe w woj. łódzkim. Obserwacje te pozwoliły na wyodrębnienie 7 typów zbiorowisk roślinnych

powstałych na odłogach oraz zanotowanie na nich 46 gatunków macromycetes. Roślinność obserwowanych odłogów wykazywała znaczne różnice strukturalne. Były tu pionierskie powierzchnie porośnięte jedynie porostami z rodzaju *Cladonia*, inicjalne stadia muraw, z dominacją traw (np. *Agrostis capilllaris, Festuca ovina*) oraz powierzchnie o znacznej liczbie gatunków roślin zielnych, zarówno łąkowych, jak i leśnych. Odrębną grupę stanowiły powierzchnie porośnięte w przeważającej części gatunkami inwazyjnymi obcego pochodzenia, głównie *Solidago canadensis* i *Padus serotina*. Funga obserwowanych powierzchni wykazywała zróżnicowanie związane z typem roślinności. Najuboższe w grzyby okazały się powierzchnie inicjalnych muraw z chrobotkami lub roślinnością trawiastą oraz powierzchnie z dominacją *Solidago canadensis.* Występowały tu nieliczne owocniki gatunków z rodzaju *Bovista*, *Lycoperdon, Calvatia*. Natomiast najbogatsze w gatunki grzybów były powierzchnie porośnięte drzewami, głównie sosnami, gdzie zanotowano gatunki grzybów mykoryzowych z rodzajów: *Amanita, Boletus, Inocybe, Laccaria, Suillus, Xerocomus*. Na odłogach z dużym pokryciem mszakami, obserwowano grzyby wykorzystujące mchy, np. *Rickenella fibula* i *Arrhenia lobata*. Dotychczasowe badania macromycetes na odłogach woj. łódzkiego dostarczają jedynie wstępnej wiedzy o różnorodności gatunkowej grzybów na tego typu siedliskach. Wydaje się celowe prowadzenia dalszych badań na tych samych powierzchniach i obserwowanie zmian, jakie będą zachodzić w strukturze macromycetes wraz z postępującą na odłogach sukcesją.

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Microbiological hazard in buildings based on the example of dwelling houses and public utility buildings in Warsaw

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ABSTRACT

This paper contains preliminary results of mycological research in residential and public buildings in Warsaw and its surroundings. The study focuses on the qualitative survey of fungi in buildings of various categories. It includes research objects divided into six categories depending on the age of the buildings and their use: new buildings and the objects of about twenty years and older, as well as twenty-year single-family houses. Two houses that are in the registry of monuments (National Heritage Institute) were also in the scope of the analysis. There have also been attempts to classify the identified species of fungi into groups of BSL (Biological Safety Level), in order to identify their potential allergenic impact on buildings users. In more than half of the studied objects the most frequently isolated species was *Alternaria alternata* (55% of cases) and fungi of the genera *Aspergillus* and *Penicillium*. *Aspergillus niger* was identified in 33% of the objects, exclusively in the neglected buildings, including those abandoned for at least one year.

KEY WORDS: moulds, mycology, building, allergy

Introduction

Mould fungi commonly exist in dwelling houses, not only in old, neglected tenement houses or ones that have not been renovated for a significant amount of time. This problem often and increasingly concerns new buildings, including the undressed ones. Currently, apart from more than 100-year-old historic buildings, new constructions, including ones that have not been commissioned, are also becoming affected (Wiejak 2011). There are numerous causes of mould fungi occurrence, such as the optimal humidity of air or walls due to construction defects. Other factors include the increased temperature and, along the pH value, poor air circulation and the access to light required for sporulation (Piotrowska 2012). The fungi spread to dwelling houses from the outside, their spores are suspended in the air and

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become a constant element of the bioaerosol of the rooms, alongside the bacteria and viruses (Zyska 2010). Unfortunately, the occurrence of mould fungi in dwelling houses and public utility buildings is a health hazard for the residents and users. A common cause of this problem is faulty ventilation system resulting from both construction defects and the wrong use of the building. Additionally, wrong horizontal and vertical insulation or freezing of the wall barriers create favourable conditions for mould fungi development. Then, the most often occurring species are primary and secondary colonizers which need humidity 0,75 to 0,9 aw (water activity) (Twarużek 2006).

The aim of the present study was to evaluate and to compare microbiological hazard in residential and public buildings in the Warsaw area.

Material and methods

Thirty six buildings were divided into six categories based on the purpose of the building, age and type (one-family, multifamily or public utility) and surveyed between 2008 and 2014. Apart from buildings in Warsaw, there were also three in Konstancin-Jeziorna, and one in Jabłonna, Łomianki and Brwinów. Sixteen buildings were over 20-year-old multifamily houses. There were construction defects in all the surveyed buildings, such as damp plasters. In six of the buildings there was faulty vertical or horizontal insulation, in other six buildings walls were prone to frost. Five buildings had insufficient ventilation. In two there was flooding caused by a failure of the water supply network or another defect. Collected microbiological material in the form of microbiological contact plates (three samples for each building and scraping) was incubated. Mould samples were taken from wall barriers using Sabouraud dextrose 2% agar imprint plates or inoculated directly on agar plates (different media supplemented with chloramphenicol in order to eliminate bacterial growth) with the use of a laboratory needle. The fungi were incubated in 27° C for approx. 2 weeks. Microscopic (shape, size of conidiophores, conidia and ascospores, diameter of hyphae) and macroscopic features (colour, shape and consistency of colony) were considered during identification. The morphological characters (ascospores, conidia, etc.) were observed and measured (at least 30 elements) under an Olympus D40 microscope with transmitted light. Photographs were taken with an Olympus DP25 camera and CellSense software. Several identification keys were used and fungal names are given according to them (Thom & Raper 1945, Raper *et al*. 1949, Navi *et al*. 1999, Piontek 1999, Watanabe 2002).

Results

Sixteen species of fungi were isolated and identified in analyzed buildings (Table 1). In some buildings, several species of fungi occurred and almost all the species identified were present in public utility buildings. The collected data for residential buildings relate to the period of autumn (from October to December), while the public buildings were analysed in summer and autumn (from July to September). Cross marker (+) indicates the identification of a single species in a given category of buildings, in at least one room.

Table 1. Fungal species, their origin and classification to the BLS group.

Species	Apartments in			One-family houses		Public utility buildings	BLS group
	new buildings	over 20 years old buildings	tenement houses over 50 years old	over 20 years old	over 50 years old		
Acremonium charticola (Lindau) W. Gams						+	1
Acremonium strictum W. Gams	+					+	1
Alternaria alternata (Fr.) Keissl.	+++	++++	++++	++	+++	+++	1
Aspergillus fumigatus Fresen.						+	3
Aspergillus niger Tiegh.	++	++	+++	+	+++	+	1
Aspergillus ustus (Bainier) Thom & Church		+					2
Aspergillus versicolor (Vuill.) Tirab.						+	2
Botryotrichum piluliferum Sacc. & Marchal						+	1
Cladosporium herbarum s.l. (Pers.) Link						++	1
Fusarium oxysporum s.l. (Schltdl.) emend. Snyder & Hansen						+	1
Fusarium sambucinum Fuckel							

Species	Apartments in			One-family houses		Public utility buildings	BLS group
	new buildings	over 20 years old buildings	tenement houses over 50 years old	over 20 years old	over 50 years old		
Mucor hiemalis Wehmer		++				+	1
Penicillium expansum Link		++				+	1
Penicillium meleagrinum Biourge	+	+				+	1
Penicillium verrucosum Dierckx						+	1
Thamnidium elegans Link						+	1
total number of species in the buildings	4	6	2	2	2	16	

Discussion

In our study the representatives of *Alternaria alternata* and fungi from genera *Aspergillus* and *Penicillium* were the most common taxa. They were isolated and identified in 55% of the buildings. Although Zyska (1999, 2001) mentions that *Cladosporium* s.l. spores are among the most common in the air, their number in our study was low. However, this research is in its initial phase and the results may be different in the further stages. Only two samplings made in the period of intense pollen occurrence showed significant presence of *Cladosporium* s.l. spores. In contrast, mould fungi belonging to *Aspergillus* genus have a very long period of sporulation, which is a likely cause of their intermingled presence with other taxa. *Penicillium* and *Aspergillus* representatives are among the most common and most cosmopolitan indoor species (Twarużek 2006), hence they were also widespread in buildings analysed in this study. *Alternaria alternata* was most commonly isolated in places that had damp plasters with humidity of 1–2%, or more, or walls prone to frost. This fungus occurs also in all buildings which experienced flooding or had problems with insulation (Piontek 1999, Twarużek 2006, Ważny 2001). About 33% of taken samples contained conidiophores and conidia of *Aspergillus niger*. However, that fungus occurred only in over 50-year-old, neglected and

unoccupied buildings. *Penicillium* fungi were mainly present (in 14% of analyzed buildings) on wallpapers or wooden elements of construction or decorative elements as well as on the particle board furniture located in flooded rooms. *Thamnidium elegans* representatives, that are usually isolated from gardens, were isolated only from one-family houses (with gardens) or public utility buildings situated in parks. The spores get inside the apartments from the outside, thus the richer microflora occurrs outdoors, the more species are found in dwelling houses (Zyska 2001). *Alternaria*, *Aspergillus* and *Penicillium* fungi were detected mostly in apartments located in cities far from green areas. *Acremonium* spp. were identified in two apartments. *Fusarium* fungi occurred less commonly in public utility buildings. Pląskowska *et al*. (2012) mention that up to 35% of identified species in public utility buildings belong to *Penicillium* genus and up to 28% to genus *Aspergillus*. *Alternaria* fungi are most often isolated from the air in air-conditioned rooms, followed by the representatives of genera: *Fusarium* and *Acremonium*. On the other hand, the representatives of genus *Cladosporium* are rather typical for not air-conditioned places (Pląskowska *et al*. 2012).

The most allergenic species among the identified fungi was *Aspergillus fumigatus* classified to BSL-3 group, which means that even healthy people may potentially suffer from serious fungal infections (Grajewski *et al*. 2006). Other allergenic fungi are *Aspergillus versicolor*, *A. ustus*, *A. niger* and *Alternaria alternata*, which may trigger asthma.

Conclusions

The preliminary results allow presenting a list of 16 species of fungi occurring in residential and public buildings in the Warsaw area. The most frequently identified genera of fungi were *Alternaria*, *Aspergillus* and *Penicillium*. Some of the species belonging to these genera are classified as dangerous to the health of people exposed to contact with them. They include *Aspergillus fumigatus* and allergenic effect fungi – *A. ustus* or *Alternaria alternata*.

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Streszczenie

W pracy zawarto wstępne opracowanie badań mykologicznych w budynkach mieszkalnych i gmachach użyteczności publicznej w Warszawie i okolicy. Badania dotyczą jakościowego rozpoznania fungi w obiektach z sześciu kategorii, które wydzielono w zależności od wieku budynków oraz ich przeznaczenia: mieszkania w tzw. „nowym budownictwie" oraz w obiektach około dwudziestoletnich i starszych, a także w dwudziestoletnich domach jednorodzinnych. W zakres analiz weszły również próby pochodzące z dwóch zabytkowych kamienic umieszczonych w rejestrze zabytków Narodowego Instytutu Dziedzictwa. Dokonano również próby zaklasyfikowania stwierdzonych gatunków grzybów pleśniowych do grupy BSL (Biological Safety Level), w celu wskazania ich potencjalnego oddziaływania alergogennego na użytkowników budynków.

W ponad połowie obiektów najczęściej izolowano *Alternaria alternata* (55% przypadków) oraz grzyby z rodzajów *Aspergillus* i *Penicillium*, w tym *Aspergillus niger*, który zidentyfikowano w 33% badanych obiektów: w budynkach zaniedbanych, w tym niezamieszkałych od przynajmniej roku.

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The impact of plant growth promoting bacteria (PGPB) on the development of phytopathogenic fungi

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ABSTRACT

The main purpose of this study was to evaluate impact of plant growth promoting bacteria (PGPB) on the development of phytopathogenic fungi and correlate it with a potential effects on the growth of plants under unfavorable conditions, in order to improve the efficiency of a phytoremediation process. The conducted research focused on the antifungal properties of PGPB. In this study, 51 isolates of bacteria were obtained after diversified disinfection time from plants growing on soil after sewage sludge amendment. The results revealed that some isolated bacteria, mainly endophytic ones, inhibited the development of *Fusarium oxysporum*, *F. culmorum* and *Alternaria alternata*.

KEY WORDS: plant growth promoting bacteria (PGPB), soil, pathogenic fungi

Introduction

Free-living soil bacteria are actually divided into three functional groups: plant growth promoting bacteria (PGPB), biocontrol PGPB and plant stress homeoregulating bacteria (PSHB). PGPB can promote plant growth and development by indirect or direct means (Cassán *et al.* 2009), that can facilitate plant growth of plants under optimal, biotic or abiotic stress conditions. They can also produce phytohormones and synthesize some less well characterized low molecular mass compounds or enzymes (Glick *et al.* 2007). Indirect plant growth promotion induced by biocontrol PGPB includes a variety of mechanisms by which the bacteria prevent phytopathogen deleterious effects. The beneficial effect of the bacteria varies significantly depending on individual bacterial strains, plant genotype, and growth conditions. Production of antibiotics by endophytes from plants, in particular the role of numerous species of *Pseudomonas*, whose antibiotic substances effectively limit the growth of plant pathogens is of particular importance (Klama 2004). The main purpose of the study was to evaluate impact of plant growth

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promoting bacteria (PGPB) on the development of phytopathogenic fungi and correlate it with the potential effect on the growth of plants under unfavorable conditions, in order to improve the efficiency of phytoremediation process. The experiment was carried out to isolate strains of bacteria with the antifungal activity from plants grown on soil contaminated and fertilized with sewage sludge.

Material and methods

Root samples were collected from plants of *Festuca rubra* L., *Morus alba* L. and *Arabidopsis thaliana* (L.) Heynh growing on fields contaminated with heavy metals and fertilized with municipal sewage sludge (one year after application) near zinc smelter in Miasteczko Śląskie (S Poland). The roots were washed, cut and treated with 70% ethanol. In this study different time of disinfection was used to isolate rhizobacteria (disinfection time 0, only sterile distilled water was used) and endophytic bacteria (2.5 and 10 min disinfection followed by rinsing to eliminate ethanol). The disinfected roots were tested for surface sterility via incubation on solid nutrient agar medium for 3 days at 28° C. The roots were homogenized, serially diluted and placed in differentiating media: Congo-Red agar (CRA) or nitrogen-free base (NFb) media to isolate free-living diazotrophic bacteria (Döbereiner 1995), in Luria agar (LA) to isolate nutritionally demanding bacteria, and Yeast-Extract-Manitol agar (YEMA) to isolate *Rhizobiaceae* bacteria. Next, plates were incubated at 30° C for 2 (CRA, NFb and LA) or 7 days (YEMA) to isolate bacteria. A total number of isolates of *F. rubra* was 9, of *M. alba* was 16 and of *A. thaliana* was 26. Isolated bacteria were tested for gram coloration and screened for possible antifungal activity. The following fungi strains were used: FO – *Fusarium oxysporum* Schltdl., FC – *F. culmorum* (W.G. Sm.) Sacc., AA1 – *A. alternata* (Fr.) Keissler. 1, AA2 – *A. alternata* 2. Strains of *A. alternata* came from the collection of the Department of Forest Pathology University of Life Sciences in Poznan and were identified by microscopic analysis of the mycelium and conidia using available mycological keys. All isolated strains were assayed for antifungal activities using Potato Glucose Agar (PGA) medium. The bacteria were inoculated on PGA medium (1 μL), then at the center of a plate a 3 cm-diameter mycelium with pathogenic fungi was inoculated. Antifungal activity was examined for 10 days of incubation at 25° C. Fungal mycelium without bacteria inoculation was used as a control. Mycelium growth inhibition was calculated as: (a-b/a) x 100% (a: mycelium diameter in control, b: mycelium diameter in plates inoculated with bacteria).

Results

The potential biocontrol PGPB activity was evaluated based on antifungal activity *in vitro*, and results are summarized in Figures 1 to 3 for particular investigated plant species.

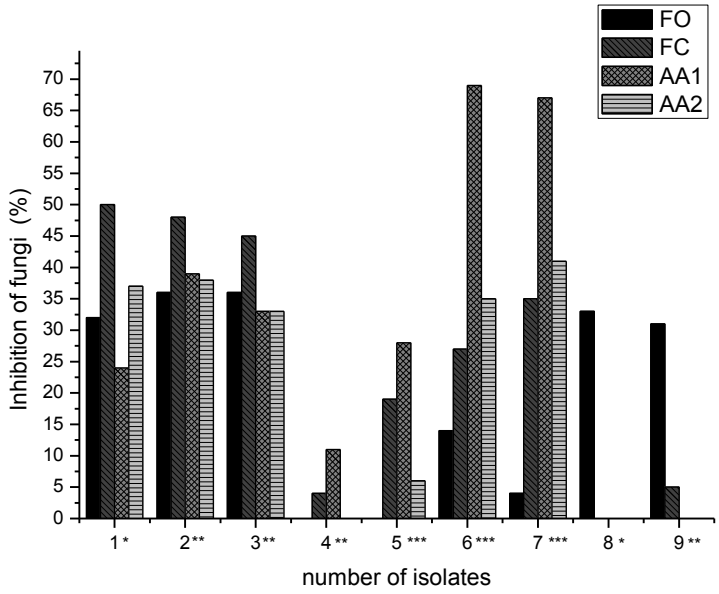

Figure 1. Antifungal activity of bacteria isolated from *Festuca rubra* (disinfection time: * - 0 min; ** - 2.5 min; *** - 10 min).

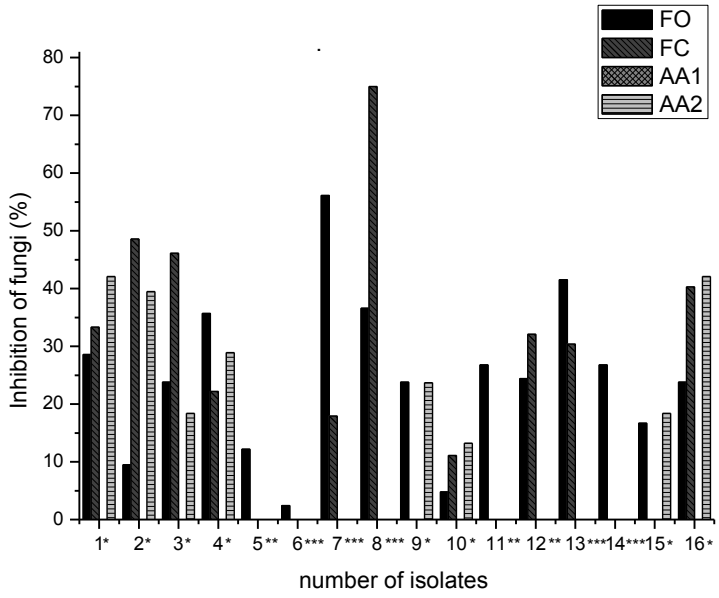

Figure 2. Antifungal activity of bacteria isolated from *Morus alba* (disinfection time: * - 0 min; ** - 2.5 min; *** - 10 min).

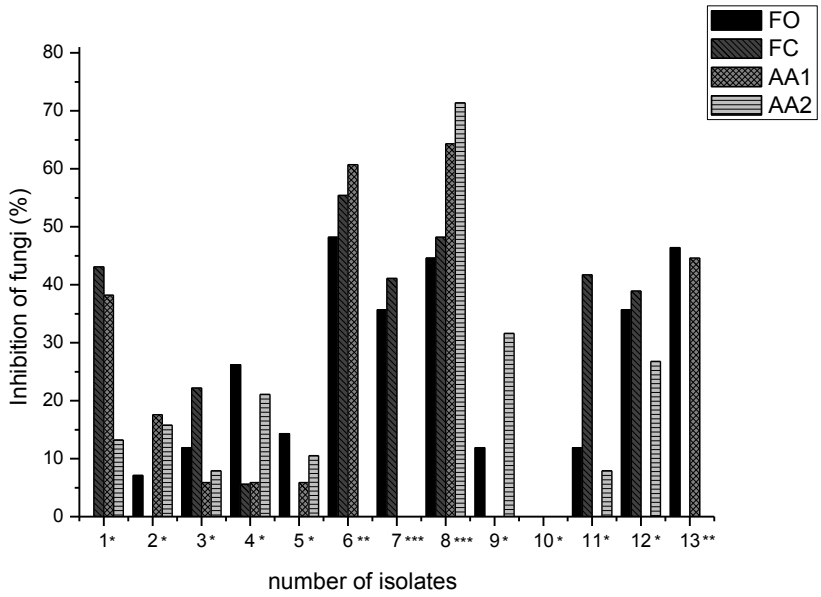

Figure 3A. Antifungal activity of bacteria strains (1-13) isolated from *Arabidopsis thaliana* (disinfection time: * - 0 min; ** - 2.5 min; *** - 10 min).

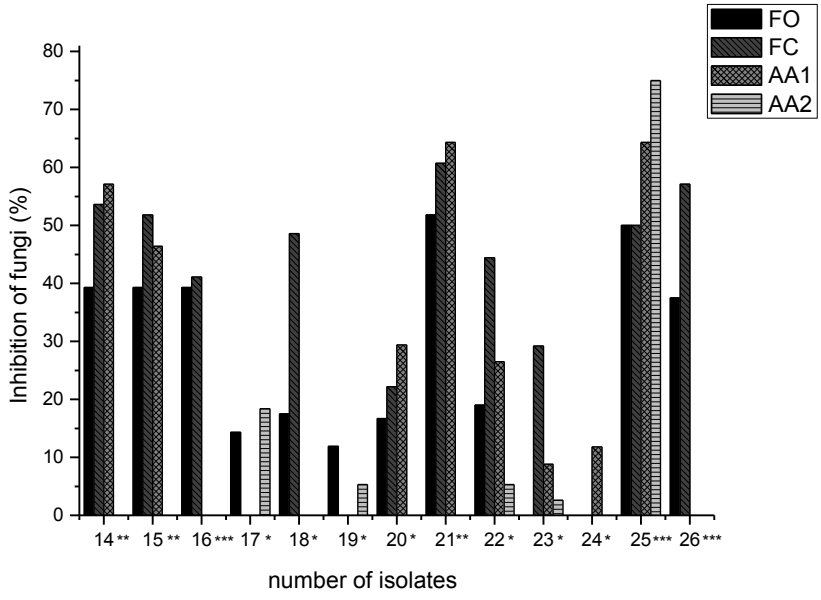

Figure 3B. Antifungal activity of bacteria strains (14-26) isolated from *Arabidopsis thaliana* (disinfection time: * - 0 min; ** - 2.5 min; *** - 10 min).

Discussion

Knowledge concerning different interactions and antagonistic effects between microorganisms can be used to control plant pathogens causing root diseases or penetrating into plants through some roots. Bacteria inhibit fungal growth releasing antifungal substances and/or cell wall degrading enzymes released by the bacteria into the culture medium (Ligon *et al*. 2000). In this study most of the bacteria isolated from the investigated plants exhibited antifungal activity. From nine strains isolated from *F. rubra* (Fig. 1), five had the ability to simultaneously inhibit the growth of *F. oxysporum, F. culmorum, A. alternata* 1, *A. alternata* 2. Two isolates from *F. rubra*: 2 (YEMA, 2.5 min.), 3 (YEMA, 2.5 min.), potential endophytes, inhibited the growth of pathogenic fungi by over 30 %. The results of the conducted research are similar to Cho *et al*. (2007) and confirm that mainly endophytic bacteria have antifungal properties. These strains were isolated on a YEMA medium, specific for *Rhizobiaceae*. Moreover two of the isolated strains: 6 (NFb, 10 min.) and 7 (NFb, 10 min.) were characterized by a very high degree of growth inhibition of *A. alternata* 1, more than 65%.

Among 16 strains isolated from *M. alba* (Fig. 2), the highest but overall modest inhibition of the pathogenic fungi growth (*F. oxysporum, F. culmorum* and *A. alternata* 2) was found for isolates: 1 (NFb, 0 min.) and 16 (LA 0 min.) expressing more than 20% inhibition. Strain 8 (NFb, 10 min.) isolated on a non-nitrogen medium after 10 min. disinfection time (potential endophyte), had the highest capacity to inhibit the growth of *F. culmorum* by 75%.

Moreover, the highest number of isolates (26) were obtained from the roots of *A. thaliana* (Fig. 3A, 3B). The widest spectrum of antifungal activity above 45 % was observed for two strains isolated on minimal media: strain 8 – potential endophyte (isolated on NFb, 10 min) () and 25 (CRA, 10 min.), also apparently an endophyte. Strain 8 had the highest antifungal activity 64% for *A. alternata* 1 and 71% for *A. alternata* 2. The following strains: 21 (LA, 2.5 min.) for *F. oxysporum, F. culmorum* and *A. alternata* 1 - more than 50 % and 25 (CRA, 10 min.) for *F. oxysporum, F. culmorum* and *A. alternata* 2 exhibited high antifungal properties. The other strains inhibited the growth of pathogenic fungi to a small extent.

In the conducted experiment it was shown that the strains isolated from the roots disinfected for 2.5 min and 10 min had the highest antifungal activity. As shown in other research, some of isolated bacterial strains can increase plant growth due to the inhibition of plant pathogenic fungi (Petatán-Sagahón *et al*. 2011, Weller 2007). Despite this, complementary experiments should be carried out with other fungal genera, like *Pythium*, *Phytophthora*, or *Sclerotinia,* to confirm the biocontrol capacity of each strain.

Acknowledgements

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Streszczenie

Bakterie promujące wzrost roślin (PGPB) to wolno żyjące organizmy glebowe, które mogą bezpośrednio lub pośrednio ułatwiać wzrost roślin w biotycznych lub abiotycznych warunkach stresowych. Mogą także produkować fitohormony i syntezować związki lub enzymy, które mogą modulować wzrost i rozwój (Glick *et al*. 2007). Pośrednie promowanie wzrostu roślin obejmuje wiele mechanizmów, dzięki którym bakterie mogą ograniczać rozwój patogenów roślinnych. Korzystny wpływ na wzrost roślin zmienia się znacznie w zależności od indywidualnych szczepów bakteryjnych oraz warunków wzrostu. Ważnym zjawiskiem jest produkcja antybiotyków przez bakteryjne endofity roślinne, w szczególności obserwowane u licznych gatunków *Pseudomonas*. Substancje te skutecznie ograniczają wzrost patogenów roślinnych (Klama, 2004).

Celem badań było wyizolowanie bakterii strefy korzeniowej i ocena inhibicji wzrostu grzybów będących patogenami roślin wobec wyizolowanych szczepów bakteryjnych.

W przeprowadzonych badaniach wykorzystano rośliny (*Festuca rubra, Morus alba*, *Arabidopsis thaliana*) rosnące w pobliżu huty cynku w Miasteczku Śląskim na glebie zanieczyszczonej metalami ciężkimi i nawożonej komunalnymi osadami ściekowymi. Bakterie izolowano z korzeni roślin stosując trzy długości czasu dezynfekcji (0, 2.5, 10 min). Po uzyskaniu czystych kultur bakteryjnych prowadzono testy zahamowania wzrostu w warunkach *in vitro* wobec czterech szczepów grzybów: FO – *Fusarium oxysporum*, FC – *F. culmorum,* AA1 – *Alternaria alternata* 1, AA2 – *A. alternata* 2.

Wyniki eksperymentu wskazują, że bakterie izolowane po czasie 2.5 i 10 minut sterylizacji, czyli głównie bakterie endofityczne, hamowały rozwój badanych grzybów – potencjalnych patogenów roślin. Badania wykazały także, że z rzodkiewnika pospolitego (*Arabidopsis thaliana*), znanego halofita, pozyskano najwyższą liczbę mikroorganizmów, które także wykazywały najlepsze właściwości inhibitujące wzrost grzybni wszystkich badanych patogenów.

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Colonization of decomposing *Sphagnum* moss litter by mycorrhizal roots in two types of peatland ecosystems

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ABSTRACT

During a 35-month study on the decomposition of *Sphagnum* moss litter in poor fen and pine bog forest, an intensive colonization of litter-bags by mycorrhizal roots was observed during the decomposition process. Content of mycorrhizal roots in litter-bags, expressed as % mass of roots, was generally increasing during the decomposition in pine bog forest, and fluctuating during decomposition on poor fen, although in both cases the results were statistically insignificant. Two morphotypes of ericoid roots and two morphotypes of ectomycorrhizal roots were recorded from litter-bags on poor fen during the decomposition experiment, while in pine bog forest one morphotype of ericoid and nine morphotypes of ectomycorrhizal roots were recorded. Molecular identification of mycorrhizal roots succeeded only in the case of one ericoid and six putatively ectomycorrhizal morphotypes. Most morphotypes were recorded only once during the whole 35-month decomposition period, and only one ericoid and one ectomycorrhizal morphotypes were shared between the poor fen and pine bog forest communities.

KEY WORDS: peatlands, *Sphagnum* decomposition, roots, mycorrhizal colonization, mycorrhizae

Introduction

The mycorrhizal fungi are well known for their symbiotic relationships with plants. Their main role is assumed to increase the uptake of scarcely distributed nutrients such as various forms of nitrogen and phosphorus, which is of particular importance in ecosystems where these substances are limiting factors for plants, e.g. peatlands or boreal forests (Smith & Read 2008 Deckmyn *et al.* 2014). The mycorrhizal fungi are able to play their role owing to the great absorptive and adsorptive surface of their extraradical mycelium, and to the

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production of a wide variety of ecto- and endoenzymes, which enables them to hydrolyze and then assimilate various plant-derived organic substances, e.g. peptides, proteins, aminoacids, phospho-monoesters and phospho-diesters (Chalot & Brun 1998, Read *et al*. 2004). In addition to that, some mycorrhizal fungi were shown also to possess the capability to degrade more complicated and resistant molecules present in plant cuticle or secondary cell walls, and this occurred owing to the secretion of e.g. esterases, polygalacturonases, xylanases, cellulases, thyrosinases, peroxidases, poly- and monophenol oxidases, laccases, and even ligninases. Thus, the saprotrophic role of ectomycorrhizal fungi in ecosystems is also recently considered (Talbot *et al*. 2008, Unestam 1991, Deckmyn *et al*. 2014). There are also more and more reports on mycorrhizal fungi colonizing decomposing leaf-litter (Unestam 1991) or experimental works on the decomposition rate and changes in chemistry of decomposing litter in the presence and absence of mycorrhizal fungi (e.g. Zhu & Ehrenfeld 1996) or on the enzymatic activity in colonized plant litter (e.g. Conn & Dighton 2000). However, there are still few papers about mycorrhizal colonization of leaf litter in peatlands, while it is known that this group of fungi is also important for the functioning of these ecosystems (e.g. Thormann *et al*. 1999, Thormann 2006).

During the research on the decomposition of *Sphagnum fallax* Klinggr. litter in Puszcza Piska forest, the intensive colonization of plant debris by mycorrhizal roots was observed in litter-bags in 2011–2013. Thus, the aims of the present study were: (1) to quantify the content of mycorrhizal roots colonizing moss litter during decomposition process, and (2) to identify the mycorrhizal fungi associated with these roots during subsequent stages of leaf litter decomposition.

Material and methods

The samples were taken from two research-plots in Szeroki Bór mire complex, Pisz district, Warmian-Masurian Voivodeship: 1) poor fen with 100% of *Sphagnum* spp. cover and *Eriophorum angustifolium*, *Vaccinium oxycoccos*, *Carex limosa*, *Andromeda polifolia* in the herb layer, with moribund pine trees on the banks and pine seedlings in poor condition growing all over the fen; 2) pine bog forest with the dominance of *Sphagnum* spp. on the ground, with *Vaccinium oxycoccos*, *V. myrtillus*, *Rhododendron tomentosum* in herb layer. In late autumn 2010, litter-bags with ~10 g of air-dried *Sphagnum fallax* litter (top 10 cm of plant with removed capituli) were planted at both research plots. The bags were then collected in the spring, summer and autumn of 2011 and 2012, as well as in the spring and autumn of 2013, covering approximately 35 months of decomposition.

Three bags per collection time per sample site in 2011–2012 and two in 2013 were collected (in total, 22 bags per sample site), all ingrowing roots were extracted mechanically, and leaf-litter and roots were dried at 60^{o} C for 72 hours to constant weight and weighed to assess % ratio of roots in relation to litter mass remaining.

One additional bag per collection time was collected from both sites, for identification of mycorrhizal fungi associated with roots (in total 16 bags=samples, no replication was

possible). All bags were stored frozen until analyses. From each bag, mycorrhizal roots were isolated and divided, first into tree-roots and *Ericaceae*-roots, and then into morphotypes (according, e.g., to Aučina *et al*. 2011), using a Nikon SMZ800 stereomicroscope. Total genomic DNA was extracted from each morphotype using the GeneMATRIX Plant & Fungi DNA Purification Kit (EURx Ltd., Poland) following the manufacturer's instructions. The full ITS region, which is considered as general barcode marker for fungi (Schoch *et al*. 2012), was amplified using the primer pair ITS1f and ITS4 (White *et al*., 1990). DNA was amplified and sequenced as described by Budziszewska *et al*. (2011). The morphotypes were assigned to putative species by comparison of their ITS rDNA sequences with UNITE database (http://unite.ut.ee/) (Kõljalg *et al*. 2013), using the galaxieBLAST algorithm.

Results and discussion

It has to be noted that all tree-roots extracted from the litter-bags at all stages of decomposition were 100% mycorrhizal, although the viability of mycorrhizal tips was not quantitatively assessed, and therefore some of the samples used subsequently for molecular identification could already be dead and colonized by non-mycorrhizal fungi. As mycorrhizal associations are frequent among *Ericaceae*, all extracted roots belonging to this plant group were also considered 100% mycorrhizal. Absolute contents of roots (g) in the litter-bags during the decomposition period on the two sample sites are shown in Figs 1–2, and % contents of roots in these bags are shown in Figs. 3–4. Only in the case of the pine bog forest, a pattern of increasing content of root colonization of plant litter during decomposition could be observed. On the poor fen site, only fluctuations were recorded. However, the differences in root content between stages of decomposition for both sample sites were statistically insignificant (Kruskal-Wallis test, p=0,06476 for pine bog forest, and p=0,3288 for poor fen).

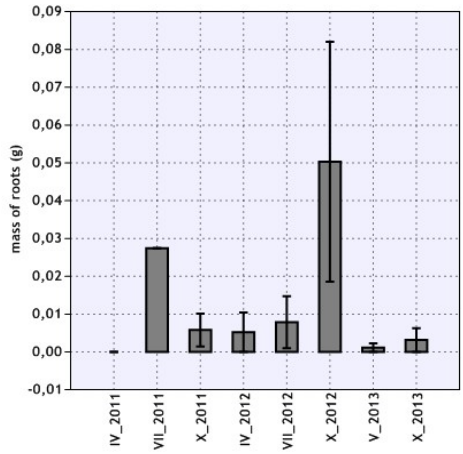

Figure 1. Mass of roots (g) colonizing *Sphagnum* litter during decomposition on poor fen. N=3 for 2011-2012, and n=2 for 2013.

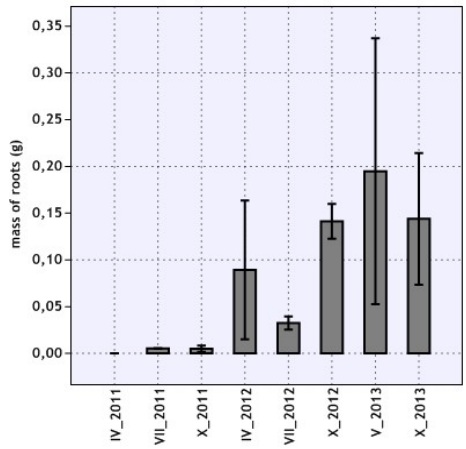

Figure 2. Mass of roots (g) colonizing *Sphagnum* litter during decomposition in pine bog forest. N=3 for 2011-2012, and n=2 for 2013.

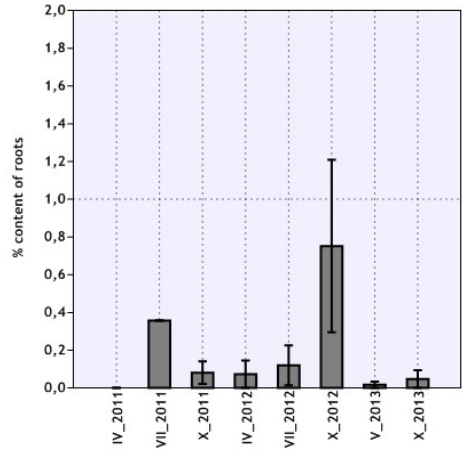

Figure 3. Content of roots (%) colonizing *Sphagnum* litter during decomposition on poor fen. N=3 for 2011-2012, and n=2 for 2013.

The results of the morphological study of mycorrhizal roots from the poor fen and from the pine bog forest are presented in Tab. 1, and Tab. 2, respectively. In total, two ericoid and 10 ectomycorrhizal morphotypes were recorded, comprising 29 samples of mycorrhizal roots (and these were subsequently used for molecular study). On the poor fen, there were two morphotypes of ericoid roots, and only two morphotypes of ectomycorrhizal roots recorded. Not surprisingly, in the pine bog forest the diversity of mycorrhizal roots was much greater, with nine ectomycorrhizal and one ericoid

morphotype recorded. Worth noting is the fact that, in total, 7 out of 10 ectomycorrhizal morphotypes were recorded only once during the whole period of decomposition, and that only one ericoid and one ectomycorrhizal morphotypes were shared between the research plots.

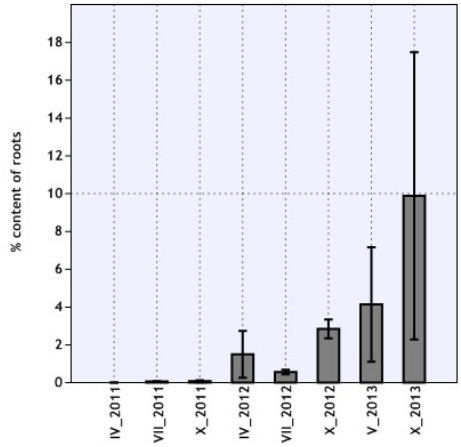

Figure 4. Content of roots (%) colonizing *Sphagnum* litter during decomposition in pine bog forest. N=3 for 2011-2012, and n=2 for 2013.

Table 1. Mycorrhizal morphotypes recorded during decomposition of *Sphagnum* litter on poor fen ("eric" – ericoid morphotypes, "m" – ectomycorrhizal morphotypes).

	IV 2011	VII 2011	X 2011	IV 2012	VII 2012	X 2012	V 2013	X 2013
ericA	-	-	+	+	+	+	+	+
ericB	-	-	+	-	-	+	+	-
mB	-	-	-	-	-	-	+	-
mC	-	-	+	-	-	-	-	-

In total, 17 out of all 29 samples of mycorrhizal roots yielded DNA of quality appropriate for molecular identification, including majority of the designated morphotypes (1 ericoid and 9 ectomycorrhizal). However, there were no simple relationships between the morphotypes and the identified sequences, i.e. where the morphotype was recorded more than once during the study, molecular identification yielded different results on different sampling seasons (data not shown). Only 7 obtained sequences could be regarded, with some caution, as mycorrhizal (Table 3). The caution is necessary in the case of wood-inhabiting saprotrophic *Ascocoryne sarcoides* and *Hypholoma*

radicosum, which could be, e.g., secondary invaders of dead or moribund roots. Some caution should also be exercised in the case of *Sebacina vermifera*, because although *Sebacinales* are well known mycorrhizal associates of Ericaceae (e.g. Weiß *et al*. 2004, Selosse *et al*. 2007), they are also known as root endophytes (Selosse *et al*. 2009, Weiß *et al*. 2011). The rest of the sequences (not shown) belonged probably to the saprotrophic secondary colonizers of roots or endophytic fungi (Geomyces sp., 7 sequences; *Cryptococcus gastricus* Reiersöl & di Menna, *Mortierella sossauensis* E. Wolf and one unidentified ascomycete; e.g. Summerbell 2005), and their invasive growth probably accounted for the variability of results of molecular identification mentioned above. Worth noting is the fact that the results of the molecular study are in accordance with field observations of macrofungal fruit-bodies (data not shown): although on the poor fen site we observed different, bryophilous species of *Hypholoma*, all species identified from pine bog forest were observed by us also as fruit-bodies.

Table 2. Mycorrhizal morphotypes recorded during decomposition of *Sphagnum* litter in pine bog forest ("eric" – ericoid morphotypes, "m" – ectomycorrhizal morphotypes).

	IV 2011	VII 2011	X 2011	IV 2012	VII 2012	X 2012	V 2013	X 2013
ericA	-	+	+	+	+	+	-	+
m1A	-	+	+	-	-	-	-	-
m2A	-	-	-	-	-	-	+	-
mC	-	-	-	-	-	-	+	-
mD	-	-	-	-	+	-	-	-
mE	-	-	-	-	+	-	-	+
mF	-	-	-	-	+	-	-	-
mG	-	-	-	-	-	+	-	-
mH	-	-	-	-	-	+	-	+
mI	-	-	-	-	-	-	-	+

Summary

Our study indicated that *Sphagnum* moss litter, decomposing in peatland ecosystems, is intensively colonized by mycorrhizal roots associated with both ericoid and ectomycorrhizal fungi. The role of these fungi in decomposition of moss litter is unknown, and was outside the scope of this study, although they certainly influence the process. In the light of the observed high variability in the colonization by mycorrhizal roots (expressed as mass or % content in

relation to remaining plant litter mass), the sample size was too small to conclude that this colonization increases with advancing stage of decomposition. The preliminary results of morphological and molecular study on mycorrhizal communities associated with roots colonizing *Sphagnum*-litter showed the presence of well-known species, occurring on the research sites also as fruit-bodies; the difference in those communities between the two sites was driven by the differences in vegetation composition. Majority of the morphotypes were recorded only once during the whole decomposition period, which could potentially indicate the existence of succession or directional changes in associated mycorrhizal communities, however the lack of replicates prevents us from giving any firm conclusions regarding this hypothesis.

Table 3. Molecular identification of putative mycorrhizal fungi recorded from roots colonizing *Sphagnum* litter during decomposition on the research sites.

IV 2011	-	-	-	-	-	-	-
VII 2011	-	-	-	+	-	-	-
X 2011	-	-	+	-	-	-	-
IV 2012	-	-	-	-	-	-	-
VII 2012	-	-	-	-	-	-	-
X 2012	-	-	-	-	-	+	+
V 2013	+	+	-	-	+	-	-
X 2013	-	-	-	-	-	-	-
species	*Sebacina vermifera* Oberw.	*Ascocoryne sarcoides* (Jacq.) J.W. Groves & D.E. Wilson	*Hypholoma radicosum* J.E. Lange	*Laccaria laccata* (Scop.) Cooke	*Thelephora terrestris* Ehrh.	*Russula emetica* (Schaeff.) Pers.(1)	*Russula emetica* (Schaeff.) Pers.(2)
site	poor fen			pine bog forest			

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Streszczenie

Podczas blisko trzyletnich badań nad rozkładem mchu torfowca na torfowisku przejściowym oraz w borze bagiennym w północno-wschodniej Polsce zaobserwowano kolonizację materiału roślinnego w woreczkach ściółkowych przez silnie zmykoryzowane korzenie. Procentowa zawartość tych korzeni, wyrażona jako stosunek ich suchej masy do suchej masy rozkładających się szczątków roślinnych, generalnie zwiększała się wraz z upływem czasu w borze bagiennym, natomiast w przypadku torfowiska przejściowego nie wykazała wyraźnych tendencji; z racji niewielkiej liczby powtórzeń zaobserwowane różnice nie były jednak istotne statystycznie. W materiale roślinnym rozkładającym się na torfowisku przejściowym odnotowano obecność dwóch morfotypów korzeni wrzosowatych oraz dwóch morfotypów korzeni ektomykoryzowych, natomiast w borze bagiennym odnotowano jeden morfotyp korzeni wrzosowatych i 9 morfotypów korzeni ektomykoryzowych. Tylko jeden morfotyp wrzosowatych i jeden ektomykoryzowy były odnotowane w obu typach siedlisk; różnice wynikały z zasadniczych różnic w składzie zbiorowisk roślinnych pomiędzy badanymi powierzchniami. Większość (7 z 10) morfotypów ektomykoryz pojawiła się tylko raz podczas całego okresu trwania eksperymentu. Badania molekularne uzyskanych morfotypów powiodły się jedynie w siedmiu przypadkach: zidentyfikowano jeden gatunek tworzący mykoryzę erikoidalną, trzy gatunki tworzące ektomykoryzy (w tym jeden tworzący dwa morfotypy) oraz dwa gatunki grzybów wielkoowocnikowych znanych jako saprotrofy, prawdopodobnie wtórnie infekujących korzenie. Sekwencje uzyskane z pozostałych badanych morfotypów należały do grzybów mikroskopijnych najprawdopodobniej kolonizujących korzenie jako saprotrofy lub endofity. Pomimo że badania niniejsze stanowią jedynie szkicowe studium, to jednoznacznie wskazują na możliwość udziału grzybów mykoryzowych w procesach rozkładu materii roślinnej w ekosystemach torfowiskowych.

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The possibilities of using data on national populations of fungal species to determine the Red List Category (based on the IUCN Criteria)

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ABSTRACT

The paper refers to the current issue of determining the threat category for fungal species based on the widely used IUCN criteria and presents the information concerns the possible usability of data on populations of fungal species, especially macroscopic ones occuring in Poland, in order to make the best possible extinction risk assessment.

The data is heterogeneous due to the various reasons, e.g. eneven mycological recognition of different parts of Poland. Therefore, the need to collect and analyze all currently available data for those taxa is urgent. The further discussion on the subject focusing on the development of a uniform national standard for such assessments is one of the most relevant issues for the near future. The arguments being presented in this paper are intented to be an additional voice in this discussion.

KEY WORDS: national red listing, threat category, IUCN, data management, macrofungi

Introduction

Data considering national populations of fungal species is heterogeneous. The sources of information about the numbers and location of previous (and historical) sites are summarized in the published checklists (e.g. Wojewoda 2003, Chmiel 2006), which were based on available information and scientific data which could be found in the literature and the mycological herbaria, but since then the tools for data management have been strongly developed. Data on the current localities is also gathered in online databases (e.g. a countrywide register of protected and endangered fungal species called GREJ; Snowarski 1997–2014) and various other online databases (often of a local range, e.g. set up only for national or landscape park areas). The existent data has been collected not only according to various, not fixed guidelines

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(e.g. uneven intensity of explorations in different parts of the country and different time intervals), but is also scattered. Therefore, the key factor in assessing species status is gathering the data itself and its thorough analysis.

Material and methods

Description of available data

Checklists and online databases

Checklists of macrofungi are characterized by lengthy time intervals during which ones the data itself was collected (e.g. about 50 localities of *Fistulina hepatica* have being reported during the period of 126 years: 1876–2002 acc. to Wojewoda 2003) and a small number of data providers, mainly professional mycologists (in the case of *Fistulina hepatica* ca. 20 authors were mentioned). It should be also noticed that many scientists, due to their full time engagements at their local universities often located in major cities, had very limited time allotted to conduct their field research. The existing data is also characterized by relatively low accuracy when it comes to locating the particular fungal sites (especially when they are compared with the current capabilities offered by GPS devices) and very limited photographic documentation (presenting the number and the state of the fruit bodies or the qualitative characteristics of the habitat). Additionally, the access to this information is very limited and often restricted as it requires a thorough study of original literature and mycological herbaria resources.

On the other hand, most currently available online databases (in Poland GREJ database) are built and supported by large numbers of informants (in case of GREJ – 236 people at present time) of whom most are considered to be amateurs and data obtained in such way is verified by experts in order to gain a scientific value.

The dispersal of informants increases the possibility of fairly well penetration of a huge area (the whole country), and their ability to temporarily reside in the particular area being studied is often incomparably greater than many professional scientists.

The photographic documentation is maintained at a high level (which is usually the basis for the preliminary determinations). Additionally, specimens are also collected and stored. In the case of the *F. hepatica* mentioned earlier, during 13 years time period (between 1999 and 2012) 145 sites were reported (all being located in 71 AtPol grid squares 10 × 10 km), and data was obtained from approximately 50 people.

Main problems and the need for future research

Considering all available data on fungal localities in Poland, the main problem concerns the assessment of the actual size of the population and the determination of how it changed in (at least) two time periods. The various degrees of involvement in the search for localities result in varying degrees of underestimation of the actual population size (IUCN methodology calls for the need to evaluate the population size going a hundred years in the past as well as to foresee it in a hundred years from now).

There is an urgent need to perform analyzes (comparative, statistical) of the data on fungal populations, that could answer the key question: how the increase in the number of people reporting new localities and a significant improvement in the communication technology (Internet tools, digital

photography, GPS) and the verification process of obtained data translates into the increase in the number of known localities of fungal species? The answer to this question is crucial for the objective assessment of population trends over time. Boddy *et al*. (2011) suggest that the sudden increase in the number of known localities of rare and endangered species is due to the tremendous growth in activity of local amateur groups, the development of the Internet and the creation of online databases. Barron (2011) states that possibly even up to 99% of the European records on fungal species come from amateurs. This is especially true in case of better-known species, e.g. protected by law for many years or forming characteristic fruit bodies.

Discussion

Usability of the IUCN Criteria in case of data deficiency

For the assessment of the risk of fungal species extinction, four main IUCN criteria (A – D) are available (Dahlberg & Mueller 2011). If, during the assessment, it is found that the test species does not meet one of the criteria (e.g. D – extremely small population size), it is necessary to go to the next criterion (all others refer to the changes of specific indicators over time). Species gets a threat category (NT-CR) based on the criterion that shows the strongest threat. If, under only one criterion, no significant risk is found, since there is currently insufficient data to conduct an overall assessment but there are reasons to believe that the species might be endangered, the correct category is DD (Data Deficient).

Assessment based on habitat quality

Criterion A

In the absence of reliable information on the population trends over time, the criteria based on the knowledge of the condition and the degree of habitat fragmentation can be used. The choice of such method of assessment seems to be the most appropriate in the case of fungi, that show a strong association with a specific habitat (e.g. *Hericium coralloides* and natural or semi-natural beech forests). In such cases, the changes in population conditions over time can be inferred from the changes in the area and the quality of the relevant habitats (criteria: A2c, A3c, A4c).

Criterion B

Another possibility of assessment based on habitats are: criterion B1ab(iii) and especially B2ab(iii). The last one concerns the total area occupied by subpopulations which is much more accurate measure than the geographical range area. IUCN (2001) recommends that the Area of Occupancy (AOO) should be calculated based on the smallest surface units which ensure the survival of subpopulations at all stages of development and are appropriate to the biological aspects of the taxon, the nature of threats and the available data. The AOO can be also interpreted as the area of (occupied) patches of habitat.

In the case of forest, wood-inhabiting fungi (especially rare, associated with the presence of old-growth stands and large diameter dead wood), spatial distribution of relevant patches of habitat is highly dependent on the forest management regime. The subpopulations of such species are usually confined to the oldest fragments of forest. The greatest threat to individuals of the species is a strong modification of habitat by economic activities (cutting old trees, dead wood removal or the movement of heavy

equipment). The planning units for such threatening events are forest subdivisions and divisions (of the average area of 25 ha = 0.25 km^2). Such territories are relatively easy to distinguish in the field and, in many cases, large enough for the individuals to survive (especially in the case of the division area). Therefore, they meet the IUCN requirements for the AOO counting units. IUCN (2013) recommends the use of grid of squares (or equivalent polygons) of a larger area (approximately 4 km^2), but this is a general guideline concerning all groups of organisms (including animals) and the measuring scale adjustments to the needs of a particular group of organisms assessment is allowed. The selection of an appropriate scale of the AOO measurement is very important since it carries a direct impact on the resulting extinction risk assessment, and the scale itself, once accepted, should be maintained for the entire group of organisms. For this reasons, this issue should be the subject of further discussion.

If the resulting AOO is located within the given thresholds: <4 000 km^2, as proposed by Dahlberg and Mueller (2011), it is necessary to determine if the range of the species (also: extent of habitats) is subject to a strong fragmentation, under subcriterion (a). The box-counting fractal dimension D can be a useful tool for that purpose (according to Hartley & Kunin 2003, Kędra 2013). The final step is the assessment under the subcriterion b(iii) by answering the question: 'whether the quality of the species habitat is continuing to decline?' (without the need of giving a specific, percentage value).

The aforementioned possibilities of assessment under the IUCN criteria: A2c, A3c, A4c, B1ab(iii), B2ab(iii) and D, seem to have the best use with the currently available data on national populations of fungi. The remaining criteria should be used after a critical assessment of how the increased activity in exploration of fungal localities impacts their known number.

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Streszczenie

Wstęp

Dane o krajowych populacjach gatunków grzybów mają charakter niejednorodny. Źródła informacji o lokalizacjach i liczbie wcześniejszych (także historycznych) stanowisk są zebrane w opublikowanych krytycznych listach (checklistach) (np. Wojewoda 2003, Chmiel 2006), które zostały opracowane na miarę ówczesnych możliwości, na podstawie dostępnych publikacji i zasobów zielników mykologicznych. Natomiast dane o aktualnych stanowiskach gromadzone są dodatkowo, także w bazach internetowych (np. ogólnopolski rejestr gatunków grzybów chronionych i zagrożonych – GREJ; Snowarski 1997–2014) oraz w innych elektronicznych bazach danych (często o zasięgu lokalnym, np. dla obszaru parku narodowego lub krajobrazowego). Dane te nie tylko gromadzone były i są według różnych wytycznych (np. niejednolita intensywność poszukiwań stanowisk w różnych rejonach kraju i różnych przedziałach czasowych), ale także są rozproszone – jedną z trudności przy ocenie gatunku jest ich zgromadzenie i krytyczna analiza.

Materialy i metody

Zakres wymaganych informacji

Ocena stopienia zagrożenia gatunku wg kryteriów IUCN wymaga zebrania danych, przede wszystkim w zakresie: liczebności populacji, zasięgu geograficznego [km^2], zajmowanej powierzchni przez subpopulacje [km^2], a także – co bardzo ważne – o trendach zmian tych wielkości w czasie (najlepiej w postaci wartości procentowych). Niezwykle istotne są też informacje o cechach biologicznych i ekologicznych ocenianych gatunków, stopniu fragmentacji ich populacji oraz typowych zagrożeniach (IUCN 2012).

Charakterystyka dostępnych danych

Krytyczne listy a bazy internetowe

Krytyczne listy grzybów wielkoowocnikowych charakteryzują się bardzo dużym przedziałem czasowym, z którego pochodzą dane (np. dla ozorka dębowego *Fistulina hepatica* podaje się ok. 50 stanowisk, stwierdzonych w okresie 126 lat: 1876–2002) (Wojewoda 2003), a także niewielką liczbą osób, od których pochodzą dane, głównie profesjonalnych mykologów (w tym wypadku było to ok. 20 nazwisk autorów źródeł). Należy także zauważyć, że wielu naukowców ma silnie ograniczone możliwości czasowe przebywania w terenie, co wiąże się z ich codzienną pracą na uczelniach –

często zlokalizowanych w większych miastach. Kolejną cechą tych danych jest stosunkowo niewielka dokładność podawania lokalizacji stanowisk (zwłaszcza w porównaniu z aktualnymi możliwościami GPS) oraz nikła dokumentacja fotograficzna obrazująca liczbę i stan owocników oraz cechy jakościowe siedliska. Dostęp do tych informacji nie jest bezpośredni i należy ich szukać w źródłowych publikacjach i profesjonalnych zielnikach mykologicznych.

Natomiast aktualne bazy internetowe (w Polsce – baza GREJ) są wspierane przez dużą rzeszę informatorów (obecnie 236 osób), głównie amatorów, których zgłoszenia weryfikowane są przez specjalistów – przez co zyskują wartość naukową. Rozproszenie miejsc zamieszkania informatorów zwiększa możliwość penetracji ogromnego obszaru (całego kraju), a ich możliwości czasowe przebywania w terenie są często nieporównywalnie większe niż wielu naukowców. Dokumentacja fotograficzna utrzymywana jest na wysokim poziomie (zwykle jest to podstawą do wstępnych oznaczeń), dodatkowo zbierane i przechowywane są eksykaty. W przypadku wspomnianego wcześniej *F. hepatica*, w ciągu 13 lat (w okresie 1999–2012) zebrano w ten sposób informacje o 145 stanowiskach (zlokalizowanych w 71 kwadratach AtPol 10 × 10 km), a dane pochodzą od ok. 50 osób.

Główne problemy i cel przyszłych badań

Rozpatrując łącznie wszystkie dostępne dane o stanowiskach grzybów, podstawowy problem dotyczy oceny rzeczywistego rozmiaru populacji i porównania tej wielkości w (przynajmniej) dwóch okresach czasowych, gdyż różny stopień zaangażowania w poszukiwania stanowisk skutkuje różnym stopniem niedoszacowania wielkości populacji rzeczywistej (metodyka IUCN przewiduje potrzebę oceny w perspektywie do stu lat wstecz, a także przewidywania na sto lat do przodu).

Istnieje pilna potrzeba wykonania analiz (porównawczych, statystycznych) danych o populacjach gatunków grzybów, które odpowiedzą na pytanie: jak wzrost liczby osób zgłaszających nowe stanowiska grzybów oraz znaczna poprawa możliwości komunikacji i weryfikacji stanowisk (narzędzia internetowe, fotografia cyfrowa, GPS) przekłada się na wzrost liczby znanych stanowisk grzybów? Odpowiedź na to pytanie jest kluczowa dla obiektywnej oceny trendów populacji w czasie. Boddy i in. (2011) dopatrują się przyczyn nagłego wzrostu liczby znanych stanowisk gatunków rzadkich i zagrożonych w kolosalnym wzroście aktywności lokalnych grup amatorów, w rozwoju Internetu i w tworzeniu baz danych. Natomiast Barron (2011) podaje, że prawdopodobnie nawet do 99% informacji o europejskich stanowiskach gatunków grzybów pochodzi od amatorów. Zjawisko to dotyczy przede wszystkim gatunków dość dobrze znanych, np. od wielu lat objętych ochroną gatunkową i/lub tworzących charakterystyczne owocniki.

Dyskusja

Użycie kryteriów IUCN w przypadku niedostatku danych

W przypadku oceny stopnia zagrożenia gatunków grzybów, do dyspozycji są cztery główne kryteria IUCN (A-D) (Dahlberg & Mueller 2011). Jeśli w trakcie oceny okaże się, że badany gatunek nie spełnia któregoś z kryteriów (np. D – skrajnie niskiej liczebności), należy rozpatrywać według kolejnych kryteriów (pozostałe: A, B i C – bazują na zmianach określonych wskaźników w czasie). Gatunkowi przyznaje się kategorię zagrożenia (NT-CR) na podstawie kryterium, które wskazuje na najsilniejsze

zagrożenie. Jeśli brak istotnego zagrożenia określono na podstawie tylko jedno kryterium, gdyż nie ma aktualnie wystarczających danych do przeprowadzenia całościowej oceny, a są podstawy żeby sądzić, że gatunek może być zagrożony – właściwą kategorią gatunku jest DD (brak danych).

Ocena stopnia zagrożenia w oparciu o stan siedlisk

Kryterium A

W przypadku braku wiarygodnych informacji o trendzie liczebności populacji w czasie, można użyć kryteriów opierających się o ocenę stanu i stopnia fragmentacji siedlisk. Wybór takiego sposobu oceny wydaje się najtrafniejszy w przypadku gatunków grzybów, które wykazują silny związek z określonymi siedliskami, a których przeszłość jest znana (np. soplówka bukowa *Hericium coralloides* związana z buczynami o charakterze naturalnym). W takich przypadkach, zmiany kondycji populacji w czasie można ocenić na podstawie zmian areału i stanu właściwych siedlisk (kryteria: A2c, A3c, A4c).

Kryterium B

Kolejną możliwość oceny w oparciu o stan siedlisk stanowią: kryterium B1ab(iii), a zwłaszcza B2ab(iii) – gdyż to ostatnie dotyczy także sumarycznej powierzchni, zajmowanej przez poszczególne subpopulacje gatunku (co jest znacznie dokładniejszą miarą niż powierzchnia zasięgu geograficznego). IUCN (2001) zaleca, aby zajmowany obszar (Area Of Occupancy, AOO) obliczony był na podstawie najmniejszych jednostek powierzchni, zapewniających trwanie subpopulacji we wszystkich stadiach rozwojowych, o rozmiarze dostosowanym do biologii gatunku, natury zagrożeń i dostępnych danych. Dopuszcza się także obliczenie tej wielkości na podstawie powierzchni (zajętych) siedlisk gatunku.

W przypadku leśnych gatunków grzybów (zwłaszcza rzadkich, związanych z obecnością starodrzewu i martwego drewna), rozmieszczenie właściwych siedlisk jest silnie uzależnione od reżimu prowadzonej gospodarki leśnej. Subpopulacje takich gatunków najczęściej ograniczają się do powierzchni najstarszych fragmentów drzewostanu. Natomiast największe zagrożenie dla osobników gatunku polega na możliwości silnej modyfikacji siedliska przez działania gospodarcze (wycięcie starodrzewu, usunięcie martwego drewna, ruch ciężkiego sprzętu). Jednostkami planowania działań stanowiących zagrożenie są wydzielenia i oddziały leśne (średnio o pow. 25 ha = 0,25 km^2), są to powierzchnie stosunkowo łatwe do wyodrębnienia w terenie i jednocześnie wystarczająco duże, aby osobniki mogły przetrwać (zwłaszcza w przypadku oddziału leśnego), więc spełniają wymagania dla jednostek zliczania powierzchni AOO. IUCN (2013) zaleca użycie siatki kwadratów (lub równoważnych poligonów) o większej powierzchni: ok. 4 km^2, jest to jednak ogólne wskazanie, dla wszystkich grup organizmów (także zwierząt) i dopuszcza się dostosowanie skali pomiaru do potrzeb oceny określonej grupy organizmów. Dobór właściwej skali pomiaru AOO jest bardzo istotny, gdyż bezpośrednio rzutuje na uzyskaną ocenę stopnia zagrożenia, a raz przyjęta skala powinna być utrzymana dla całej grupy organizmów. Z tego powodu, to zagadnienie powinno być przedmiotem dalszej dyskusji.

Jeśli obliczona powierzchnia AOO mieści się w podanych przedziałach liczbowych (< 4 000 km^2; wg Dahlberg & Mueller 2011), należy przejść do podkryterium (a) i

ustalić czy zasięg występowania gatunku (także: zasięg siedlisk) podlega silnej fragmentacji (do czego przydatne może być obliczenie wymiaru fraktalnego D; wg Hartley & Kunin 2003, Kędra 2013). Ostatnim krokiem jest ocena wg podkryterium b(iii), przy którym należy odpowiedzieć na pytanie: „czy jakość siedlisk gatunku ulega ciągłemu pogorszeniu?" (bez konieczności podawania wartości procentowej).

Podsumowanie

Przedstawione możliwości oceny stopnia zagrożenia gatunków grzybów (kryteria IUCN: A2c, A3c, A4c, B1ab(iii), B2ab(iii) oraz D) wydają się mieć najlepsze zastosowanie przy aktualnym stanie dostępnych danych o ich krajowych populacjach. Pozostałe kryteria powinny być użyte po krytycznej ocenie wpływu wzrostu aktywności poszukiwań grzybów na znaną liczbę ich stanowisk.

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Gasteroid fungi – the morphological characteristics of selected endangered and rare species noted in Poland

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ABSTRACT

The aim of the work was to present the characteristics of selected species from *Disciseda, Geastrum* and *Tulostoma* genera which due to the small differences in morphology of their fruit bodies may pose some identification problems. The selected species of gasteroid fungi of these genera are described based on the materials collected during the course of our studies. All materials were gathered during the research into macromycetes in xerothermic habitats located in the Nida Basin. Taxa noted by us are considered to be very rare in the mycobiota of Poland and are highly endangered.

KEY WORDS: Gasteromycetes, thermophilic fungi, endangered species of fungi, Agaricaceae, Geastraceae, Lycoperdaceae

Introduction

The gasteroid fungi (formerly Gasteromycetes) are polyphyletic group of fungi which currently belong to different taxa in the *Agaricomycetes* class (Hibbett & Thorn 2001, Binder & Bresinsky 2002). Biological and morphological properties as well as the development, maturation and dispersal of spores are characteristic features of the *Gasteromycetes*. Gasteroid fungi are usually spherical, piriform and clavate. The hymenium is enclosed inside the fruit body until spores mature. Fruit bodies consist of three basic parts: peridium (the wall), gleba (the fertile area) and trama (sterile hyphae that form pseudoparenchyma; Pilát 1958, Rudnicka-Jezierska 1991).

Fruit bodies of gasteroid fungi develop underground (hypogeously) and are spherical. As they mature they emerge over ground becoming epigeous. Gasteroid fungi are mainly saprobionts that grow in woodless areas, xerothermic, sandy and steppe sites, in forests, but also in wet places and even on the moors (Pilát 1958, Rudnicka-Jezierska 1991).

Species that differ by small macro- and micromorphological characters of the fruit body's structure in individual genera were selected for morphological analysis. A scrupulous submicroscopic and molecular examination of the structure of

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similar taxa helps to identify fruit bodies correctly and reduces the risk of determination errors (e.g. Tomaszewska *et al*. 2011).

The aim of this study was to present the characteristics of selected species from *Disciseda, Geastrum* and *Tulostoma* genera, which due to the small differences in morphology of their fruit bodies may pose some identification problems.

Material and methods

Species were collected during the research into macromycete fungi in xerothermic habitats located in the Nida Basin (south of Poland) between 1991 and 2013. The studies were intensified from 2010 until 2013. The investigations were conducted in protected areas such as nature reserves (Krzyżanowice, Skorocice), landscape parks and Natura 2000 sites (Nida Landscape Park – PLH260003 Ostoja Nidziańska, Szaniec Landscape Park – PLH260034 Ostoja Szaniecko-Solecka, Kozubów Landscape Park – PLH260029 Ostoja Kozubowska). The examined plant communities are protected under the Habitats Directive. The investigations conducted in these respected areas also provided data about their functioning and the interactions between fungi and xerothermic vegetation.

The mycological investigations were conducted using permanent research plots and were supplemented with the route method. A total of 30 plots (each of them of 100 m^2) were established in six communities of xerothermic vegetation from *Festuco-Brometea* class, such as: *Adonido-Brachypodietum pinnati, Festucetum pallentis, Inuletum ensifoliae, Sisymbrio-Stipetum capillatae, Seslerio-Scorzoneretum purpureae* and *Thalictro-Salvietum pratensis* (names according to Matuszkiewicz 2012). The observations and collections of fruit bodies were carried out at intervals. The number of species fruit bodies, the organoleptic properties, i.e. the shape, size and colour of the endoperidium, the colour of the exoperidium and the manner in which it flakes, and also the pigmentation and the structure of the stem surface, were all noted during collections.

The laboratory examinations were conducted using light microscopy (LM) and scanning electron microscopy (SEM). The structure, size and shape of the capillitium and the spores were measured using standard reagents and light microscope. The measurements were performed using 400x and 1000x magnification. The episporium sculpture was investigated using SEM.

The material (gleba samples with spores) was mounted on an aluminium stub and coated with 24-carat gold (Karcz 1996, 2009). The electron micrographs were taken at the magnifications of: 3000, 5000, 10000 and 12000x. The studies with the help of the scanning electron microscope (SEM) were carried out in the Department of Environment Protection of Jan Kochanowski University in Kielce and in the Laboratory of Field Emission, Scanning Electron Microscopy and Microanalysis at the Institute of Geological Sciences of Jagiellonian University in Kraków.

The following studies were used for taxonomic identification: Pilát (1958), Wright (1987), Rudnicka-Jezierska (1991), Sarasini (2005) and Sunhede (1990). The nomenclature of the taxa is given after Index Fungorum (2014).

Results

Eight selected species of gasteroid fungi were examined. Macro- and micromorphological characters were used to describe the species. Species descriptions are based on material collected in our investigations. A list of similarities and differences between the described species is given in Tables 1, 2 and 3.

Disciseda bovista (Klotzsch) Henn.

Mature fruit bodies globose, rarely flattened. Exoperidium white, whitish yellow, mature brownish ashen. Endoperidium is rigid, pergameneous and nut-like coloured. Spores globose, (5–)6.5–7.8(–8.6) µm in diam. (according to Lizárraga *et al*. 2010: 4–7 µm in diam.), distinctly strongly verrucose, verrucae 1–1.5 µm, without sterigmata (Fig. 1a). Capillitium is light yellow, hyaline, quite thick-walled. Capillitium threads are wavy, brittle and 2.7–3.5 µm thick.

Disciseda candida (Schwein.) Lloyd

Mature fruit bodies are loaf-like. Exoperidium is dirty whitish yellowish, mature, earth brown. Endoperidium strong, leathery, matt, brown-grey to ashen in colour. Spores globose, punctate, delicately verrucose or glabrous, (3.8–)4.5–5 µm (according to Bates *et al*. 2009: 4.0–5.6(–6.4) × 4.0–5.6(–6.4) µm in diam.), without sterigmata (Fig. 1b). Capillitium light yellow, hyaline and thin-walled. Capillitium threads wavy, brittle and 2.5 µm thick.

Geastrum campestre Morgan

Exoperidium splits into 5 to 12 triangular segments that are hygroscopic or subhygroscopic. It is beige-coloured, grey-brown, light brown to dark brown and when expanded 3 to 5 cm in diam. on average. Endoperidium globose is 0.5 to 2 cm in diam. and only partially with apophysis. The granulately rough, farinaceous surface of the endoperidium is a characteristic feature of the species. Peristome cristate with delimited bulge, from 12 to 20 ridges (Fig. 2a). Gleba dark brown. Spores globose, finely verrucose, 4.8–7(–8) µm. It should be noted that there is a possibility of mistaking this species with *Geastrum berkeleyi* Massee which also has granulately surface of the endoperidium and cristate peristome.

Geastrum minimum Schwein.

Exoperidium splits into 6 to 12 not hygroscopic segments that reach 3 to 4 cm in diam. when expanded. Endoperidium globose is 0.4 to 1.2 cm in diam., grey-brown, ochraceous-brown or grey-white. A white layer of fine crystals of calcium oxalate on the endoperidium is the main diagnostic trait of the species. Peristome sericeous-fimbriate is lighter than the remainder of theendoperidium, with a collar delimited by a bulge (Fig. 2b). Gleba dark brown. Spores globose, 3.5–5.5(–7) µm in diam., minutely verrucose.

Geastrum schmidelii Vittad.

Exoperidium splits into 5 to 10 entirely not hygroscopic segments that reach 1-3 cm in diam. when expanded. Endoperidium globose is brown-grey to brown at the bottom, whitish at the top, especially in young fruit bodies. Peristome sulcate, from 10 to 19 ridges, delimited by a furrow, covered with farinose coating in young fruit bodies (Fig. 2c). Gleba dark brown. Spores globose, 4.7–7.5 µm (according to Sarasini 2005: (4–)4.2–4.8(–5.5) µm in diam.), distinctly thickly verrucose.

Tulostoma brumale Pers.

Exoperidium whitish, membranous, soon flaking away. Mature endoperidium ochraceous-white, sometimes with rusty-brown stains, also yellowish brown. Peristome tubular, mouth area darker

coloured, yellowish or dirty brown. Stem fibrillose, ochraceous-fawn, minutely and very delicately squamulose, straight (Fig. 3a). Spores (4.2–)4.7–5.64 µm in diam. (according to Sarasini 2005: (3.5–)4.2–4.7(–5.5) µm in diam.), globose, light yellow, minutely verrucose (Fig. 3b). Capillitium hyaline, thick-walled, lumen small, coloured at septa and swollen in septa, branched, external surface covered with fine crystals.

Tulostoma melanocyclum Bres.

Exoperidium whitish or light ochraceous, hyphal, persisting quite long. Endoperidium ashen-ochraceous or ochraceous-rusty. Peristome tubular, dark, dirty brown. Stem ochraceous-brown to dark brown, sulcate, covered with fine, adherent squamulae (Fig. 3c). Spores are globose and subglobose (Fig. 3d), brown, spinulose, (6.58–)7.52–8.46 µm (according to Hansen & Knudsen 1997: 4.5–5 µm, without the ornamentation). Capillitium subhyaline, thick-walled, well visible lumen, colourless and not thickened at septa, moderately branched, without crystals.

Tulostoma squamosum Gmelin

Exoperidium dark, sometimes whitish, thin-walled, persisting longer. Endoperidium pergameneously rigid, light yellow, white-ochraceous, becoming chestnut-coloured. Peristome tubular, mouth area concolourous with the head, stem cinnamon-coloured or brown-red, unevenly covered with protruding, sharp squamulae (Fig. 3e). Spores globose and subglobose, yellow-brown, small spiny, (4.5–)5.64–7.52 µm (Fig. 3f). Capillitium hyaline, thick-walled, lumen visible, slightly yellowish and often broader at septa, branched, without crystals.

Table 1. The comparison of selected morphological and anatomical characteristics in similar *Disciseda* species.

Feature	*Disciseda bovista*	*Disciseda candida*
Exoperidium	White, whitish yellow, mature brown pale grey	Dirty whitish yellowish, mature from brownish to earthy coloured
Endoperidium	Rigid, pergameneous, hazel nut like coloured	Strong, leathery, pergameneous, brown-grey
Peristome	Frayed	Frayed, frimbrillate
Gleba	Red brown	Brightly brown, rusty brown
Spores	(5–)6.5–7.8(–8.6) µm in diam. Distinctly strongly verrucose (verrucae 1-1.5 µm)	(3.8–)4.5–5 µm in diam. Smooth or puncticulate, very fine verrucose
Sterigmata	Absent	Absent
Capillitium	2.7–3.5 µm thick, wavy, fragile	2.5 µm thick, wavy, fragile

Discussion

The new information regarding many rare and endangered species of gasteroid fungi in Poland was collected in our study. Only one species, *Tulostoma brumale* has a larger number of localities, and for this reason it can be considered for more frequent. Other species are very rare and are considered as endangered (E) in Polish Red List (Wojewoda & Ławrynowicz 2006).

The aim of the study was to draw the attention to the selected features of fungi fruit bodies from the *Disciseda, Geastrum* and *Tulostoma* genera which due to their variability are difficult to identify. Full descriptions of the taxa are based on macro- and micromorphological characters recorded in field studies and laboratory examinations. The important differences between fruit bodies within genera and species may be noticeable but are strongly influenced by the sample size being collected and how well the morphology is being preserved. They also become less distinct as fruit bodies mature and become old. Consequently, our research has shown the need to conduct parallel complementary studies with the use of LM and SEM to carry out the correct determination of these fungi. The climatic and habitat factors can also impact phenotypic characters (Tomaszewska *et al*. 2012, 2014). The identification of taxa based only on observations of features by using the LM may contribute to erroneous determinations. Further complex investigations into gasteroid fungi are needed in order to verify fully the morphological structure and also to identify taxa correctly.

Table 2. The comparison of selected morphological and anatomical characteristics in similar *Geastrum* species.

Feature	***Geastrum campestre***	***Geastrum minimum***	***Geastrum schmidelii***
Exoperidium	Splits into 5–12 triangular segments; hygroscopic or subhygroscopic; Beige-coloured, grey-brown, light brown to dark brown	Splits into 6–12 segments; not hygroscopic; Grey brown, ochraceous brown or grey white	Splits into 5–10 segments; not hygroscopic; Grey white to brown
Endoperidium	Globose, 0.5–2 cm in diam; Granulately rough, farinaceous surface	Globose, 0.4–1.2 cm in diam; Presence of crystals of calcium oxalate on the surface	Globose, 0.3–2.5 cm in diam; Smooth surface
Peristome	Cristate, with delimited bulge, 12–20 ridges	Sericeous-fimbriate, lighter than the remainder of the endoperidium, with a collar delimited by a bulge	Sulcate, delimited by a furrow, covered with farinose coating in young fruit bodies, 10–19 ridges
Gleba	Dark brown	Dark brown	Dark brown
Spores	4.8–7(–8) µm in diam, globose, finely verrucose	3.5–5.5(–7) µm in diam, globose, minutely verrucose	4.7–7.5 µm diam, globose, distinctly thickly verrucose

Table 3. The comparison of selected morphological and anatomical characteristics in similar *Tulostoma* species.

Feature	*Tulostoma brumale*	*Tulostoma melanocyclum*	*Tulostoma squamosum*
Exoperidium	Whitish, membranous, early flaking	Whitish or pale ochraceous, relatively long-term	Dark, sometimes whitish, thinly membranous, more durable
Endoperidium	White ochraceous, yellowish brown	Grey and ochraceous, cinereous ochraceous	White ochraceous
Peristome	Tubular, around the mouth dark coloured – yellowish or dirty brown	Tubular, dark, dirty brown	Tubular, around the mouth colored as the peridium
Gleba	Pale ochraceous	Pale ochraceous, ferrugineous	Pale ochraceous, ferrugineous
Stem	16–29 × 2–2.8 mm; fibrous, pale ochraceous, covered with tiny and very delicate scales	23–33 × 2–3 mm, ochraceous brown to dark brown, furrowed, covered with	18–24(–28) × 2.5–4.5 mm; cinereous or brown red, uniformly covered with coarse
Spores	(4.2–)4.7–5.64 µm in diam; globose, pale ochraceous, with small verrucae	(6.58–)7.52–-8.46 µm in diam; globose, brown, delicate spiny	(4,5–)5.64–7.52 µm in diam; globose or subglobose, yellow brown, small, spiny
Capillitium	Width of capillitium: 3.7–5.64 µm, width of septa: (6.11–) 8.64–9.4 µm. Hyaline, thick-walled with small lumen, swollen at the coloured septa, branched, external surface covered with crystalline plaques	Width of capillitium: (4.7–)5.64–6.58 µm, width of septa: 5.64 µm. Almost hyaline, thick-walled with a visible lumen, not swollen at the uncoloured septa, branched, without crystalline plaques	Width of capillitium: 4.7–6.58 µm, width of septa: 4.7–5.64 µm. Hyaline, thick-walled with a visible lumen, slightly swollen at the yellowish septa, branched, without crystalline plaques

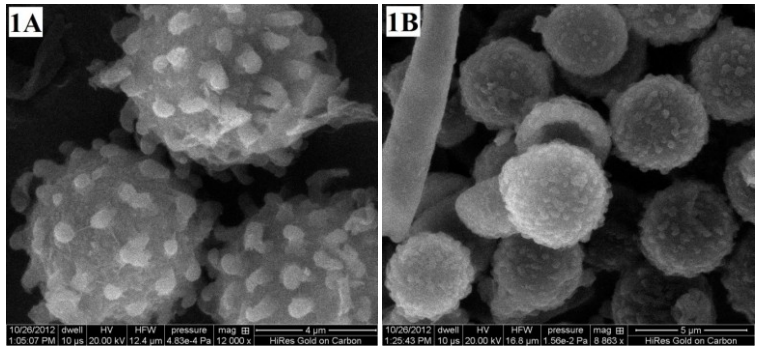

Figure 1. Spores of *Disciseda* species in SEM: A – *D. bovista,* B – *D. candida*.

Figure 2. The morphology of the peristome and the structure of the endoperidium surface in *Geastrum* species (photo by A. Tomaszewska): A–*G. campestre*, B–*G. minimum*, C – *G. schmidelii*. Scale bars = 5 mm.

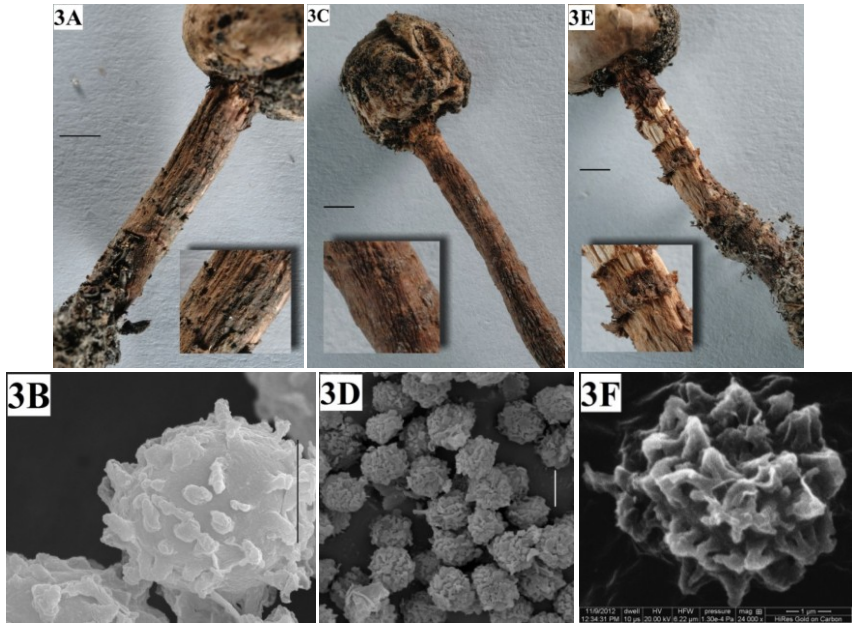

Figure 3. The morphology of the fruit body stem and the spore texture (in SEM) of *Tulostoma* species: A, B – *T. brumale*; C, D – *T. melanocyclum;* E, F – *T. squamosum*. Scale bars: A, C, E = 3 mm, B = 3 µm, D = 5 µm, F = 1µm, (photos A, C, E by G. Wołczyk).

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Streszczenie

W pracy opisano osiem wybranych gatunków grzybów gasteroidalnych należących do rodzajów: *Disciseda, Geastrum* oraz *Tulostoma.* Były to następujące gatunki: *Disciseda bovista, D. candida, Geastrum campestre, G. minimum, G. schmidelii, Tulostoma brumale, T. melanocyclum* oraz *T. squamosum*. Wszystkie gatunki zebrano podczas badań nad grzybami wielkoowocnikowymi na siedliskach kserotermicznych Niecki Nidziańskiej głównie w latach 2010-2013. Badania prowadzono w sześciu

zbiorowiskach roślinności kserotermicznej – *Adonido-Brachypodietum pinnati, Festucetum pallentis, Inuletum ensifoliae, Sisymbrio-Stipetum capillatae, Seslerio-Scorzoneretum purpureae* and *Thalictro-Salvietum pratensis* (nazewnictwo za Matuszkiewicz 2012). W fitocenozach tych zespołów wyznaczono trzydzieści powierzchni badawczych, na których prowadzono obserwacje w regularnych dwutygodniowych odstępach czasu.

Zanotowane grzyby należą do gatunków silnie zagrożonych w naszym kraju. Tylko jeden z nich – *Tulostoma brumale* – posiada większą liczbę stanowisk i z tego powodu można uznać go za częstszy. Pozostałe należą do grzybów bardzo rzadkich, a także do gatunków wymierających w Polsce, kategoria E (Wojewoda & Ławrynowicz 2006).

Do analizy morfologicznej wybrano gatunki, które w obrębie rodzajów wykazują niewielkie różnice w budowie owocników. Na podstawie cech makro- i mikromorfologicznych, które zaobserwowano podczas badań terenowych i laboratoryjnych, sporządzono pełne opisy zanotowanych taksonów. Istotne różnice w budowie owocników poszczególnych taksonów wynikają ze stopnia rozwoju i zachowania cech poszczególnych owocników (Tomaszewska *et al*. 2011). Cechy te zacierają się w miarę dojrzewania i starzenia się tych struktur. Na wykształcenie cech fenotypowych mogą także wpływać między innymi czynniki pogodowe i siedliskowe (Tomaszewska *et al*. 2012). Otrzymane wyniki wskazują na potrzebę dalszych badań tej grupy grzybów, w celu poznania pełnej zmienności cech budowy morfologicznej i poprawnej identyfikacji taksonów.