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Detoxification and adaptation mechanisms of *Trichoderma atroviride* to antifungal agents

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Abstract: Filamentous fungi are crucial for recycling of organic material in nature. In natural habitats, they cope with many stress factors and therefore their adaptation ability to various conditions is very high. *Trichoderma* sp., fungi used in agriculture as biocontrol agent, are exposed to a variety of toxic molecules including pesticides and fungicides. They have to fight with toxic molecules using stress adaptation mechanisms known as the stress response. Adaptation of fungi to stress, especially to chemical stress, is not well studied in environmental fungal strains. Moreover, the adaptation process presents a risk of resistance mechanism induction to antifungal agents. Such resistant strains could be spread in the environment. This work aims to contribute to the knowledge of the adaptation process spread throughout the fungal kingdom. Transcriptional response of ABC transporters, the main detoxification efflux pumps of subfamily B and G in presence of antifungal agents, is shown. On the other hand, as azoles are the most commonly used antifungal structures in clinical practice and agriculture, changes in important fungal ergosterol biosynthesis genes as a result of their exposure to various azoles structure are highlighted.

Keywords: adaptation and tolerance mechanisms of filamentous fungi, detoxification, *Trichoderma atroviride*, antifungal agents

Introduction

Filamentous fungi are naturally found in environmental niches such as decaying wood, plants and soil. Their role in nature is crucial for recycling of organic material, hence for the whole ecology. Many of them are pathogens of plants and humans. In natural habitats, fungi must cope with many abiotic and biotic stress factors, therefore their adaptation ability to various conditions is very high (Hagiwara et al., 2016). Filamentous fungi induce stress response leading to their adaptation to various conditions. Stress responses leading to adaptation mechanisms often result in mechanisms of tolerance, which can be fixed during the adaptation process and then lead to true resistance mechanisms. Fungal resistance is a big problem that complicates the treatment of fungal infections not only in clinical practice, but also in agriculture. Also, the available arsenal of antifungal agents is limited due to the similarity of host and pathogen cells, even with ongoing research for new antifungal compounds (Pierce and Lopez-Ribot, 2013). The possible target sites for antifungal compounds, i.e., (a) cell wall synthesis enzymes; (b) ergosterol, as the main sterol component of fungal membrane, and its biosynthesis; (c) enzyme enabling the transformation of flucytosine (5-fluorocytosine) to antimetabolite 5-fluorouracil, which results in RNA synthesis inhibition; are related to structural differences between fungal and host cells (Robbins et al., 2017). The limited number of specific target sites, toxicity of many active structures, and the indisputable decrease in fungal susceptibility linked with the increased resistance to the previously active compounds complicates the fungal disease treatment. Therefore, the key point in antifungal research is not only to search for new antifungal active structures and possible novel target sites, but also to study the adaptation fungal mechanisms often leading to resistance development, and the prevention of resistance development. There are many mechanisms of acquired resistance resulting from adaptation to stress: (I) changes in the accumulation of the drug in the cell, (II) modification of specific target site, (III) modification of biosynthetic pathway, (IV) creation of fungal biofilm (V) or (VI) their combination (Perez-Cantero et al., 2020; Ghannoum and Rice, 1999). The most common mechanism of resistance to antifungal agents is the activation of efflux pumps systems, which seems to be the first defense of fungal cells to toxic compounds. The major players in this mechanism are ABC transporters of family B (MDR transporters) and family G (PDR transporters) involved in the fungal tolerance mechanisms as well as in fungal resistance (Paul and Moye-Rowle, 2014, Víglaš and Olejníková, 2021). In this work, transcriptional response of ABC transporters of subfamily B and G in the presence of antifungal agents of various structures is shown. As azoles are the most commonly used structures in clinical practice

and agriculture, the changes in important fungal ergosterol biosynthesis genes were highlighted.

Material and methods

Strains and culture conditions

Model filamentous fungi *Alternaria alternata* CCM F-128, *Botrytis cinerea* CCM F-16, *Fusarium culmorum* CCM F-21, *Microsporum gypseum* CCM F-8342 (Czech Colletion of Microorganisms), *Trichoderma atroviride* P1 ATCC 74058 (American Type Culture Collection), *Trichoderma reesei* QM6a (Institut für Mikrobiologie, Universität Innsbruck), *Trichophyton terrestre* (Slovak Medical University in Bratislava) were cultivated on Potato dextrose agar growth medium (PDA) (5 g/l potato extract, 20 g/l glucose, 22 g/l agar, pH 6.4, Biolife, Italy). The culture was grown at 28 °C for 48 h in the light. For inoculation (susceptibility testing of filamentous fungi to antifungal agents), spore inoculum was used in this study except for the inoculation of *B.cinerea*. For inoculation of *B.cinerea,* CCM F-15 (Ø 5 mm) mycelial disk were used as this strain does not produce enough spores for spore inoculum preparation. The spore inoculum was prepared by pouring 0.01 % Tween 80 detergent solution on the surface of grown fungal culture and the spores were released using inoculation loop. The spore solution was filtered through three-times folded sterile gauze into a sterile tube. A Bürker counting chamber was used to determine the number of spores in the suspension and the spore inoculum was adjusted to 10^6 spores/mL. For transcriptomic analysis, an agar plug (Ø 5 mm) of the actively growing colony margin of *T. atroviride* were used as the starting material for inoculation.

Determination of susceptibility to antifungals by macrodilution method under in vitro conditions

Amphotericin B, Benomyl, Cyclosporine, Fluconazole, Mancozeb, Terbinafine, Cycloheximide, Voriconazole, 4-Nitroquinoline N-oxide, Dichlorane, and Prochloraz were dissolved in dimethyl sulfoxide (DMSO). Then, 50 µL of antifungal agent solution was aseptically added directly into the Petri dish (Ø 5 cm) diluted with freshly autoclaved PDA medium (5 mL) to the final concentration before cooling down. Final concentration volumes, after dissolving the antifungal agents in the agar media, were in the range of 0.1—100 $\mu g \cdot mL^{-1}$. A sterile paper disc (Ø 5 mm) was placed in the middle of the PDA medium and inoculated either with 5 µl of spore inoculum (10^3 conidia/disc) model filamentous fungi (*Alternaria alternata, Fusarium culmorum, Microsporum gypseum, Trichoderma atroviride, Trichoderma reesei, Trichophyton terrestre*) or mycelial discs (*Botrytis cinerea)* placed in the middle of Petri dish. The results were evaluated by measuring the colony diameter in regular time intervals until the control (without the antifungal agent) reached the margin of the Petri dish. The values obtained by measuring the fungal growth in the presence of antifungal agents were compared to the control (DMSO only) added and evaluated as IC_{50} and MIC_{80} values.

RNA isolation

The whole equipment for RNA isolation was treated with 0.1 % diethyl pyrocarbonate (DEPC) for RNAse inhibition and sterilized in an autoclave (120 °C, 20 min, 120 kPa) before the use. Then, 20 mL of sterile PDA medium (Ø 10 cm Petri dish) covered with cellophane was inoculated with a disc of *Trichoderma atroviride* mycelium. Mycelial mat formed on the surface of the medium after 48 h of stationary incubation at laboratory temperature in the dark. After 48 h of cultivation, the cellophane with mycelial mat was aseptically transferred to the Petri dish with 10 mL of PDA in combination with antifungal agents. Antifungal agents used during this experiment included: Amphotericin B, Benomyl, Cyclosporine, Fluconazole, Mancozeb, Terbinafine, Cycloheximide, Voriconazole, 4-Nitroquinoline N-oxide, Dichlorane, Prochloraz. Their concentrations were picked based on the fungal susceptibility results and are summarized in Tab. 1. The transferred mycelium was incubated in the presence of an antifungal agent for 24 h in the dark.

Mature mycelium was harvested and homogenized using liquid nitrogen in sterile mortar with pestle. The powder was transferred into a chilled sterile microtube, steeped with 700 µL of TRI reagent (RNAzol® RT Sigma-Aldrich, St. Louis, USA), mixed well using a vortex and left to incubate for 15 min (laboratory temperature). From then on, all the reagents were kept on ice. The mixture was

Tab. 1. Antifungal agent concentrations for RNA isolation.

	A	C	F	T	V	X	B	D	M	P	4
c ($\mu g \cdot mL^{-1}$)	2.5	100	100	1	0.5	5	0.5	10	100	0.5	2.5

A = Amphotericin B, C = Cyclosporine, F = Fluconazole, T = Terbinafine, V = Voriconazole, X = Cycloheximide, B = Benomyl, D = Dichlorane, M = Mancozeb, P = Prochloraz, 4 = 4-Nitroquinoline N-oxide.

centrifugated for 10 min at 12 000 rpm and 4 °C. The supernatant was transferred into a new microtube and 200 µL of chloroform were added to the mixture, which was vortexed, left to incubate for 5 min (laboratory temperature) and centrifugated for 15 min at 12 000 rpm and 4 °C. The supernatant was transferred to a clean microtube using a pipette, and 500 µL of isopropyl alcohol were added. The mixture was left to incubate for 10 min at laboratory temperature, centrifugated for 10 min at 12 000 rpm and 4 °C, the supernatant was decanted, and the sediment was washed twice with 70 % ethanol (centrifugation for 10 min, 12 000 rpm, 4 °C). Ethanol was carefully decanted, and the sediment was left to dry in a scientific thermos-block at 65 °C. Then, 50 µL of sterile tempered RNase free H_2O were added to the dried sediment until it was fully dissolved. Finally, 1 µL of STOP RNase inhibitor was added to the mixture.

DNAse treatment of isolated RNA samples

RNA samples were incubated with 2.5 µL of DNase, 10 µL of Digestion Buffer and 37.5 µL of H_2O at 37 °C for 150 min, and finally the DNase was inactivated for 10 min at 70 °C. The whole procedure was realized according to the RNase free DNase Set protocol (5Prime GmbH, Hamburg, Germany) and all the samples were stored in the freezer at –70 °C.

Transcriptomic analysis

RNA samples were then transcribed into the cDNA sequence by reverse transcriptase. The reaction mixture was prepared as follows: 7 µL of RNA (approximately 1 µg); 1 µL of oligo(dT); 4 µL of 5x RevertUP buffer; 1 µL of reverse transcriptase; 5 µL of H_2O, and the conditions were: 42 °C for 60 min (cDNA synthesis), and at 99 °C for 5 min (reverse transcriptase inactivation) according to the RevertUP II Reverse Transcriptase protocol (biotechrabitt, GmbH, Hennigsdorf, Germany).

Gene expression of ABC transporters (*Taabc2—Taabc9, Tamdr1—Tamdr8*) and *erg* genes (primers are summarized in Tab. 2) was performed; *β-actin* and *gpdh* were used as housekeeping genes. PCR was performed according to the 5Prime HotMaster Taq DNA Polymerase protocol. The reaction mixture was prepared as follows: 2 µL of cDNA (50 ng); 1 µL of forward primer; 1 µL of (10 $\mu mol \cdot L^{-1}$) reverse primer; 1 µL of (10 $\mu mol \cdot L^{-1}$) 10 $mmol \cdot L^{-1}$ dNTP mix; 5 µL of 10x Mg^{2+} buffer; 39.5 µL of H_2O; 0.5 µL of Hot Master Taq DNA polymerase at the conditions of: 94 °C for 2.5 min (initial denaturation), 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), 68 °C for 1 min (elongation), 68 °C for 10 min (final elongation), number of cycles: 30. The obtained PCR products, representing the transcribed genes, were detected via gel electro-

Tab. 2. Primers sequences used in this study.

Amplification region	***Forward** primer*	***Reverse** primer*
β-actin	AGAGCTCCAGCTTGGAGAAGT	GAGCAAGAGCAGTGATCTCCTTCTG
GPDH	CCAGAACATCATCCCCAGCAGC	GATGGAAGAGTTGTTGTTGCCGAG
Taabc2	TTCGTGGCTCCATCCAGGTTGAC	CAACGGCAGCGAGCTTTCGTAAG
Taabc3	GTCAGTTGATGGTATTGTCCG	GTTCCTGCTTAGGCCTTGAGG
Taabc4	TGCCTGCATTGCCATCACCGACA	CGCTGGTGGCATTGCTATCCTTG
Taabc5	TGTCTTTACACTGGCATTGGAC	CCTGGGTATATTGACACACACG
Taabc6	CACAAGTCCTTTGGCTTCTACC	ACAGCTCCAGAAACCAGTTCTT
Taabc7	CCACCATCGTTCAGCAGATTCA	TGCTCATCAACACCAGGAACGT
Taabc8	ACGTATCTCAACCTGGACGACT	TTGTGATGAAAGGATCGAACTG
Taabc9	ACTACATCGTGGGCAAGTTCAT	CCACCAGCAGTTGAAGTAGATG
Tamdr1	TATGGATGTCGTCTTGTCTCCTT	GGATATGAAAAGTGAACGCTGTC
Tamdr2	GAGTGGAAGCGTATGATATCTGG	TGATACGGTGAATAAGGTGCTCT
Tamdr3	ACCCAAAGATTCTTCTCTTGGAC	ACCTGGTACGTCTTCTTTTAGGG
Tamdr4	GAACACGAGAGAGACAATGTTCC	TTCTTGTGCTACGAGTGAAAGGT
Tamdr5	CTTCACCATGGACTTTTCTCATC	ATTACAACTCCACCGAGGAAGAT
Tamdr6	CTCAAGAGTCTTCGCTGTTTCAT	CAGAATGGCTTCCCTTTTCTAGT
Tamdr7	TGGATCTCAGACTAGTTGGTCGT	TACGAAACTCCAACTCCTATTGC
Tamdr8	GAGTTGAAGACCTTGTTTGATCG	GAGGGTGCTAATCTCCTTACCAT
Erg3	CATGACGCGAGACAACATCT	GCTTGTGCAGCGTCTTGTAG
Erg4	TACCTGGCTAACCACGATCC	ATCATGCAGGCGAAGAAGAC
Erg5a	TGATATCAATGCCGACCTCA	AGAGGCTTTTCCGATCATCA
Erg5b	TTCCTGCAAGCAATCTACCC	CGTTGGGGATTTGAACACTT
Erg6a	CGAGTATGCCACTCTGACGA	GGTGTTTTCAGGGAAGGACA
Erg6b	CATGAAGGTGTTGGATGTCG	GACCGTCTTCGACTTTGAGC
Cyp51a	CGAGCCGCCTACTGTATTTC	TGCCAAGAAGAATGAACGTG
Cyp51b	CTTGGCCCAGCTGTACTTTC	TCAGCGTTGACATCCTTGAG

phoresis (electrophoresis conditions: 80 V, 200 mA, 50 W, 2 h) in 1 % agarose in TAE buffer (Tris base, acetic acid, and EDTA) using Gel-red (Biotum Inc., Hayward, CA, USA) as a dye for PCR products visualization. PCR products were visualized under UV light. Gene expression in the presence of an antifungal agent was compared to control condition (1 % DMSO).

Results and Discussion

Bioinformatic analysis of T. atroviride ABC transporter (family B and G) genes and ergosterol biosynthetic genes

Gene candidates for ABC transporters were identified by proteinBlast searching in *Trichoderma atroviride* protein sequences using the *S. cerevisiae* PDR5 and PDR12 efflux pumps (ABC G-family) as queries. PDR5 and PDR12 transporters, and their homologues in other yeasts and fungi, are the main ABC transporters contributing to the resistance of fungal cells to antifungal agents (Sanglard et al., 2009). Another family of ABC transporters, the ABC-B family (known as MDR transporters), is also related to the resistance mechanisms of fungi. *Aspergillus fumigatus* AfuMDR1, AtrC, and AtrB were used as the queries for ABC-B transporters. Results of *T. atroviride* protein blast are listed in Tab. 3. Eight homologues were found for PDR5 and PDR12 with full length and reverse topology typical for the G-family of ABC transporters, and eight MDR transporters with membrane topology typical for the B-family. ABC-G transporters were indicated in this study as Taabc2—Taabc9 and those of the B-family as Tamdr1—Tamdr8 (Tab. 3).

Tab. 3 Results of bioinformatics analysis of ABC transporters *T.atroviride*.

T. atroviride protein ID	Label in this work	Similarity of protein sequence with other fungal transporters	Description of known similar transporters
		ABC-B family, predicted transporter topology [$(TMD)_6$-$NBD]_2$	
134080 XP_013946837	Tamdr1	66 % ATRC, *Emericella nidulans*	group III, activated by natural toxic compounds and xenobiotics in fungal germlings and cycloheximide (Andrade et al., 2000; Angermayr et al., 1999)
161068 XP_013946523	Tamdr2	59 % ABC4, *Venturia inaequalis* 58 % atrD, *Emericella nidulans*	Pmd1 like protein of *S. pombe*, group IV of ABC-B transporters (Kovalchuk and Driessen, 2010), protection against cytotoxic compounds, production of secondary metabolites (Andrade et al., 2000)
161084 XP_013943859	Tamdr3	54 % ABC, *Ajellomyces capsulatus* 52 % ABC3, *Venturia inaequalis* 52 % atrH, *Emericella nidulans*	transporter present in gene cluster of the production of hydroxamate-containing siderophores (Hwang et al., 2008)
206230 XP_013939251	Tamdr4 Tamdr2*	39 % atrD, *Emericella nidulans*	Pmd1 like protein of *S. pombe*, group IV of ABC-B transporters (Kovalchuk and Driessen, 2010), protection against cytotoxic compounds, production of secondary metabolites (Andrade et al., 2000)
268603 XP_013938227	Tamdr5	28 % Ste6p, *Saccharomyces cerevisiae*	a-factor transporter (Kuchler et al., 1989)
269904 XP_013948946	Tamdr6	37 % atrD, *Emericella nidulans*	Pmd1 like protein of *S. pombe*, group IV of ABC-B transporters (Kovalchuk and Driessen, 2010), protection against cytotoxic compounds, production of secondary metabolites (Andrade et al., 2000)
		36 % TrAtrD, *Trichophyton rubrum* 36 % ABC4, *Venturia inaequalis*	resistance against azoles (Scorzoni et al., 2017)
293752 XP_013940732	Tamdr7	38 % AfuMDR1 *Aspergillus fumigatus*	resistance against azoles (Wiliams et al., 2016)
88870 XP_013944359	Tamdr8 Tamdr1*	62 % atrD, *Emericella nidulans*	Pmd1 like protein of *S. pombe*, group IV of ABC-B transporters (Kovalchuk and Driessen, 2010), protection against cytotoxic compounds, production of secondary metabolites (Andrade et al., 2000)
		62 %, AfuMDR1, *Aspergillus fumigatus*	resistance against azoles (Scorzoni et al., 2017)

Tab. 3 (continue) Results of bioinformatics analysis of ABC transporters *T.atroviride*.

ABC-G family, predicted revese transporter topology [NBD — $(TMD)_6$]$_2$			
53468 XP_013946842	Taabc2 Taabc2/ pdr3*	known TAABC2 transporter, *T. atroviride*	highly expressed under nitrogen starvation and in the presence of culture filtrates of *Botrytis cinerea*, *Rhizoctonia solani* and *Pythium ultimum*, appears to be coregulated with some CWDE-encoding genes, correlated with mycoparasitism (Ruocco et al., 2009)
131379 XP_013942767	Taabc3	68 % abc1, *Magnaporthe grisea*	inducible by toxic drugs and rice phytoalexin (Lee et al., 2005)
		64 % abc 1, *Gibberella pulicaris*	necessary for tolerance to phytoalexins and virulence on potato tubers (Fleißner et al., 2002)
		63 % PMR1, *P. digitatum*	decreased susceptibility to thiabendazole and some DMI-fungicides (Hamamoto et al., 2001; Nakaune et al., 2002)
		63 % AtrG, *A. nidulans*	involved in azole resistance (Miura et al., 2018)
246378 XP_013940893	Taabc4 Tapdr2*	73 % abc1, *Gibberella pulicaris*	tolerance to phytoalexins and virulence on potato tubes (Fleißner et al., 2002)
		68 % ABC1, *Magnaporthe grisea*	inducible azole compounds miconazole and metconazole (Urban et al., 1999) and rice phytoalexin (Lee et al., 2005)
88381 XP_013943680	Taabc5 Tapdr7*	64 % ABC1, *Venturia inaequalis*	constitutively expressed and therefore supposed to have basic metabolic functions, strongly enhanced expression by treatment with several drugs, including antibiotics, azole fungicides and plant defense toxins (Reddel, 2015)
		61 % atrA, *Aspergillus nidulans*	multidrug resistance (del Sorbo et al., 1997)
10258 XP_013947537	Taabc7	79 % BMR5, *Botryotinia fuckeliana*	expressed constitutively (del Sorbo et al., 2008)
		65 % MfABC1 *Monilinia fructicola*	involved in resistance against fungicide resistance to DMI-fungicides (Schnabel and Paradkar, 2003)
314979 XP_013947572	Taabc6	65 % ATRB, *Aspergillus nidulans*	resistance to fungicides (Andrade et al., 2000)
		64 % MFABC1, *Monilinia fructicola*	resistance to DMI-fungicides (Schnabel and Paradkar, 2003)
		65 % TABC2, *T. atroviride*	highly expressed under nitrogen starvation and in the presence of culture filtrates of *Botrytis cinerea*, *Rhizoctonia solani* and *Pythium ultimum*, appears to be coregulated with some CWDE-encoding genes, correlated with mycoparasitism (Ruocco et al., 2009)
293326 XP_013942263	Taabc8 Tapdr5*	66 % BMR1 *Botryotinia fuckeliana*	resistance to polyoxin and iprobenfos involved in self-defence, contributes to pathogenicity constitutively expressed (del Sorbo et al., 2008)
314045 XP_013949023	Taabc9 Tapdr4*	43 % atrF, *A. fumigatus*	increased expression in the presence of itraconazole (Slaven et al., 2002)
		42 % BcatrB, *Botrytis cinerea*	influences the activity of phenylpyrrole fungicides, involved in virulence and interaction with other organisms' resistance to toxic compounds and fungicides (Schoonbeek et al., 2002)
		43 % MfABC1, *Monilinia fructicola*	fungicide resistance determinant (Schnabel and Paradkar, 2003)

*Based on the triat protein ID, Tamdr8, Tamdr4, Taabc2, Taabc4, Taabc5, Taabc7, Taabc8, Taabc9 (this study) are otherwise indicated as Tamdr1, Tamdr2, Tapdr3, Tapdr2, Tapdr7, Tapdr5, Tapdr4 (Zhang et al., 2015).

Phylogenetic relationship of *T. atroviride* ABC-G transporters to *S. cerevisiae* PDR5 and PDR12, and the main multidrug-resistance transporters of *Aspergillus fumigatus* AtrA, AtrF, AtrB is shown in Fig. 1. In this phylogenetic analysis of MDR transporters, AfuMDR1, AtrC, AtrB and the transporter PMD1 of *Schizosaccharomyces pombe* were included. Result of the phylogenetic analysis is shown in Fig. 2. The phylogenetic analysis was performed by online available bioinformatics tools. Sequences found in the NCBI database were aligned by the software Clustal Omega and the phylogenetic trees were

constructed with PhyloTree. As it is clearly shown in Fig. 1, the homologues of PDR5 are clustered in more clades. In the PDR5 clade, there are Taabc3, Taabc4, Taabc5, AtrF and AtrA transporters. As the cladogram indicates, Taabc3 and Taabc4 are closely linked to AtrF and Taabc5 to AtrA. In the clade with AtrB, there are Taabc2, Taabc6, Taabc7. ABC-G transporters Taabc8 and Taabc9 are each

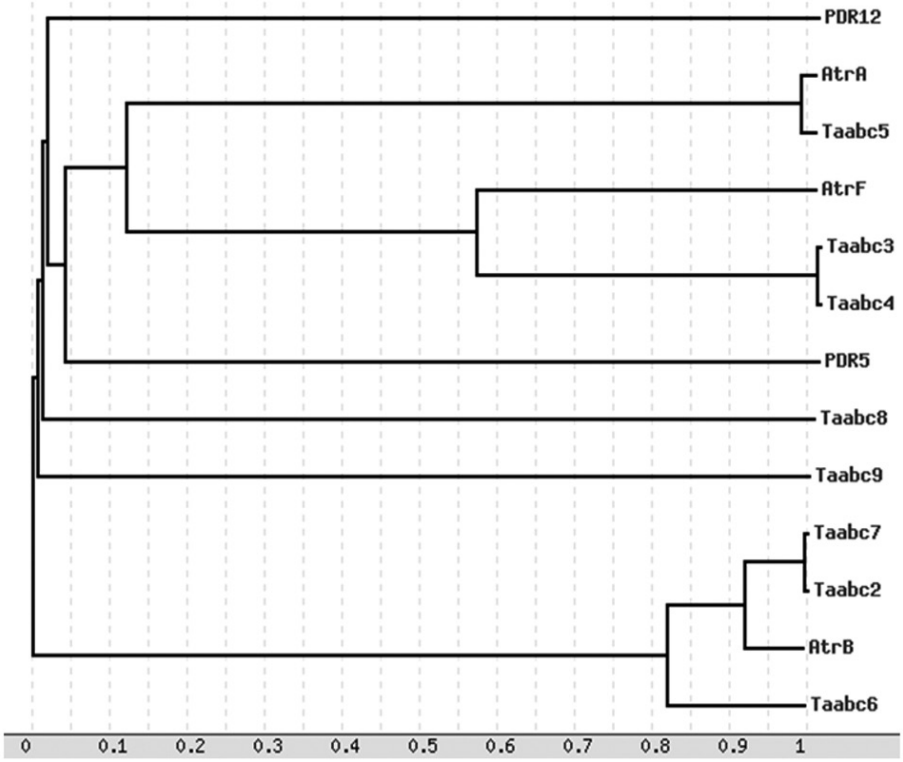


Fig. 1. Phylogenetic analysis of *T. atroviride* ABC-G transporters, the scale reflects their phylogenetic proximity.

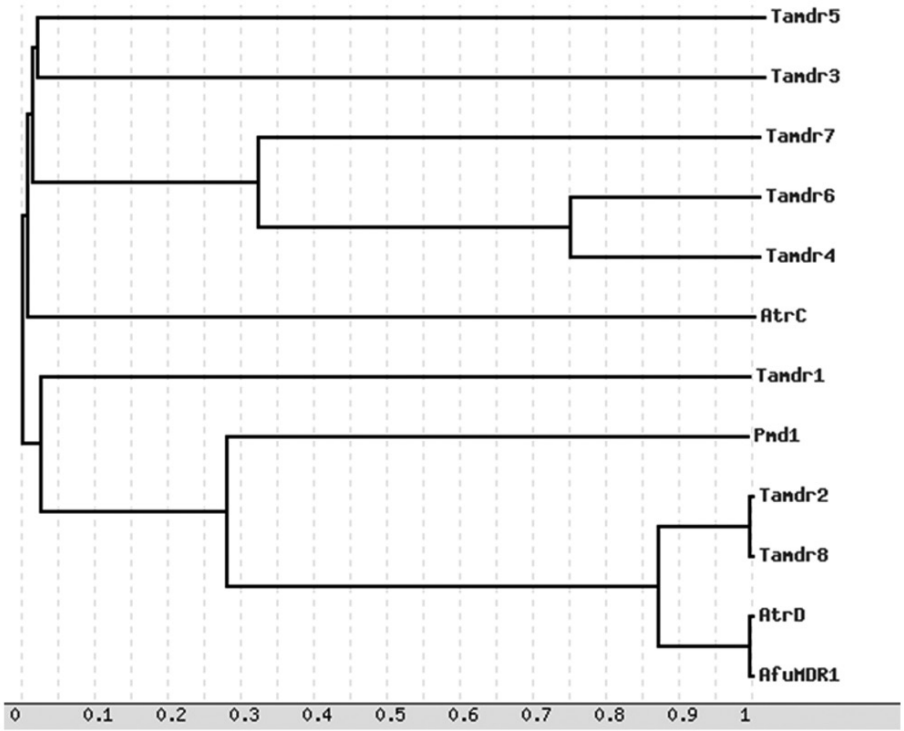


Fig. 2. Phylogenetic analysis of *T. atroviride* ABC-B transporters, the scale reflects their phylogenetic proximity.

Tab. 4. Homologues of ergosterol biosynthetic genes found in *T. atroviride* genome.

TRIATDRAFT Protein ID/NCBI Accession No.	Label in this work	similarity with *Aspergillus fumigatus proteins* NCBI Accession No.	enzyme
48253 XP_013937523	Tacyp51a	63 % Afu4g06890 (Cyp51A) XP_752137	14-alpha sterol demethylase predicted in ergosterol biosynthesis
254651 XP_013948124	Tacyp51b	62 % Afu7g03740(cyp51B) XP_749134	14-alpha sterol demethylase predicted in ergosterol biosynthesis
149835 XP_013940996	Taerg6a	75 % Afu4g03630 erg6 XP_746550	sterol 24C-methyltransferase
83118 XP_013940996	Taerg6b	63 %Afub_099400 (erg6A) XP_746550	sterol 24C-methyltransferase
298475 XP_013946486	Taerg5a	68 %Afu1g03950 erg5 KMK_57343	cytochrome-P450 sterol-C22 desaturase
43598 XP_013944443	Taerg5b	55 %Afu1g03950erg5 KMK_57343	cytochrome-P450 sterol-C22 desaturase
54251 XP_013946692	Taerg3	52 % Afu2g00320(Erg3A) XP_749168	sterol delta 5,6-desaturase
151594 XP_013943383	Taerg4	63 % Afu5g14350 (erg4A) XP_753221	C24(28)-sterol reductase

in their own clade. The phylogenetic analysis of ABC-B transporters indicates TAMDR proteins to be divided into two clades. Transporters Tamdr1, Tamdr2, Tamdr8 are in the clade with Pmd1, AtrD and AfuMDR1. Other *T. atroviride* ABC-B transporters are in the clade with AtrC.

Since triazoles are the most applied antifungal agents in clinical practice and agriculture, the expression level of ergosterol biosynthetic genes has been studied. Candidates for genes encoding enzymes in the ergosterol biosynthetic pathway in the *T. atroviride* genome were identified as the homologues of *A. fumigatus erg*-genes. The expression level of *Cyp51A* (Afu4g06890), *Cyp51B* (Afu7g03740), *erg*3 (Afu2g00320), *erg*4 (Afu5g14350), *erg*5 (Afu1g03950) and *erg*6 (Afu4g03630) homologues was also investigated. The homologues of ergosterol genes found in *T. atroviride* protein sequences are listed in Tab. 4. Two homologues were found: XP_013937523 (Cyp51A) and XP_013948124 (Cyp51B) encoding 14-alpha sterol demethylase, XP_013940996 (Erg6a) and XP_013940996 (Erg6b) encoding sterol 24C-methyltransferase, XP_013946486 (Erg5a) and XP_013944443 (Erg5b) encoding cytochrome-P450 sterol-C22 desaturase, XP_01394669 (Erg3) encoding sterol delta 5,6-desaturase, and XP_013943383 (Erg4) encoding C24(28)-sterol reductase. Protein function was predicted using the UniProt database and protein similarities are listed in Tab. 4 with values exceeding 50 %.

Comparison of T. atroviride and other model filamentous fungi susceptibility to antifungal agents with different modes of action

During evolution, filamentous fungi have acquired protective mechanisms against many toxic chemical structures. This natural resistance seems to be a typical characteristic of filamentous fungi due to their presence in natural niches; they are able to resist toxic compounds if these are secondary metabolites of other microorganisms or synthetic pollutants in the environment. Universal mechanisms of various microorganisms coping with toxic compounds are very similar and dependent on the detoxification capability of the fungal species. Detoxification mechanisms are often related to the adaptation mechanisms, including the activity of efflux systems or detoxification enzymes decreasing the toxicity of compounds by modifying their structure (Zeilinger et al., 2016). In this work, detoxification ability of *Trichoderma atroviride*, known as a very active mycoparasitic fungus used in biocontrol (Ruocco, 2009), was investigated. Soil fungal species, especially when used in agriculture, fight many toxic compounds in their natural environment. Moreover biocontrol fungi produce endogenous toxic compounds to fight phytopathogenic fungi, which could be the reason behind the very effective detoxification system of *Trichoderma sp.*

Susceptibility of *T. atroviride* and other filamentous fungi to antifungal agents with different modes of action as well as to fungicides used in agriculture are summarized in Tab. 5 and Tab. 6.

The results were evaluated by means of IC_{50} and MIC_{80} values. Fungal susceptibility results shown in Tab. 5 and Tab. 6. indicate phytopathogens (*A. alternata*, *B. cinerea*, *F. culmorum*) to be less susceptible to fungicides used in agriculture, mainly to mancozeb and dichlorane. *T. terrestre* and *M. gypseum*, representatives of clinically important fungi causing topical fungal infections, show similar susceptibility to the tested antifungals. Their susceptibility to terbinafine, the major agent used for

Tab. 5. Susceptibility of model filamentous fungi to fungicides used in agriculture expressed by IC_{50} and MIC_{80} values ($\mu g \cdot mL^{-1}$).

Fungicide	Benomyl		Dichlorane		Mancozeb		Prochloraz		4-NQO	
Filamentous fungus	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}
Alternaria alternata	R*	R*	25	R*	R*	R*	0.5	10	1	8
Botrytis cinerea	0.06	0.6	4	9	R*	R*	0.8	2.5	8	19
Fusarium culmorum	1	2.5	R*	R*	R*	R*	0.1	0.4	10	R*
Microsporum gypseum	1	2	25	R*	R*	R*	0.5	0.8	3	5
Trichoderma atroviride	0.5	1	10	R*	R*	R*	<0.5	1	3	4
Trichoderma reesei	1	2	3	R*	85	R*	0.2	0.2	0.7	2
Trichophyton terrestre	2	15	5	10	7	20	0.1	0.2	0.5	1.5

*R = MIC80 > 100 $\mu g \cdot mL^{-1}$, 4-NQO = 4-Nitroquinoline N-oxide.

Tab. 6. Susceptibility of model filamentous fungi to antifungal agents of clinical importance (different modes of action) expressed by IC_{50} and MIC_{80} values ($\mu g \cdot mL^{-1}$).

Antifungal agent	Amphotericin B		Cyclosporine		Fluconazole		Terbinafine		Voriconazole		Cycloheximide	
Filamentous fungus	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}	IC_{50}	MIC_{80}
Alternaria alternata	1	R*	1	5	15	R*	0.5	2.5	0.4	1	7	25
Botrytis cinerea	1	R*	0.6	38	23	R*	0.9	6	0.6	1	24	50
Fusarium culmorum	7.5	R*	2.5	R*	R*	R*	0.5	2.5	0.2	0.4	5	50
Microsporum gypseum	100	R*	1	25	R*	R*	0.005	0.04	0,1	1	R*	R*
Trichoderma atroviride	5	R*	R*	R*	R*	R*	0.5	1	0.6	1	5	R*
Trichoderma reesei	0.6	1	0.5	2.5	R*	R*	0.3	0.8	0.4	0.6	10	50
Trichophyton terrestre	5	R*	0.1	5*	10	R*	0.005	0.01	0.04	0.1	R*	R*

*R = MIC80 > 100 $\mu g \cdot mL^{-1}$.

the treatment of local mycoses, has been detected. Interestingly, the results of tested *Trichoderma spp.* differ, with *T. reesei* (biotechnological producer of celullolytic enzymes) being more susceptible than *T. atroviride* (biocontrol fungus used in agriculture) (Tab. 5, Tab. 6). Considering the high MIC_{80} values, it is clear that *T. atroviride* is less susceptible compared with other fungal species (Tab. 5, Tab. 6) and it has the highest tolerance to dichlorane, mancozeb, amphotericin B, cyclosporine, and cycloheximide. Therefore, it really seems that *T. atroviride,* thanks to its decreased susceptibility to antifungal agents, presents a suitable model for the study of the fungal adaptation process and the response of filamentous fungi to the presence of antifungal compounds.

Expression analysis of T. atroviride genes involved in adaptation to antifungal agents

Our results confirmed the decreased susceptibility of this model organism to various antifungal agents. When compared to other fungal species, *T. atroviride* proved to be the most resistant to antifungals used clinically as well as in agriculture. The detoxification system of this fungal species involves various mechanisms, which are not fully known yet. Some of the defensive mechanisms of *Trichoderma sp.* relying on the activity of enzymes modifying active structures or of the efflux system are mentioned in literature (Tripathi et al., 2013; Kovalchuk and Driessen, 2010). Only two ABC transporters are described in literature (Ruocco, 2009; Sun et al., 2019; Zhang et al., 2015). When considering the number of genes encoding ABC-G and ABC-B transporters found in the genome of *T. atroviride* by bioinformatics analysis (Tab. 3), this scientific topic still requires a lot of attention.

Figs. 3, 4 and 5 show the results of ABC transporters gene expression of *T. atroviride* when exposed to antifungal agents. Genes encoding MDR transporters (ABC-B), family G of ABC transporters (ABC-G) were studied using *β-actin* and *gpdh* as housekeeping genes. Expression analysis shows the induction of *Tamdr1*, *Tamdr2,* and *Tamdr3* genes in the presence of most antifungal agents, though the expression of *Tamdr3* is lower compared with that of *Tamdr1* and *Tamdr2*. Dichlorane does not induce *Tamdr1*, *Tamdr2,* and *Tamdr3* expression, nor *Tamdr1* is induced after filamentous fungus cultivation in the presence of cycloheximide. Genes encoding efflux pumps *Tamdr5, Tamdr6,* and *Tamdr7* were not expressed. The expression of *Tamdr4* and *Tamdr8* after cultivation in the presence of tested agents is elevated; however, the expression can also be noticed in control (Fig. 3, K12) cultivated without antifungal agents or fungicides.

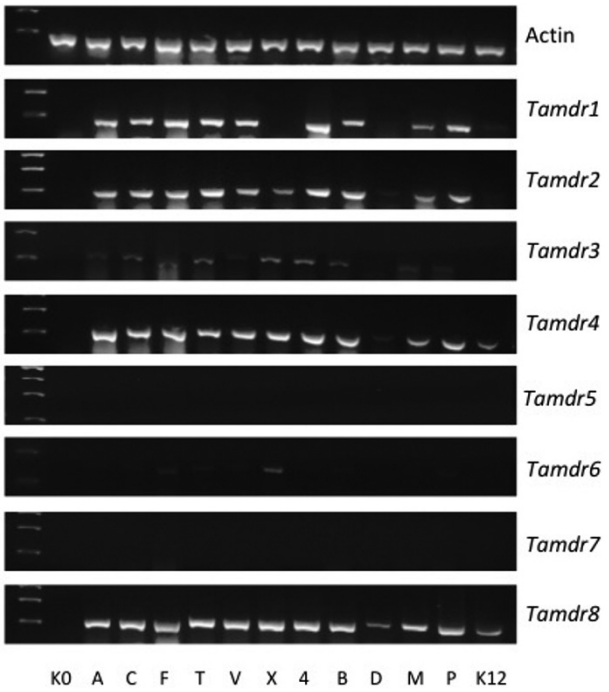


Fig. 3. Expression profile of ABC-B transporter genes in the presence of antifungal agents and fungicides.

K0 = control (in time of transfer), A = Amphotericin B, C = Cyclosporine, F = Fluconazole, T = Terbinafine, V = Voriconazole, X = Cycloheximide, B = Benomyl, D = Dichlorane, M = Mancozeb, P = Prochloraz, 4 = 4-Nitroquinoline N-oxide, K12 = control (after transfer), bands on the left side of the are molecular weight mark's bands.

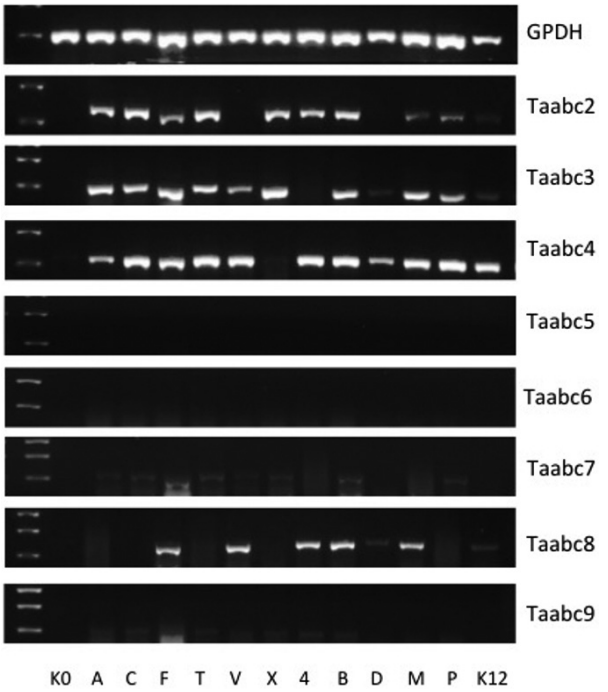


Fig. 4. Expression profile of ABC-G transporter genes in the presence of antifungal agents and fungicides.

K0 = control (in time of transfer), A = Amphotericin B, C = Cyclosporine, F = Fluconazole, T = Terbinafine, V = Voriconazole, X = Cycloheximide, B = Benomyl, D = Dichlorane, M = Mancozeb, P = Prochloraz, 4 = 4-Nitroquinoline N-oxide, K12 = control (after transfer), bands on the left side of the are molecular weight mark's bands.

Fig. 4 demonstrates higher expression of *Taabc2*, *Taabc3,* and *Taab8* after their exposition to antifungal agents. Dichlorane and voriconazole do not induce expression of the *Taabc2* gene, 4-nitroquinoline N-oxide does not induce the expression of *Taabc3*. Fluconazole, voriconazole, cycloheximide, 4-nitroquinoline N-oxide and mancozeb induce *Taabc8* gene expression. There is no noticeable expression of other ABC transporter genes of the G family. Expression of the *Taabc4* gene in the presence of tested agents is comparable to that of control (K12, Fig. 4).

The results show both ABC-B and ABC-G transporters to be involved in the *T. atroviride* detoxification system. This fact can be understood as the contribution to its resistance mechanisms. As shown in Figs. 3 and 4, gene expression of *Taabc2, Taabc3, Taabc8*, *Tamdr1,* and *Tamdr2* was detected in the presence of most antifungal agents. So, the activity of these efflux proteins contributes to *T. atroviride* decreased susceptibility to various antifungal agents. In fungal cells resistant to a variety of antifungal agents (as is the case of *T. atroviride*, Tab. 5, Tab. 6*),* the genes of ABC transporters possess increased expression levels (Sanglard et al., 2009). Elevated gene expression leads to increased protein levels in the plasma membrane and their activity. As shown in Figs. 3 and 4, *Taabc8* (known also as *pdr5*) expression increased in the presence of fluconazole, voriconazole, 4NQO, benomyl, and mancozeb. Increased expression of ABC-B (MDR) *Tamdr1* and *Tamdr2* in the presence of most antifungals was detected (Fig. 4). These two transporters are not expressed in control samples, only in the presence of antifungal agents. *Taabc2* and *Taabc3* seem to be the most effective ABC-G transporters in *T. atroviride* as they are expressed after the exposure to most antifungals. The *Taabc3* transporter has the highest similarity to PDR5 of *S. cerevisiae* and is homologous to various ABC-G transporters involved in antifungal resistance in other fungi (Tab. 3). *T. atroviride* has two homologues very closely related to PDR5 (phylogenetic analysis, Fig. 1), *Taabc4* and *Taabc3*. The expression analysis results show that both these transporters genes are expressed when the fungus is exposed to antifungal agents. However, *Taabc4* is expressed also in the control sample without the presence of toxic antifungal compounds. This efflux pump probably serves as a backup system for the fast defensive reaction or pro-

vides an important physiological role in modelling lipids of the plasma membrane, as it is known for its yeasts homologues PDR5 of *S. cerevisiae* and CDR1, CDR2 of *C. albicans*. These transporters serve as flippases and transport sterols and sphingolipids into the plasma membrane; their role in the lipid homeostasis was uncovered as their overexpression has shown the compensatory mechanisms under azole stress (Rizzo et al., 2019). In several yeasts and fungi, the relationship between the overexpression of ergosterol biosynthetic genes (due to azole stress) and increased level of ABC-G transporters was observed suggesting their mutual regulation (Vu et al., 2019). Thus, the constitutive expression of one of these homologues supports the role of *Taabc4* in maintaining the physiology and homeostasis of fungal cells. The *Taabc3* transporter, as the second homologue of PDR5, is induced as a response to the presence of toxic compounds (in this case antifungal agents with diverse chemical structure). These two transporters ensure the homeostasis as well as the detoxification of *T. atroviride*. Based on the data found in literature, *Taabc4* is one of the transporters with approximately ten-times increased gene expression level after 6 h of incubation in the presence of biocide dichlorvos (Zhang et al., 2015). The most remarkable expression changes were detected in the *Taabc2* transporter in the presence of the same fungicide. After successful preparation of *Taabc2* mutant, *Taabc2* was confirmed as the key ABC-G transporter involved in dichlorvos detoxification. Our results are in accordance with this finding, as *Taabc2* was expressed also in the presence of all antifungals except for voriconazole (Fig. 3). The relationship of ABC-G transporter and the levels of ergosterol was shown after deletion of *Taabc2* of *T. atroviride*. The results show ergosterol content to decrease in the presence of biocide dichlorvos, indicating that *Taabc2* is important to maintain lipid homeostasis and ergosterol content under stress conditions (Sun et al., 2019). Such result indicates the unknown role of ABC transporters in stress signalling as the deletion of the ABC transporter leads to ergosterol depletion in plasma membrane under stress conditions. The role of ABC transporters is also important in stress response not only as an effective efflux system but, as a signal molecule leading to the induction of mechanisms reducing the damage caused by toxic compounds, as are antifungal agents for fungi. Therefore, the roles of fungal ABC transporters and the lipid homeostasis seem to be related not only in the sense of physiology but, also in the context of adaptation to antifungals.

As azoles are the most used antifungals in clinical practice and agriculture, the response of *T. atroviride* to some azoles was investigated. Fluconazole, voriconazole and prochloraz were assayed. The biosynthetic pathway of ergosterol in filamentous fungi differs in the sequence of its steps and is more branched compared to that described in yeasts (Alcazar-Fuoli et al., 2007). Ergosterol biosynthesis in fungi is well described in *A. fumigatus* and *N. crassa*. Experimental data show that ergosterol is synthesized via the eburicol branch in both these fungi, involving C-24-metyl transferase (Erg6) as a synthesis step before Cyp51 (azole target) (Alcazar-Fuoli et al., 2007; Hu et al., 2018). Based on the bioinformatic comparison with *A. fumigatus*, *T. atroviride* has two paralogues of the *Erg5*, *Erg6* and *Cyp51* genes in the genome. Fig. 5 shows the induction of the second paralogue of *Erg5* and *Cyp51* gene after the exposition to azoles. The presence of azoles in the cultivation medium induced also the expression of one *Erg6* paralogue. According to the expression profile of control gene, ergosterol synthesis via zymosterol branch is assumed, in physiological conditions as it is described in yeast (Parks and Casey, 1995). The alternative step of ergosterol biosynthetic pathway, lanosterol conversion to eburicol by *Erg6* is therefore activated after the addition of azoles to filamentous fungi.

Filamentous fungi seem to have more by-pass possibilities inducing alternative branches and offering various possibilities of final product

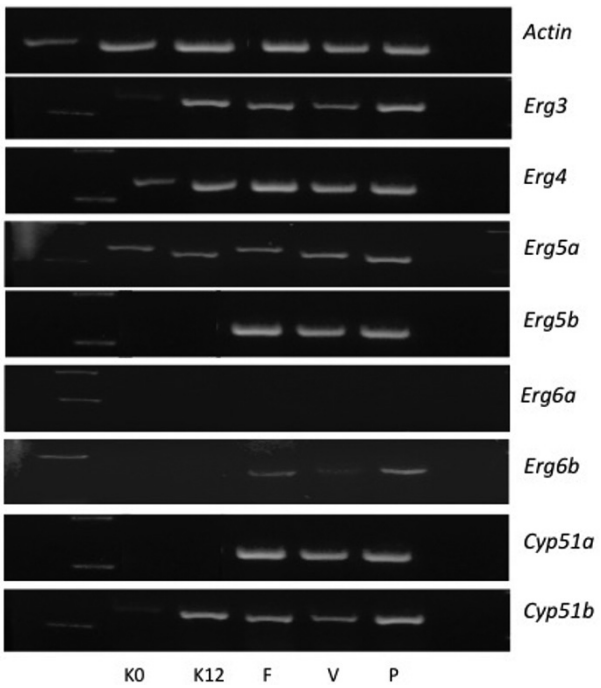


Fig. 5. Expression profile of ERG genes in the presence of antifungal agents.

K0 = control (in time of transfer), K12 = control (after transfer), F = Fluconazole, V = Voriconazole, P = Prochloraz, bands on the left side of the are molecular weight mark's bands.

synthesis. Following the bioinformatics analysis of *T. atroviride,* this fungus has clearly undergone the duplication of *erg* genes during its evolution. *T. atroviride* has two paralogues of *Cyp51*, *Erg5,* and *Erg6* in the genome (Tab. 4). *Erg* genes of *Aspergillus fumigatus* were the queries for *T. atroviride erg* genes as these are known and well annotated. *A. fumigatus Cyp51* genes are evolutionary differentiated in two lineages of two paralogue proteins (CYP51A and CYP51B). As gene duplication is the source of the genetic adaptation mechanism, it seems that filamentous fungi with more paralogues of *erg* genes have higher tolerance to azoles compared to fungi with only one copy of the gene (Perez-Cantero et al., 2020). *T. atroviride* seems to have two paralogues of the most important *erg* genes contributing to its tolerance. It is possible that azoles can bind enzyme with cytochrome P450 in the active site encoded by the *Erg5* gene to ensure less azole binds to the active site of the *Cyp51* gene. Two paralogues of the *Erg6* gene in the *T. atroviride* genome were detected; further research is necessary to determine whether both are functional. The *erg* genes expression profile has shown that only one, *Erg6b,* is induced in the presence of azole compounds. The expression profile of the *Cyp51* and *Erg5* gene has shown that *Cyp51b* and *Erg5a* show constitutive expression and the paralogues *Cyp51a* and *Erg5b* are induced after the exposure of the fungus to azoles. When both paralogues are expressed, higher enzyme amount and increased concentration of the azole drug are needed to inhibit the activity of both enzymes. Therefore, the question is whether azole compounds are able to bind both paralogues with the same affinity as paralogues have usually different binding capacity to active structures. Ascomycetes with more paralogues in the genome are more tolerant to azole compounds. In *A. fumigatus*, Cyp51B binds the active structure whereas the second paralogue Cyp51A sustains the enzymatic activity. The most discussed resistance mechanism against azoles is the gain of function (GOF) mutation in the promoter sequence in *Cyp51,* which leads to enhanced gene expression. Mutation in the active site leads to decreased binding ability of the active azole structure (Perez-Cantero et al., 2020). Considering the results, induction of the second gene paralogue in the presence of azole combined with the active efflux seems to be the mechanism responsible for *T. atroviride* tolerance to antifungal azoles.

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