PH.D. THESIS

CHARACTERISATION OF OPPORTUNISTIC PATHOGENIC ISOLATES OF *COCHLIOBOLUS*

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INTRODUCTION

Members of the genus *Cochliobolus* belong to the ascomycetous order *Pleosporales* and can be isolated from graminiculous hosts and grasses. These melanized fungi are able to produces several types of secondary metabolites, which have important roles in the infections of plants. Asexual stages of the genus *Cochliobolus* are placed in the two anamorphic genera *Bipolaris* and *Curvularia*.

The species *B. australiensis*, *B. hawaiiensis*, *B. spicifera*, *Cu. lunata, Cu. brachyspora* and *Cu. senegaliensis* can be frequently isolated from phaeohyphomycosis, which is the summarizing name of the infections caused by melanized fungi. Furthermore, these fungi are the most important causative agents of the allergic fungal sinusitis and, after *Aspergillus* and *Fusarium* spp., are the third most frequent causing agents of keratomycosis. The species are common in the tropical and subtropical regions of the world, but due to the climatic changes, they are emerging in other regions too.

Among the members of the family Pleosporaceae, the genus *Cochliobolus* can be easily differentiated based on the ascospores, while the genera *Bipolaris* and *Curvularia* can be identified based on the condial morphology. The species *B. australiensis* and *B. spicifera* are very difficult to distinguish both on the basis of the currently used morphological markers (such as the length and width of the conidia and the number of septa) and via the sequencing of the ITS region.

The taxonomy of the genera *Cochliobolus, Bipolaris* and *Curvularia* was examined in detail by the sequence analysis of the ITS region and the *gpd*, *Brn1* and LSU genes. The genus *Cochliobolus* could be separated into two groups. The *Cochliobolus* group 1 contains the highly virulent phytopathogenic *Bipolaris* species with typical, large canoe-shaped conidia, while the *Cochliobolus* group 2 is formed by *Bipolaris* species producing atypical, little or medium-sized, fusiform-shaped conidia and the *Curvularia* species together. This *Cochliobolus* group 2 also contains the studied *B. australiensis, B. hawaiiensis* and *B. spicifera* species. These opportunistic pathogenic species are poorly characterized from the aspects of extracellular enzyme production, which may be important in pathogenesis, and the carbon assimilation or antifungal susceptibility spectra.

AIMS

The objectives of our study were the investigation of the morphological characters used in the species differentiation of *B. australiensis, B. hawaiiensis* and *B. spicifera;* the identification of species specific molecular markers and the characterization of physiological

features of the human pathogenic *Cochliobolus* isolates. In addition, the investigation of the antifungal effects of ophiobolin A produced by the members of the genus *Bipolaris.*

For this purpose, the following specific objectives have been formulated:

1. Investigation of the morphological markers used in the species identification of the genera *Bipolaris* and *Curvularia*.
2. Identification of molecular markers for the differentiation of the human pathogenic *Bipolaris* species.
3. Phylogenetic analysis of the isolates.
4. Carbon source assimilation of the human pathogenic *Cochliobolus* isolates.
5. Extracellular enzyme production of the human pathogenic *Cochliobolus* isolates.
6. Antifungal susceptibility of the *Cochliobolus* isolates.
7. Inhibitory effect of antifungals combined with statins against *Cochliobolus* isolates.
8. Antifungal effects of ophiobolins produced by *Bipolaris* spp.

METHODS

DNA based techniques:
- Purification of genomic DNA
- Polymerase Chain Reaction (PCR)
- DNA sequencing

Agarose gel electrophoresis
Nucleotide and amino acid sequence assays:
- Nucleotide sequence analysis (BLAST, FASTA)
- Nucleotide and amino acid sequence alignment (ProbAlign)
- Phylogenetic analysis (MrBayes)

Carbon source assimilation tests on agar slants
Antifungal susceptibility test on 96-well microtiter plates (Checkerboard titration method by the drug combinations)
Analytical methods:
- Spectrofotometry (VIS)
- High Performance Liquid Chromatography (HPLC)
- Thin layer chromatography (TLC)

Light- and fluorescent microscopy

RESULTS

Investigation of the morphological markers used in the species identification of the genera *Bipolaris* and *Curvularia*.

Morphological characters currently used in the species identification, such as the shape, length and width of the conidia and number of the conidial septa were re-examined in the isolates derived from human keratomycosis and obtained from culture collections (CBS, BRIP). The isolates of *Curvularia* sp. could be easily differentiated from the *Bipolaris* species based on their different conidial morphology, while the isolates of *B. hawaiiensis* could be

identified by the size of the conidia. We did not find any morphological feature that clearly discerns *B. australiensis* and *B. spicifera* from each other. It is similar to the results of McGinnis (1986), who made the most comprehensive study of the human pathogenic *Bipolaris* species.

Identification of molecular markers for the differentiation of the human pathogenic *Bipolaris* species

Sequence-based identification of the species *B. australiensis, B. hawaiiensis* and *B. spicifera* was also examined. Currently the ITS region is used to verify the morphology-based identification of these fungi. However, we did not find any sequence motifs which are able to clearly differentiate the three investigated species. Moreover, similarity searches performed in international sequence databases using the ITS region gave 98-100% identity for *B. australiensis, B. hawaiiensis* and *B. spicifera*, as well as many other *Bipolaris* species. Therefore, additional sequences, such as calmodulin *(cmd)*, translational elongation factor 1α (*tef*), tubulin (*tub*) and the intergenic spacer of the rDNA (IGS) were also involved in the analysis. The type strains of *B. australiensis*, *B. hawaiiensis* and *B. spicifera* were used as reference strains during the investigations. The proposed marker motifs were identified in an alignment of the reference strains at first, then, they were tested in larger aligments of strains from keratomycoses and other sources. On the basis of *cmd, tub* and *tef*, the three *Bipolaris* species could not be differentiated, while the IGS region contained a numerous motifs and nucleotide positions useful for the identification of the species. We suggest sequencing of the IGS region for the differentiation of *B. australiensis, B. hawaiiensis* and *B. spicifera* isolates. RAPD analysis was also performed to test it as a possible method for species identification and reproducible species-specific amplification patterns could be established with certain primers.

Phylogenetic analysis

Because of the morphological and molecular similarity of *B. australiensis* and *B. spicifera*, it was questioned that they are really different species. Therefore, a phylogenetic analysis was performed based on the nucleotide sequence of the ITS region, the *tef* gene and the RAPD data (alone and in combinations) using the Bayes-algorithm, but the phylograms showed high amount of politomy and low posterior probability. Therefore the ITS + *tef* + *tub* + RAPD + IGS sequences were combined into a data matrix, which was analysed using the Bayes-method. On the estimated phylogeny, the isolates of *Curvularia* sp., *B. hawaiiensis, B. australiensis* and *B. spicifera* grouped to four distinct clades. Although *B. australiensis* and *B.*

spicifera can be separated on this phylogram, the question, whether they are two distinct species or only two varieties of the same species, remained unresolved.

Carbon source assimilation of the human pathogenic *Cochliobolus* isolates

The carbon source assimilation spectra of 64 *Bipolaris* and *Curvularia* isolates from human kertomycosis and culture collections were investigated and the morphological and physiological changes were also recorded. The isolates showed great intraspecific variability in the usage of the compounds. Generally, the carbon assimilation spectra bear no phylogenetic information.

Extracellular enzyme production of the human pathogenic *Cochliobolus* isolates

The extracellullar elastase, phospholipase, keratinase, lipase and proteinase production of the isolates from human keratomycosis were investigated. All of the isolates produced elastase and lipase, the possible role of which in the pathogenesis can be suggested. Some isolates were able to produce phospholipase and proteinase enzymes as well and none of them showed keratinase production.

Antifungal susceptibility of the *Cochliobolus* isolates

The minimal inhibitory concentration of amphoterycin B (AMB), natamycin, clotrimazole (CLZ), econazole, fluconazole (FLU), itraconazole (ITR), ketoconazole (KET), miconazole (MCZ) and terbinafine was determined against the involved *Bipolaris* and *Curvularia* isolates. The most effective drug was ITR and, in some cases, CLZ and KET proved to be effective against the isolates. Majority of the strains proved to be highly insensitive to AMB. Minimal inhibitory concentrations of atorvastatin, fluvastatin (FLV), lovastatin, rosuvastatin and simvastatin (SIM) were also determined. These drugs are primarily used as cholesterol-lowering agents, but they also have some antifungal effects. FLV and SIM were the most effective against *Bipolaris* and *Curvularia*.

Inhibitory effect of antifungals combined with statins against *Cochliobolus* isolates

Combinations of statins with AMB, FLU, ITR, KET and MCZ were tested against six different *Bipolaris* isolates. The interaction types were determined both with the FIC index and the Abbott-formula. Most of the interactions were additive, but in some cases (FLU-FLV) synergism could also be observed, while antagonism was not detected. Several additive interactions were very close to synergism.

Antifungal effects of ophiobolins produced by *Bipolaris* spp.

Ophiobolins are sesterterpene-type secondary metabolites of *Bipolaris* species; they can be extracted from the media of the human pathogenic species as well. We investigated their antifungal effects against several zygomycetous fungi, which are known to be resistant

to the majority of the currently used antifungal agents. Against isolates of *Gilbertella, Micromucor, Mucor, Rhizomucur* and *Rhizopus*, ophiobolin A and B proved to be effective in the ranges of 3.125-12.5 μg/ml and 25-50 μg/ml, respectively. In addition, aberrations in the germ tubes and hyphal development were observed due to the ophiobolin A treatment. Swollen and segmented hyphal elements, as well as plasma extrusions were also detected. Microscopy and molecular studies revealed apoptosis-like effects of ophiobolin A in *Mucor* and *Rhizopus* strains.

SUMMARY

1. On the basis of the morphological characters the *B. spicifera* and *B. australiensis* species couldn't be differentiate.
2. The three *Bipolaris* species could not be differentiated on the basis of ITS region, *cmd, tub* and *tef*. The IGS region contained a numerous motifs and nucleotide positions useful for the identification of the species.
3. The *B. spicifera* and *B. australiensis* are probably the varieties of the same species based on the results of phylogenetic analysis and the investigation of morphological and molecular markers.
4. The carbon source assimilation spectra of *Cochliobolus* isolates from human kertomycosis were investigated and the morphological and physiological changes were also recorded.
5. All of the isolates produced elastase and lipase, while some isolates were able to produce phospholipase and proteinase enzymes, but none of them showed keratinase production.
6. The antifungal susceptibility of the isolates were investigated against the most important antifungal agents. Most effective drug was ITR and, in some cases, CLZ and KET proved to be effective also. Majority of the strains proved to be highly insensitive to AMB. Among the statins FLV and SIM were the most effective against *Bipolaris* and *Curvularia*.
7. Among the interaction antifungal drugs the FLU-FLV was the most effective, and in some cases the AMB-, ITR- and KET-statin combinations proved to effective also. Most of the interactions were additive, but in some cases synergism could also be observed.
8. The minimal inhibitory concentrations of ophiobolin A and B were determined against some zygomycetous isolates, moreover the apoptotic-like effect of ophiobolin A was confirmed against *Mucor* and *Rhizopus* strains also.

LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

Journal articles:

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Nyilasi I, Papp T, Csernetics Á, **Krizsán K**, Nagy E, Vágvölgyi Cs (2008) High-affinity iron permease (*FTR1)* gene sequences-based molecular identification of clinically important Zygomycetes. *Clin Microbiol Inf* 14, 393-397. *IF: 2.980*

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