Functional characterization of the mevalonate-isoprenoid biosynthesis pathway genes in *Mucor circinelloides*

Ph.D. dissertation

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2018
Szeged

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1. ABBREVIATIONS

AmB	amphotericin B
ATMT	*Agrobacterium tumefaciens*-mediated transformation
CBS	Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands
cDNA	complementary DNA
CFU	colony forming unit
CLO	clotrimazole
DHA	dihydroxyacetone
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
FLU	fluvastatin
FPP	farnesyl pyrophosphate
GHMP	galacto-, homoserine-, mevalonate- and phosphomevalonate kinases
GGPP	geranylgeranyl pyrophosphate
GPP	geranyl pyrophosphate
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPLC	high performance liquid chromatography
hpRNA	hairpin RNA
IPP	isopentenyl pyrophosphate
ITR	itraconazole
LB	Luria – Bertani medium
Mal	maltose
MEA	malt extract agar medium
MEP	methylerythritol phosphate
miRNA	micro interfering RNA
MOPS	3-(N-morpholino) propanesulfonic acid
MVA	mevalonate pathway
Na-ac	sodium acetate
NCBI	National Center for Biotechnology Information

OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	phagocytic index
PMC	PEG – sorbitol – MOPS – calcium chloride (buffer)
qPCR	quantitative real-time PCR
RPM	revolutions per minute
SMC	sorbitol – MOPS – calcium chloride (buffer)
SIM	simvastatin
siRNA	small interfering RNA
SZMC	Szeged Microbiology Collection, Hungary
TAE	tris – acetic acid – disodium EDTA (buffer)
TF	transcription factor
Treh	trehalose
TRIS	tris(hydroxymethyl)aminomethane
YNB	yeast nitrogen base
YPG	yeast extract – pepton – glucose medium

***Mucor circinelloides* genes and encoded proteins frequently occur in the thesis:**

hmgS	HMG-CoA synthase
mvk	mevalonate kinase
dmd	diphosphomevalonate decarboxylase (pyrophosphomevalonate decarboxylase or mevalonate 5-pyrophosphate decarboxylase)
ipi	isopentenyl pyrophosphate (IPP) isomerase
isoA	farnesyl pyrophosphate (FPP) synthase
carG	geranylgeranyl pyrophosphate (GGPP) synthase
leuA	α-isopropylmalate isomerase
pyrG	orotidine 5'-monophosphate decarboxylase
gpd1	glyceraldehyde-3-phosphate dehydrogenase 1
zrt1	ZIP zinc transporter

2. OVERVIEW

Members of the subphylum Mucoromycotina, order Mucorales (such as *Lichtheimia, Mucor, Rhizomucor* and *Rhizopus* species) are saprotrophic fungi, which also have medical, industrial, biotechnological and agricultural importance. Some species may cause post-harvest damage in agriculture; or are used as producers of extracellular enzymes, organic acids and carotenoids. Several species belonging to this fungal group are also considered opportunistic human pathogens, which can cause fatal systemic infections (zygomycosis or mucormycosis) in immunocompromised patients with neutropenia, diabetic ketoacidosis or serious skin injuries.

Metabolites synthesized via the mevalonate-isoprenoid pathway (such as sterols, functional groups of proteins and carotenoids) play an important role in signal transduction, morphogenesis, apoptosis, adaptation to environmental change and protection against free radicals. Today ergosterol and its biosynthetic pathway are the major targets of the antifungal agents used in clinics to treat infections caused by Mucoromycotina fungi. The therapy of mucormycosis is still limited because of the intrinsic resistance of these fungi to the majority of the currently clinically applied antimycotics. To date limited information is available about the function and regulation of the mevalonate-isoprenoid biosynthesis pathway genes in Mucoromycotina fungi. Investigation of the fungal mevalonate-isoprenoid biosynthesis pathway may allow the identification of new potential novel drug targets that could bolster the arsenal of available options to treat fungal infections. Furthermore it provides a great opportunity to develop new therapeutic strategies with special attention paid to biosynthesis pathway that promote growth inhibition of fungi. Moreover modification of the pathway may allow to isolate β-carotene overproducing mutant strains. Thus, our aim was to characterize six genes of the *Mucor circinelloides* mevalonate-isoprenoid pathway, encoding the HMG-CoA synthase (*hmgS*), mevalonate kinase (*mvk*), diphosphomevalonate decarboxylase (*dmd*), isopentenyl pyrophosphate isomerase (*ipi*), farnesyl pyrophosphate synthase (*isoA*) and geranylgeranyl pyrophosphate synthase (*carG*). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate serves as precursors of sterols and carotenoids, respectively.

3. INTRODUCTION

3.1. Characterization and taxonomy of Mucoromycotina fungi

The phylum formerly known as Zygomycota was an artificial assemblage of fungi of uncertain taxonomic position. In a previous classification the phylum consisted of two classes, zygomycetes and trichomycetes (**Benny et al., 2001)** and is composed of fungi that form coenocytic mycelia and reproduce sexually by the production of zygospores (**Whittaker, 1969; Tanabe et al., 2004, 2005**). Hibbett and coworkers abandoned the phylum Zygomycota and classified zygomycete fungi into four subphyla, including Entomophthoromycotina, Kickellomycotina, Mucoromycotina, and Zoopagomycotina and phylum Glomeromycota (**Hibbett et al., 2007**). In addition, four years later based on a multigene analyzis, a new subphylum Mortierellomycotina was also described, which consisting Mortierellales species (**Hoffmann et al., 2011)**. A new phylogenetic classification follows the principles promoted by Hibbett and coworkers and two phyla, Mucoromycota and Zoopagomycota was circumscribed (**Hibbett et al., 2007; Spatafora et al., 2016**). The two phyla consist of six subphyla, four classes, and 16 orders. Zoopagomycota comprises Entomophthoromycotina (with classes Entomophthoromycetes, Basidiobolomycetes and Neozygitomycetes), Kickxellomycotina and Zoopagomycotina (previous orders Eccrinales and Amoebidiales have been demonstrated not to be members of Kingdom Fungi), while Mucoromycota comprises Glomeromycotina (with class Glomeromycetes), Mortierellomycotina, and Mucoromycotina subphyla (**Fig. 1, Benny and O'Donnell, 2000; Cafaro, 2005; Spatafora et al., 2016**). All taxa are either demonstrated or presumed to be monophyletic. Former phylogenetic analyzes suggested that the phylum Zygomycota is polyphyletic or paraphyletic, now Spatafora and coworkers demonstrated that the two clades, Zoopagomycota and Mucoromycota, form a paraphyletic grade from which Dikarya are derived (**Richardson, 2009; Spatafora et al., 2016**).

Most of the genera of the former phylum are currently included in Mucoromycotina, being the subphylum with the highest number of species (**Hibbett et al., 2007; Spatafora et al., 2016**). Mucoromycotina includes *Mucor, Rhizopus*, and the majority of the most common and best known zygomycetes. Fungi belonging to the subphyla are fast-growing, filamentous, non-flagellated fungi, which form coenocytic mycelia (lacking regular septation; septas are usually formed in specialized hyphae playing role in reproduction) and cell wall consisting of high level of chitin and chitosan, low level of glucan and fucose (a carbohydrate present only in few fungal taxa) (**Richardson, 2009; Mélida et al., 2015;**

Araújo and Hughes, 2016; Muszewska et al., 2018). These fungi are mainly saprobes, and it is frequently isolated from soil, dung, plant debris, and sugar-rich plant parts (e.g. fruits), but mycoparsasites, ectomycorrhizal species are also represented in the subphyla (**Spatafora et al., 2016**).

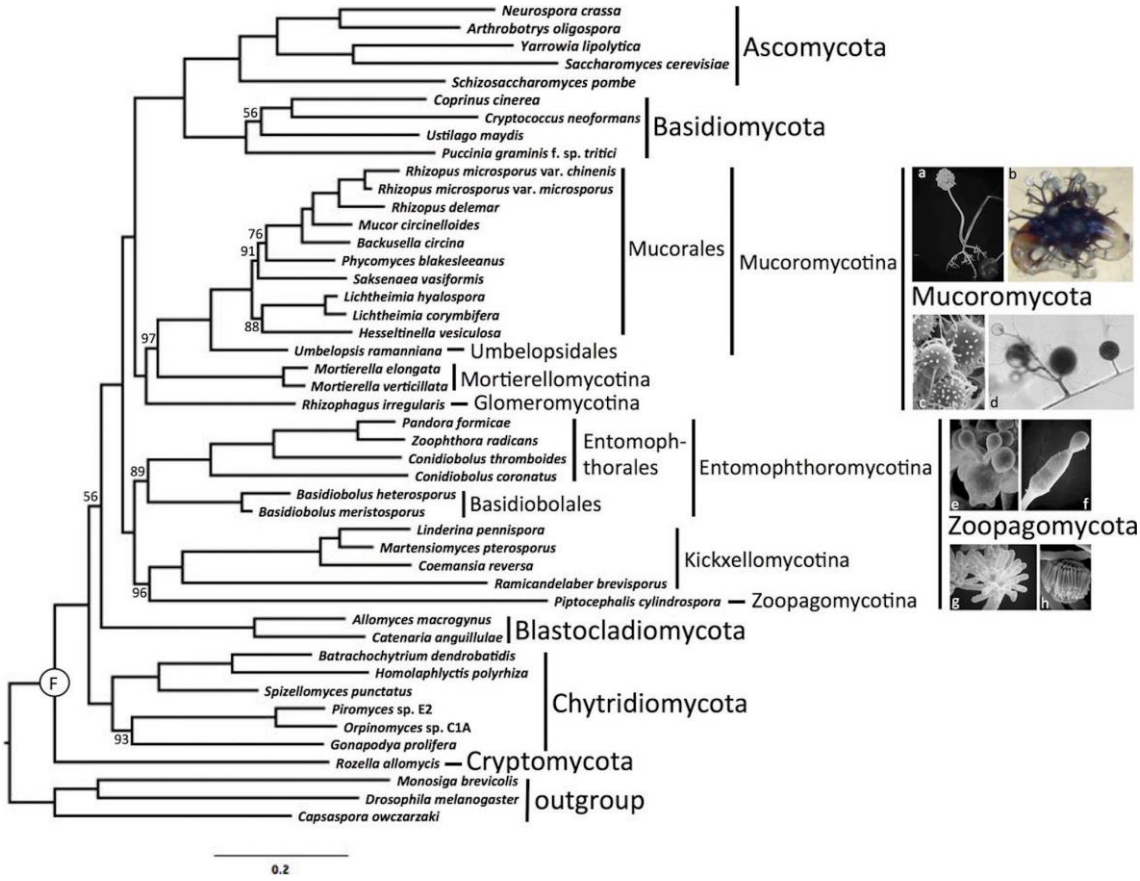


Fig 1. Phylogenetic tree of Kingdom fungi based on the concatenated alignment of 192 conserved orthologous proteins (**Spatafora et al., 2016**).

Mucoromycotina fungi have a haploidic life cycle; the only diploid cells are zygospores (**Eslava et al., 1975; James and Kerry, 2004**). Reproduction normally happens by asexually, which typically involves the production of sporangia (**Fig. 2**) and/or sporangioles at the end of sporangiophores (specialized hyphae) and uni- or multinucleate sporangiospores (**Schachtschabel et al., 2008**). Sporangiospores are typically dispersed with water or wind. Sexual reproduction takes place with zygospores, which are formed by gametangial conjugation (**Fig. 2**) (**Eslava et al., 1975; James and Kerry, 2004**). Homothallic (conjugation takes place between hyphae of same strain) and heterothallic (conjugation occurs between hyphae belong to the opposite mating type strains) species are

also occurring in the subphyla (**Idnurm, 2011**). The sporangium rises from zygospore bearing haploid sporangiospores (**Fig. 2**) (**Eslava et al., 1975; James and Kerry, 2004**).

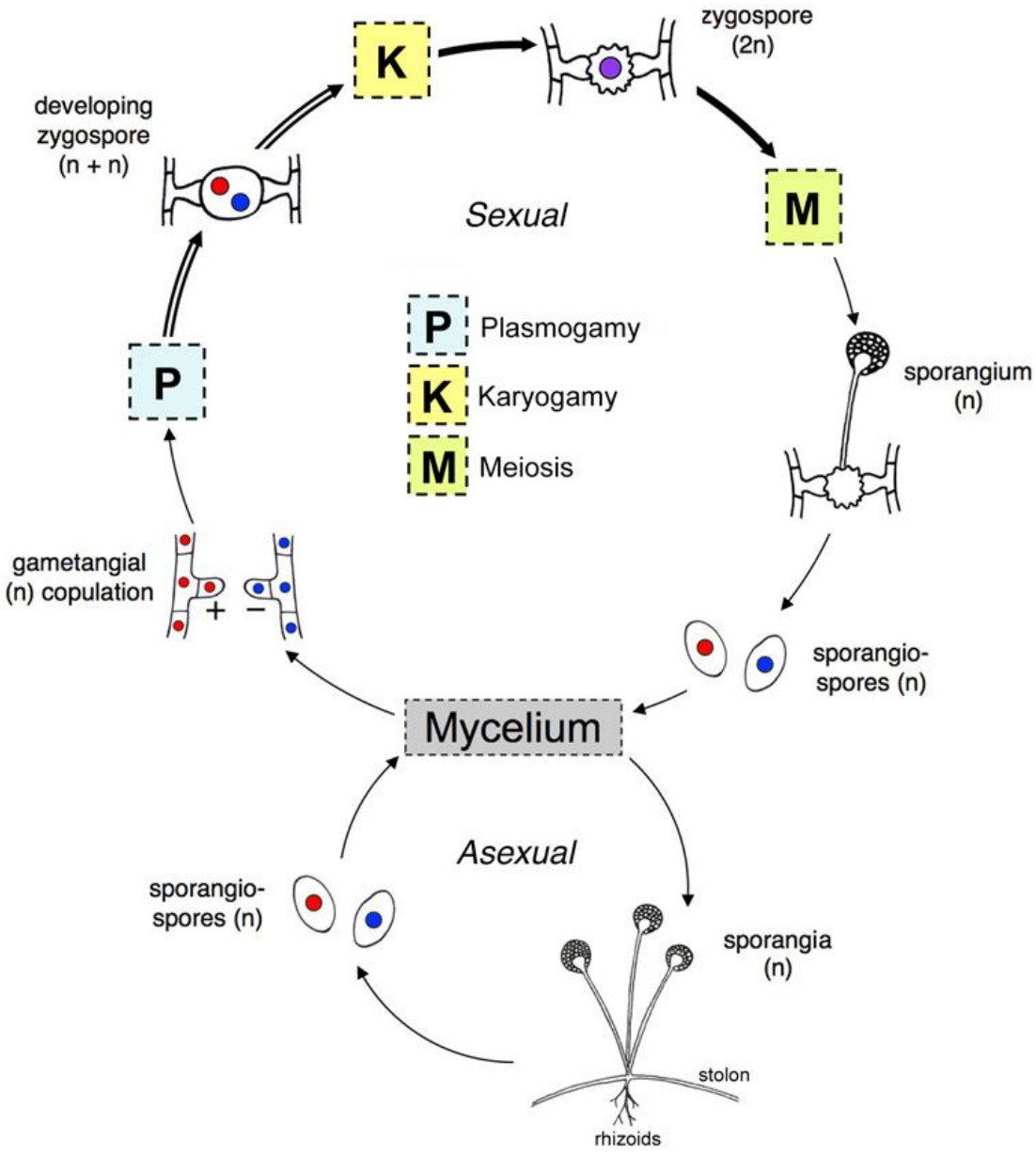


Fig 2. Reproduction cycles of Mucoromycotina fungi (**Carris et al., 2012**).

3.2. Economic and clinical importance of Mucoromycotina fungi

Fungal secondary metabolites are utilized as antibiotics, toxins, pesticides, and animal as well as plant growth factors (**Nielsen and Nielsen 2017**). Mucoromycotina fungi are recieving a growing attention due to their evolutionary distance to other fungi (**Gladieux et al., 2014**). The role of these fungi in industry, like production of

polyunsaturated fatty acids, beer and ethanol with *Mucor indicus*, degradation of oil waste, removal of heavy metal from waste water (biosorption) with *M. indicus* and biotransformation with *Mucor rouxii* are described well (**Arcidiacono et al., 1992; Harms et al., 2011; Karimi and Zamini, 2013; Simister et al., 2015**). Mucoromycotina fungi are also used as producers of organic acids (e.g. lactic, malic and fumaric acid) and hydrolytic enzymes, in the elaboration of different kinds of Asian food and in cheese ripening, and *Blakeslea trispora* for β-carotene production (**Shetty et al., 2000; Abe et al., 2004; Millati et al., 2005; Karimi and Zamini, 2013**). β-carotene is a fat soluble pigment that has great commercial value due to its diverse uses in food, pharmaceutical products, cosmetics and textiles (**Hussein et al., 2006; Britton et al., 2009; Sandmann, 2015**). Carotenoids are widely used as natural pigments mostly because of their antioxidant properties (**Bhosale and Bernstein, 2005**). They protect cells against photooxidation by quenching singlet oxygen, free radicals (e.g. prevention of lipid peroxidation) and reactive oxygen species (**Bhosale and Bernstein, 2005**).

The worldwide carotenoid market value was $1.5 billion in 2014 and is assumed to reach nearly $1.8 billion in 2019, with a compound annual growth rate of 3.9% reported by global market during 2015 (global market for carotenoids (2015)). Microbial β-carotene currently produced by the industry with alga *Dunaliella salina* or *B. trispora* (**Lamers, 2008; Papaioannou and Liakopoulou-Kyariakides, 2012**). The wild-type *B. trispora* produces carotenoids in similar amount to *M. circinelloides*, but transformation systems are not available for genetic modification to improve carotenoid production. Mixed culture of different mating types of *B. trispora* and optimized fermentation media are used industrially for carotenoid production (**Mehta et al., 2003**). In contrast, approach for efficient genetic transformation of *M. circinelloides*, such as overexpression and gene silencing, allow us to improving carotenoid production (**Csernetics et al., 2011; Torres-Martínez et al., 2012**).

Several members of Mucoromycotina and Entomophthoromycotina are considered opportunistic human pathogens; and these infections are called mucormycosis and entomophthoromycosis, respectively (formerly zygomycosis) (**Kwon-Chung, 2012**; **Riley et al., 2016**). Species of the former class Zygomycetes were firstly reported to cause disease in humans in a publication entitled Mycosis Mucorina (**Platauf, 1885**). This first case of disseminated disease has presented in a patient suffering from cancer caused by *Lichtheimia* (formerly *Absidia*) *corymbifera* (**Platauf, 1885**). *Mucor* and *Lichtheimia* are, after *Rhizopus*, the clinically most relevant genus of Mucoromycotina (**Ribes et al., 2000;**

Alvarez et al., 2009). In addition, species of *Rhizomucor*, *Apophysomyces, Saksenaea*, *Cunninghamella, Cokeromyces*, and *Syncephalastrum* have also been described as caustaive agents of mucormycosis, but less cases have been reported (**Gomes et al., 2011**).

Five major forms of mucormycosis occur, including rhino-orbito-cerebral, pulmonary, disseminated, cutaneous, and gastrointestinal, while species of Entomophthoromycotina generally cause locally manifested - cutaneous and subcutaneous - and slowly progressive infections (**Prabhu and Patel, 2004; Pfaller and Diekema, 2005**). An increase in the number of mucormycosis has been observed in the last few decades, mainly because of the modern surgical interventions, immunosuppressive therapies and irresponsible use of antimicrobial agents (**Ribes et al., 2000; Chayakulkeeree, et al., 2006; Ibrahim et al., 2012**). According to Centers for Disease Control and Prevention USA (**CDC, 2015**) the overall mortality rate of mucormycosis found to be 54%. It was 46% among people with sinus infections, 76% for pulmonary infections, and 96% for disseminated mucormycosis (**CDC, 2015**). The most vulnerable group of these infections are immunocompromised patients, the major risk factors include neutropenia, cancer, corticosteroid treatment, diabetes mellitus in ketoacidosis, deferoxamine treatment, organ transplantation, and burn injury (**Sugar, 2000; Sugar and Liu, 2000; Prabhu and Patel, 2004; Ibrahim et al., 2008**). Phagocytes - primarily macrophages and neutrophils - have the most important role in host immune defence against fungi causing mucormycosis, thus longer neutropenia is one of the major risk for developing infection (**Ibrahim et al, 2012; Morace and Borghi, 2012**). Pulmonary alveolar macrophages play important role in defence against these fungi, because inhalation of the sporangiospores is one of the most common way of infection (**van de Veerdonk et al., 2010**).

3.3. Antifungal agents applied in clinics to treat fungal infections

Only few antimycotic drugs were reported which are able to kill the Mucoromycotina fungi, whereas others merely only inhibit the growth of pathogens. The proper antifungal therapy and selection of drug should be based on several criteria, such as immune capability of the host, site of infection, characteristics of the infection (the fungal species and its susceptibility to different antifungal drugs), and pharmacokinetic characteristics of the antifungal drug (e.g., absorption, elimination, and toxicity) (**Lepak et al., 2015**). Mucorales are mostly resistant against widely used antifungal drugs used in clinics (such as against different azoles and echinocandins), thus generally, antifungal therapy need to be combined with surgical debridement of the necrotic regions.

Today, ergosterol and its biosynthetic pathway are the major targets of antifungal therapy used to treat infections caused by Mucoromycotina fungi. Based on the mode of action the drugs belong to different classes, which are as follows: (1) polyene macrolides (e.g. Amphotericin B, (AmB); **Fig. 3**); (2) azole derivatives (such as ketoconazole, fluconazole, itraconazole (ITR), posakonazole, clotrimazole (CLO) and voriconazole; **Fig. 3**); (3) allylamines (e.g. terbinafine); (4) DNA and RNA synthesis inhibitors (flucytosine); (5) echinocandins (**Selvakumar et al., 2006; Vandeputte et al., 2012; Bondaryk et al., 2013**).

Ergosterol is the most prevalent sterol in the fungal plasma membrane. The target of polyene macrolides (produced by *Streptomyces* spp.) is ergosterol; through the channels formed by the complex cell leaks K^+, resulting in a disruption of the proton gradient (**Lemke et al., 2005**). In higher concentrations, polyenes also inhibit chitin syntheses, moreover AmB causes oxidative damage to plasma membranes (**Sudoh et al., 2000**). AmB (**Fig. 3**) is effective against a wide variety of fungi, including yeasts and molds such as Mucoromycotina fungi (**Lemke et al., 2005**). Treatment with AmB is often followes by nephrotoxicity rather than therapeutic efficacy; whereas lipid formulations have reduced toxicity and it allows higher therapeutic doses (**Azanza et al., 2015**).

Azoles act as inhibitors of the cytochrome-P450 dependent lanosterol 14-α demethylase, a key enzyme in ergosterol biosynthesis (**Vandeputte et al., 2012**). The active binding site of lanosterol 14-α demethylase contains a heme domain (**Sheng et al., 2009**). Azoles bind with a particular nitrogen molecule in the azole ring core to the iron atom of the heme domain, preventing the demethylation of lanosterol (**Joseph-Horne and Hollomon, 1997; Sheng et al., 2009**). Azoles may also interact with the 3-ketosteroid reductase, an enzyme in methylsterol biosynthesis (**Sant et al., 2016**). Azoles used in high dosage may cause azole-induced toxic injury (**Lo Re V 3rd et al., 2016**). The first azole accessible for systemic use was CLO (**Fig. 3**), however, its application was restricted due to inconsistent concentrations in the blood serum. Miconazole is effective to treat systemic infections, but it could be given only intravenously. ITR, the first triazole used in humans, is available for oral (capsules and cyclodextrin solution to enhance the bioavailability of the drug) and intravenous administration (**Carrier et al., 2007**). Azoles are used for prophylaxis in transplant patients to prevent invasive candidiasis, aspergillosis and mucoromycosis (**Ibrahim et al., 2012**). Several Mucoromycotina fungi shows resistance to the most of the widely used azoles (**Caramalho et al., 2017**). Nowadays, ITR (**Fig. 3**), posaconazole and isovuconazole are used frequently to treat mucormycosis, while for

combination therapy azoles are using mainly with AmB (**Greenberg et al., 2006; Cornely et al, 2013; Pagano et al., 2013; Caramalho et al., 2015; Marty et al., 2016**).

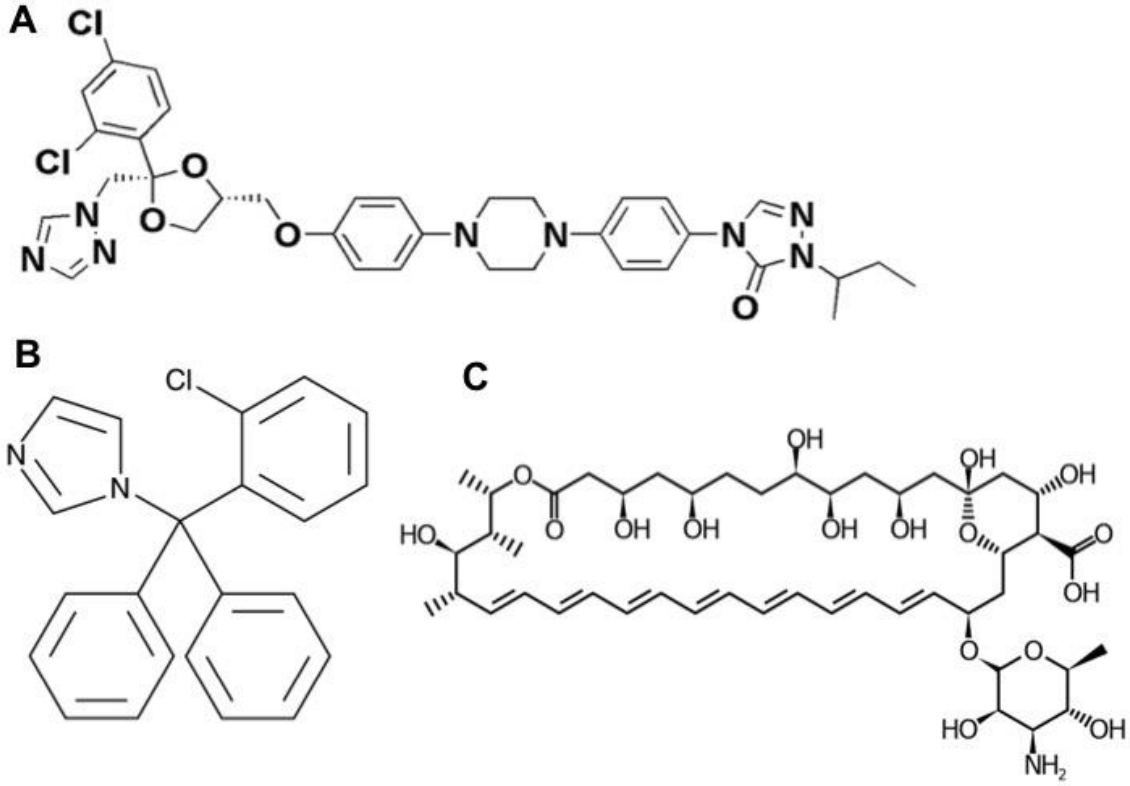


Fig 3. Biochemical structure of antifungal agents: (A) itraconazole, (B) clotrimazole and (C) AmphotericinB (**Warrilow et al., 2010; Chávez-Fumagalli et al., 2015**).

Echinocandins (such as micafungin, anidulafungin and caspofungin) are water soluble lipopeptides, which inhibit the β(1-3)-D-glucane synthase, thus reducing the cell wall integrity. No any toxic effect of these agents had been described. Echinocandins are used only parentheral, they have fungicidal activity against *Candida* and *Aspergillus* species, while Mucorales shows resistance to these antifungals (**Walker et al., 2010**).

Allylamins are inhibiting the squalene epoxydase, enzyme involved in the ergosterol biosynthesis. Mainly used topically against dermathophytes, also effective against *Candida albicans* **(Georgopapadakou et al., 1992)**.

Flucytosine act as inhibitor of the RNA and DNA synthesis in fungi. As the effect of cytosine deaminase, 5-fluorocytosine is converted to cytostatic 5-fluorouracil, which next convert into the RNA intercalating, thus protein synthesis inhibitior, 5-fluorouridine triphosphate and 5-fluorodeoxyuridine monophosphate. The latter inhibit the thymidylate synthase, and deoxythymidine triphosphate (dTTP) synthesis, thereby the DNA synthesis.

It is generally used in combination with AmB in treatment of *Cryptococcus* infections (**Dixon and Walsh, 1996; Loyse et al., 2013**).

Microorganisms develop the system to prevent the fungicidal or fungistatic effect of antifungals drugs, that is classified into three basic mechanisms, (i) try to reduce the uptake and accumulation of the drug inside the fungal cell, (ii) reducing the affinity of the drug at the target point, and (iii) moderation of metabolism to counterbalance the antifungal drug effect (**Sanglard, 2002**). Moreover in high degree resistant of clinical isolates, combination of several mechanisms was also observed (**Perea et al., 2001**). Expanding in resistance with antifungal therapy is due to the sequential acquisition of different mechanisms (**Franz et al., 1998; Marr et al., 1998; MacCallum et al., 2010**). The cause of resistance to polyenes has been studied, and is a significant alteration of the lipid composition in the plasma membrane (**Kelly et al., 1994; Lemke et al., 2005**). This may be one of the reasons to a lower affinity of AmB, probably a lack of the binding site (**Joseph et al., 1995**). Another reason for AmB protection might be a modified substance of β-1,3 glucans in the cell wall. These components, which increase the stability of the cell wall, influence the access of large molecules such as AmB to the plasma membrane (**Seo et al., 1999; Mesa-Arango et al., 2016**).

The susceptibility of *M. circinelloides* against wide range of antifungal agents and statins were tested and minimal inhibitory concentrations (MIC) were determined (**Table 1)**. The MIC of AmB was determined to *M. circinelloides* in range of 0.06 to 1.0 μg/ml, while differences were observed in the susceptibility against different azoles, for example *M. circinelloides* showed resistance against fluconazole, voriconazole and ravuconazole, but posaconazole, ITR, and ketoconazole inhibited the growth of the fungus in the investigated concentrations (**Table 1**). *M. circinelloides* also showed sensitivity to lovastatin and were resistant to different echinocandins (**Table 1**). It has to be noted that different strains belonging to the same species shows high deviation in susceptibility to antifungals; it can be also observed in case of *M. circinelloides*, resulting in large scale of MIC, such as in case of posaconazole and ITR. Our research group previously determined the MIC of different antifungal agents against *M. circinelloides* MS12 strain (**unpublished data**), following the instructions of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 (2008) protocol. The *M. circinelloides* MS12 showed resistance to echinocandins and most of the azoles involved in that study (such as fluconazole, posaconazole, ITR, voriconazole, ketoconazole and econazole), but miconazole and CLO were able to inhibit it's growth in 16 μg/ml concentration. MIC for AmB was determined in 2 μg/ml and FLU

and lovastatin were also able to inhibit the growth of *M. circinelloides* MS12 in 8 μg/ml and 64 μg/ml, respectively (**unpublished data**).

Table 1. MIC of antimycotics and statins against *M. circinelloides* determined in different studies.

Antimycotic	MIC (μg/ml)	References
Amphotericin B	0.06 - 1	Caramalho et al. 2015; Almyroudis et al. 2007; Drogari-Apiranthitou et al. 2012; Salas et al. 2012
Posaconazole	0.50 - 32	Caramalho et al. 2015; Almyroudis et al. 2007; Drogari-Apiranthitou et al. 2012; Salas et al. 2012
Itraconazole	2 - >8	Almyroudis et al. 2007; Drogari-Apiranthitou et al. 2012
Ketoconazole	16	Almyroudis et al. 2007
Fluconazole	>64	Almyroudis et al. 2007
Voriconazole	>8	Drogari-Apiranthitou et al. 2012
Ravuconazole	>8	Drogari-Apiranthitou et al. 2012
Terbinafine	8	Drogari-Apiranthitou et al. 2012
Anidulafungin	>8	Drogari-Apiranthitou et al. 2012
Caspofungin	>16	Almyroudis et al. 2007
Lovastatin	5 - 40	Galgóczy et al. 2011
Fluvastatin	>25	Galgóczy et al. 2011

3.4. The mevalonate-isoprenoid biosynthesis pathway in *M. circinelloides*

Isoprenoids (or terpenoids) are a group of functionally diverse compounds comprising of at least 50,000 different structures mostly identified from plants, bacteria and fungi (**Hemmerlin et al., 2012; Schmidt-Dannert, 2015**). These metabolites (such as carotenoids, ergosterol and functional groups of farnesylated and geranylgeranylated proteins) have several biological function: they are playing role in morphogenesis, signal transduction, apoptosis, protection against free radicals, adaptation to environmental change and contribute to membrane permeability and integrity (**Wawrzyn et al., 2012**). In fungi, metabolites are synthesized via the mevalonate-isoprenoid biosynthesis pathway, in which formation of mevalonate is one of the key steps.

In fungi the mevalonate pathway (MVA) begins with acetyl-CoA, which is the final metabolite of the glycolysis pathway. Three molecules of acetyl-CoA are sequentially condensed by thiolase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase to form HMG-CoA, which is then reduced to mevalonate by HMG-CoA reductase, expected that it

might work is a rate-constraining advance of this pathway (**Wang and Keasling, 2002; Burg et al., 2011; Liao et al., 2016**). Next, conversion of mevalonate to isopentenyl pyrophosphate (IPP) is catalyzed by three enzymes. In this part of the biosynthesis mevalonate is phosphorylated at the 5-OH position by mevalonate kinase and mevalonate-5-phosphate kinase, and then decarboxylated to IPP by diphosphomevalonate decarboxylase (mevalonate-5-diphosphate decarboxylase) (**Fig. 4**) (**Miziorko, 2011; Liao et al., 2016**). Recently, two atypical types of that part of the MVA pathway have been described in *Haloferax volcanii* and *Thermoplasma acidophilum* (**Dellas et al., 2013**). One involves the decarboxylation of mevalonate-5-phosphate to isoprenyl phosphate (IP) by mevalonate-5-phosphate decarboxylase and followed by the phosphorylation of IP to IPP by IP kinase (**Grochowski et al., 2006; Dellas et al., 2013**). The other involves phosphorylation of mevalonate at both ends (at the 3-OH and 5-OH positions) by mevalonate-3-kinase and mevalonate-3-phosphate-5 kinase to generate mevalonate-3,5-bisphosphate, which is decarboxylated to IPP by mevalonate-5-phosphate decarboxylase (**Azami et al., 2014; Vinokur et al., 2014**).

In the isoprenoid pathway, formation of dimethylallyl pyrophosphate (DMAPP) is catalyzed by IPP isomerase, and condensation of IPP and DMAPP form the intermediate geranyl pyrophosphate (GPP). The carbon chain is then increases by repetitive condensations with further IPP units. These steps are managed by prenyltransferases, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate (FPP and GGPP, respectively) synthases, and produce linear intermediate compounds with various lengths (**Fig. 4**) (**Chang et al., 2013**). The compounds formed via the isoprenoid pathway serve as precursors in many different biosynthetic side routes leading to the formation of several types of end products, such as carotenoids, ergosterol, terpenoid moiety of ubiquinones and prenyl groups of certain proteins (**Liang et al., 2002; Wawrzyn et al., 2012**).

Carotenoids belong into the big and diverse family of isoprenoid compounds. The carotenoid biosynthesis starts with the condensation of two molecules of C20 GGPP units, thus formation of phytoene. Several dehydrogenation and two cyclisation steps result in β-carotene (**Fig. 5**) - the major carotenoid produced by most of the Mucoromycotina fungi - catalyzed by phytoene synthase/lycopene cyclase (a bifunctional enzyme) and phytoene dehydrogenase, which encoded by *carRP* and *carB* genes, respectively, in *M. circinelloides* (**Velayos et al., 2000a, 2000b**). Conversion of β-carotene to xanthophylls (β-carotene with functional groups containing oxygen, such as zeaxanthin and β-cryptoxanthin) was also described in *M. circinelloides* (**Papp et al., 2006, 2013; Csernetics et al., 2011**). Induction

of carotenoid production by light to be accounted as conserved in fungi, such as in *Neurospora crassa*, *Phycomyces blakesleeanus* and *M. circinelloides* (**Silva et al., 2006; Zhang et al., 2016**). In *N. crassa wc-1* and *wc-2* genes, encoding white-collar proteins (photoreceptors), play important role in light induction of carotenoid production (**Cheng et al., 2003; He and Liu, 2005**). Three homologos of *wc-1* have been reported in *M. circinelloides* and one of them, *mcwc-1c*, was also found to be involved in the light induction of carotenoid production (**Silva et al., 2006**).

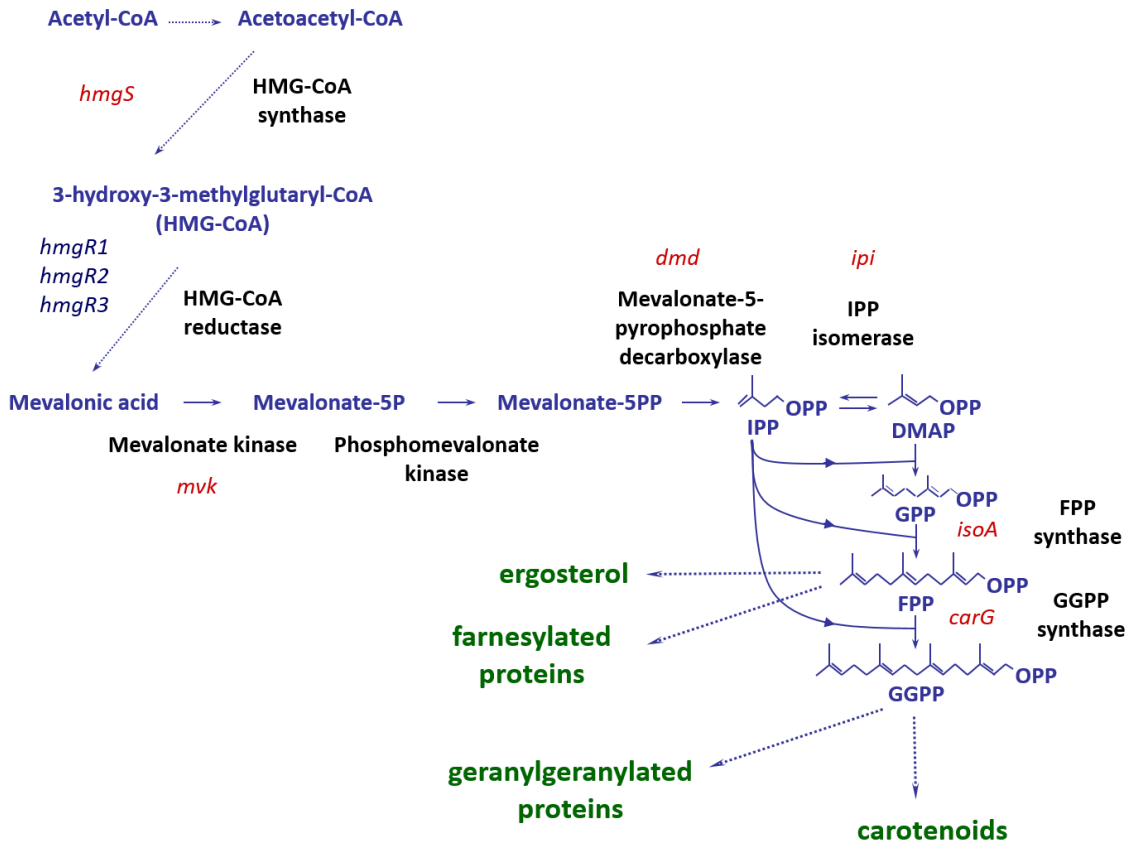


Fig 4. The mevalonate-isoprenoid biosynthesis pathway in *M. circinelloides*. The identified genes involved in the biosynthesis are shown with red and dark blue (**Csernetics et al., 2011; Nagy et al., 2014 and unpublished data**). IPP - isopentenyl pyrophosphate, DMAPP - dimethylallyl pyrophosphate, GPP - geranyl pyrophosphate, FPP - farnesyl pyrophosphate, GGPP - geranylgeranyl pyrophosphate.

Sterols are neutral lipids of eukaryotic cells (**Dupont et al., 2012**). Three predominant forms represent the main sterols found in eukaryotes like cholesterol in vertebrates, phytosterols (sitosterol, stigmasterol, campesterol) in plants, and ergosterol in fungi (**Fig. 5**). While even structural and function studies have failed to show any

advantages of ergosterol in comparision to cholesterol, moreover biosynthesis require more energy than cholesterol, the particular reason why ergosterol is present in fungi is still not clear (**Shrivastava and Chattopadhyay, 2007; Weete et al., 2010**).

Ergosterol is involved in several biological functions such as maintenance of cell membrane integrity and fluidity, furthermore in regulation and control of the cell cycle (**Alvarez et al., 2007; Abe et al., 2009**). Ergosterol biosynthesis is an oxygen-dependent process, as several enzymes of the post-squalene pathway require molecular oxygen (**Rosenfeld and Beauvoit, 2003**). In ergosterol biosynthesis pathway around 20 enzymes exist (**Ferreira et al., 2005; Alcazar-Fuoli et al., 2008**). As a result of a lack of ergosterol and/or accumulation of its precursor, the *FgERG4* deletion mutant *Fusarium graminearum* displayed several defects in plasma membrane function, such as increased sensitivity to metal cations and osmotic stresses (**Long et al., 2017**).

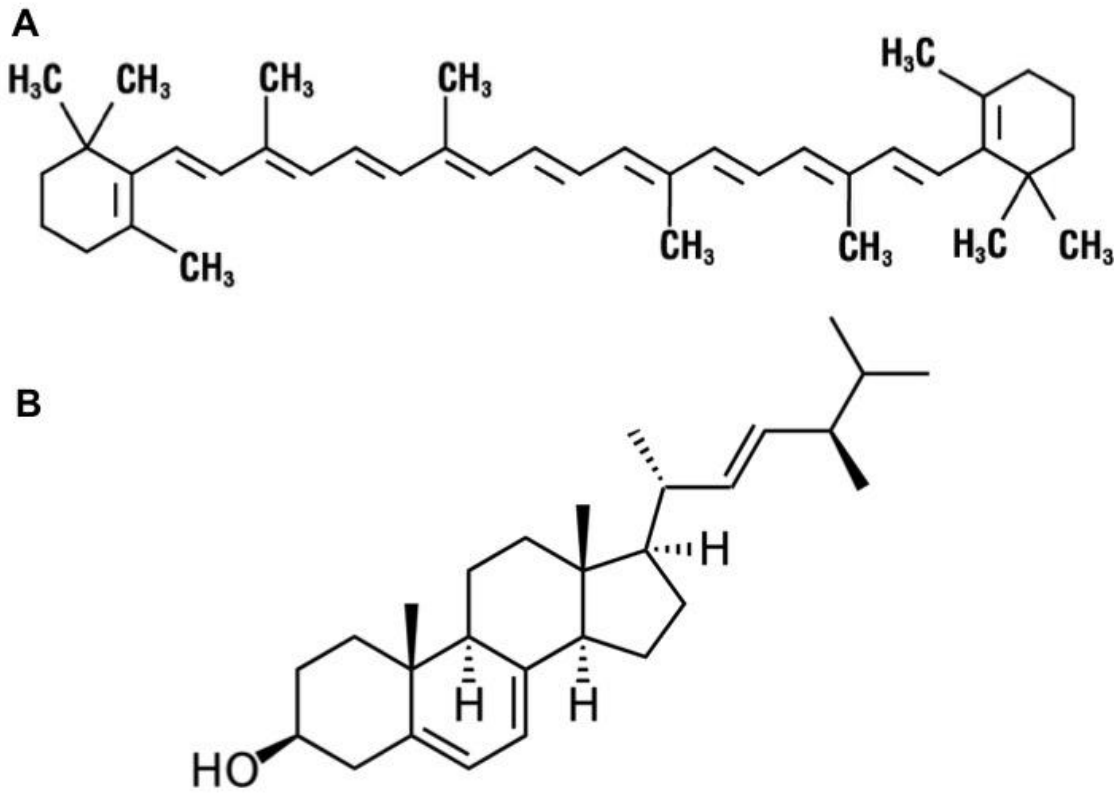


Fig 5. Biochemical structure: (A) β-carotene and (B) ergosterol (**Valcarce et al., 2001; Oh et al., 2013**).

Statins (such as lovastatin, fluvastatin (FLU), simvastatin (SIM), rosuvastatin and atorvastatin, **Fig. 6**) are cholesterol-lowering drugs. Statins are competitive inhibitors of the HMG-CoA reductase by binding to the enzyme and displace their natural substrate

(**Maciejak et al., 2013**). It was shown that statins have fungicidal activity, and also act synergistically with different antifungal agents (such as azoles) against yeasts, dermatophytes and clinically important Mucoromycotina species (**Lukács et al., 2004; Gyetvai et al., 2006; Nyilasi et al., 2014**). Statins were found to inhibit not only ergosterol, but other mevalonate-derived metabolite biosynthesis pathways, such as dolichol, heme-A, isopentenyl tRNA, carotenoids, ubiquinone, and siderophores, as well as protein prenylation (**Oide et al., 2006; Buhaescu and Izzedine, 2007; Schrettl et al., 2007**).

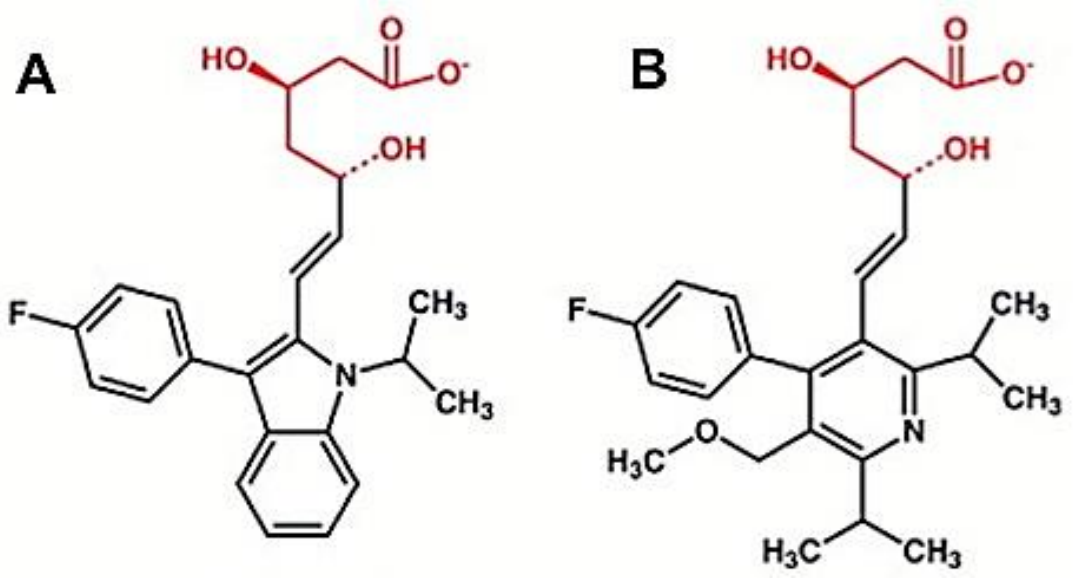


Fig 6. Biochemical structure: (A) simvastatin and (B) fluvastatin (**Istvan et al., 2003**).

3.5. Genetic background of the mevalonate-isoprenoid pathway in *M. circinelloides*

Several genes playing role in the mevalonate-isoprenoid biosynthesis in *M. circinelloides* have been cloned: *hmgS*, *mvk* and *dmd*, encoding the HGM-CoA synthase, mevalonate kinase and diphosphomevalonate decarboxylase (**unpublished data**), the *hmgR1*, *hmgR2* and *hmgR3*, encoding HMG-CoA reductases (**Nagy et al., 2014**), and the *ipi*, *isoA* and *carG*, encoding the IPP isomerase, FPP and GGPP synthases, respectively (**Velayos et al., 2003, 2004; Csernetics et al., 2011**).

The *hmgS*, *mvk* and *dmd* genes (**unpublished data**) have been cloned by our research group before the present thesis. The encoded enzymes are responsible for formation of HMG-CoA, mevalonate-5-phosphate and IPP, respectively. The HMG-CoA synthase - encoded by the 1812 bp (together with 256 bp 3'untranslated region) *hmgS* gene) - consist of 452 aa, and the molecular weight is 50.6 kDa. Two catalytic domains were

identified: the N-terminal (9 - 182 aa) and C-terminal (183 - 451 aa). Transcription factor (TF) binding sites in the promoter of *hmgS* responsible for regulation of sterol biosynthesis have also been identified (such as SRE motifs, and NF-Y, Sp1, CREB sites). The mevalonate kinase - encoded by the 1364 bp *mvk* gene - belongs to the enzyme family of galacto-, homoserine-, mevalonate- and phosphomevalonate (GHMP) kinases. The molecular weight of 409 aa protein is 44.9 kDa. The protein consists of one transmembrane domain (141 - 165 aa), thus it is anchored to the cell membrane. Several potential TF binding sites is presumed in the promoter: binding sites of ADR1 (positive regulators of peroxisomal genes in yeasts and PRARs (α-peroxisomal proliferator activated receptors **(Bemis and Denis, 1988**)). Binding sites of TFs mediating the expression of the HSF1 gene heat shock protien have also been identified (**Harrison et al., 1994**). Presumably these factors play role in regulation of ergosterol biosynthesis (**Harrison et al., 1994; Palmer et al., 1995; Bemis and Denis, 1998**). The diphosphomevalonate decarboxylase - encoded by the 1372 bp (together with 11 bp 5'- and 72 bp 3'untranslated region) *dmd* gene - also the member of the GHMP enzyme family and consist of two GHMP kinase catalytic domains (108 - 166 aa and 240 - 224 aa). Molecular weight of the 410 aa protein is 45.2 kDa. In the promoter region TF binding sites of ADR1 and HSF1, moreover ABF2 and NIT-2 have also been identified (**unpublished data**).

The *hmgR1*, *hmgR2* and *hmgR3* genes were identified in the *M. circinelloides* genome, encoding HMG-CoA reductases (**Nagy et al., 2014**). The length of the genes are 3740, 3595 and 3491 bp, while the encoded proteins are 1107, 1078 and 1115 aa, respectively. The calculated molecular mass of these proteins are 120.78, 118.45 and 120.71 kDa, respectively. HMG-CoA reductases are membrane anchored proteins and three main domains can be identified: the N-terminal hydrophobic domain containing several transmembrane segments, and a sterol-sensing domain; a short linker region and the conserved C-terminal catalytic domain (which contain the HMG-CoA binding motif). The proteins shows 47 - 57% amino acid identity to each other (**Nagy et al., 2014**). Overexpression of the *hmgR2* and *hmgR3* genes increased the carotenoid and ergosterol content in *M. circinelloides*, and decreased the sensitivity to statins at various degrees, while in the case of *hmgR1* no significant changes were found, suggesting differences in the role of the encoded proteins in isoprenoid biosynthesis (**Nagy et al., 2014**).

The IPP isomerase is responsible for the formation of DMAPP. It is encoded the *ipi* gene that was cloned and characterized in *M. circinelloides* in Csernetics and coworkers studies (**Csernetics et al., 2011**). The 910 bp gene encodes a 225 aa protein. The predicted

molecular mass of the putative IPP isomerase is 26 kDa. In the catalytic region (18 – 208 aa) the conserved „NUcleoside DIphosphate linked to some other moiety X" domain and the conserved cysteine and glutamic acid residues (C^{85} and E^{147}, respectively), important components of the active site, were also identified. The FPP synthase is a prenyltransferase, responsible for the conversion of IPP to GPP and FPP (to date GPP synthase was only identified in plants, **Szkopińska and Płochocka, 2005**). The 352 aa FPP is encoded by the 1492 bp *isoA* gene in *M. circinelloides* (**Velayos et al., 2004**). The GGPP synthase is also a prenyltransferase and is responsible for the conversion of FPP to GGPP. The GGPP synthase is encoded by the 1461 bp in *M. circinelloides* (*carG* gene). The encoded 303 aa protein has a predicted molecular mass of 34.78 kDa (**Velayos et al., 2003**). Three short APE-like (al-3 proximal element - like) sequences have been identified in the promoter, thus transcription of the gene is regulated by blue light (**Velayos et al., 2003**). Effect of overexpression of the *ipi*, *isoA* and *carG* on the carotenoid production of *M. circinelloides* was analyzed and was found that all three genes, but mainly overexpression of the *carG*, resulted significant increase in the total carotenoid content (**Csernetics et al., 2011**). The effect was more prominent, when the genes were placed under the control of the *M. circinelloides* glyceraldehyde-3-phosphate dehydrogenase 1 (*gpd1*) regulatory regions (**Csernetics et al., 2011**).

3.6. Genetic modification of Mucormycotina fungi

Genetic transformation is a biological process involving in the delivery and maintenance of exogenous DNA in the cell. The first transformation of fungi was carried out successfully 40 years ago with *S. cerevisiae* (**Beggs, 1978; Hinnen et al., 1978**), thereafter with *N. crassa* (**Case et al., 1979**), *Aspergillus nidulans* (**Balance et al., 1983; Yelton et al., 1984**) and *M. circinelloides* (**van-Heeswijck and Roncero, 1984**). These early examinations built up a fundamental protocol for protoplast transformation by incorporating them in concentrated of polyethylene glycol (PEG) in the presence of calcium ions followed by exposure to DNA. Potential impediments of PEG-mediated transformation are getting high concentration of viable protoplasts, low transformation efficiency and high rates of transient transformants. However, due to its simplicity in technical operation and equipment required, the PEG-mediated method remains the most commonly used one to conduct transformation in filamentous fungi. Additionally other methods, including *Agrobacterium tumefaciens*-mediated transformation (ATMT), electroporation and biolistic transformations were also developed for fungi, including

Mucoromycotina species (**Ruiz-Diez, 2002; Michielse et al., 2004; Nyilasi et al., 2005; Gutierrez et al., 2011; Li et al., 2017**).

The basic procedure for PEG-mediated transformation contains three major steps: protoplast preparation, uptake of the transforming DNA, and protoplast regeneration. Protoplasts or spheroplasts can be prepared through digestion of the cell walls of mycelium or germinating spores using cell wall degrading enzymes, such as helicase or glusulase from snail stomach preparation, and cellulase, β-1,3-glucanase, chitinase, and driselase from *Trichoderma* or other fungal species (**Fincham, 1989; Jung et al., 2000**). The digestion is performed in an osmotic buffer containing sorbitol or high salt concentration in order to stabilize osmotically the resulting protoplasts (**Fincham, 1989**). The uptake of DNA by protoplasts is accomplished by incubating them with high-concentrated DNA and by the addition of up to 10 volumes of a 40 – 60% PEG 4000 solution (**Fincham, 1989**). PEG has been observed to cause clumping and fusion of protoplasts, which is thought to facilitate the trapping of DNA to the fungal cells (**Fincham, 1989**). However, a current research suggested that PEG is unlikely to induce the coordination between DNA and the recipient cell surface, and the fusion of protoplasts is not the direct cause of DNA uptake; therefore, the role of PEG in DNA uptake still not described (**Kuwano et al., 2008**). Following the PEG treatment, protoplasts transferred to regeneration medium for recovery of the cell wall before being plated onto the selective medium. The selection of transformants can be achieved by using an auxotrophic complementation or drug resistance depending on the selectable marker gene built into the vector DNA.

Electroporation has been successfully used for the transformation of several fungi, including *N. crassa, Aspergillus oryzae, Aspergillus niger, Rhizopus oryzae* and *Colletotrichum* species (**Dandan et al., 2017**). By applying a high-voltage pulse that creates transitory small pores along the membrane surface, this procedure makes the cell membrane more permeable (**Batista et al., 2016**). Electroporation of protoplasts is the most widespread technique, although methods have also been developed for spores and mycelia (**Dandan et al., 2017**). The electroporation procedure for *M. circinelloides* protoplasts have been also described and successfully carried out (**Gutiérrez et al., 2011**). Biolistic transformation is also known as particle bombardment. In biolistic transformation, foreign DNA is adsorbed on the surface of tungsten or gold particles and further under the push of high pressure, the particles containing nucleic acid are injected into the host cell. Biolostic transformation has been utilized to successfully transform *A. nidulans, Trichoderma reesei* and also *M. circinelloides* (**Gonzalez-Hernandez et al., 1997; Dandan et al., 2017**). *A.*

tumefaciens is a gram-negative plant pathogenic bacterium, naturally causes crown gall tumors in plants. *A. tumefaciens* is able to transfer part of its tumor-inducing plasmid (T-DNA) into infected cells, where it integrates into the host genome. ATMT was implemented successfully to transform various fungal species, such as *Fusarium circinatum*, *Agaricus bisporus*, *Calonectria morganii*, *Fusarium oxysporum*, *Verticillium fungicola*, *Monascus purpureus* and *M. circinelloides* (**Covert et al., 2001; Mikosch et al., 2001; Malonek and Meinhardt, 2001; Mullins et al., 2001; Amey et al., 2002; Campoy et al., 2003; Nyilasi et al., 2005; Papp et al., 2013**).

In Mucoromycotina fungi artificial introduction of DNA by transformation is normally established and maintained by autonomously replicating plasmids (**van-Heeswijck and Roncero, 1984; Garre et al., 2014**). The first transformation of *M. circinelloides* was carried out by van-Heeswijck and Roncero **(van-Heeswijck and Roncero, 1984**). In this study 5'- upstream of the *leuA* gene (encoding the α-isopropylmalate isomerase) an autonomously replicating site (ARS) was described (**Roncero et al., 1989**). The exact position and the sequence boundaries of this *M. circinelloides* ARS element remain unclear (**Appel et al., 2004**), however in *M. circinelloides* ARS element is not required to maintain the plasmids as extrachromosomal elements (**van-Heeswijck and Roncero, 1984; Appel et al., 2004; Csernetics et al., 2011**). Relatively high transformation frequency can be reached with circular plasmids, at the same time the copy number of plasmid often remains low, resulting in mitotic instability (**Ibrahim and Skory, 2006**). The integration happens rarely in Mucoromycotina fungi when using circular plasmids, moreover it also difficult to achieve with linear fragments (**Papp et al., 2013**). This can be due to a presumed mechanism, which can protect the fungal genome from foreign DNA (**Ibrahim and Skory, 2006**). Successful integration was achieved with ATMT in *M. circinelloides*; in these studies random integration was observed, but transformants found to be unstable (**Nyilasi et al., 2005; Papp et al., 2013**). Stable transformants were isolated when random integration was carried out with restriction enzyme-mediated integration via PEG-mediated transformation of *M. circinelloides* protoplasts (**Papp et al., 2013**). Recently, the CRISPR/Cas9 system has been used to target and disrupt genes in *Aspergillus* spp., *T. reseii* and *S. cerevisiae* (**Fuller et al., 2015; Nødvig et al., 2015**; **Mans et al., 2015**). Moreover, it was successfully adapted to *M. circinelloides* for guided integration of the foreign DNA into the genome. This genome editing tool seems to be the most efficient for creation of knock out *M. circinelloides* mutants (**Nagy et al., 2017**).

Beside gene deletion, RNA-based gene silencing is also a frequently and successfully applied method for functional characterization of genes (**Nicolás et al., 2003; Hood, 2004; Redberry, 2006**). This approach seems to be beneficial when gene-targeting approaches fail, for example, when multiple copies of a gene of interest is present in the genome (**Meyer, 2008**). It is also a useful technique to study essential genes, when the deletion may be fatal to the organism (**Liu et al., 2002; Kadotani et al., 2003**). Three RNA-based (antisense RNA, hammerhead ribozymes and RNA interference) have been shown to be valuable tools for gene silencing in eukaryotes. All three silencing methods were already used successfully in various filamentous fungi (**Moralejo et al., 2002; Kadotani et al., 2003**). In eukaryotes, there are two mechanisms, each are related but distinct RNA silencing pathways (**Nakayashiki, 2005**). Based on the small RNA molecules involved, those are referred to as "small interfering RNA (siRNA)-directed pathway" and "microRNA (miRNA)-directed pathway". Both siRNAs and miRNAs interfere with gene expression via targeted degradation of mRNA or by the suppression of translation. The siRNAs act as guides for a siRNA-induced silencing complex to target perfectly complementary mRNAs for degradation. The second class of small RNAs, miRNAs is processed from imperfect stem-loop RNA precursors (pre-miRNAs) that are transcribed from non-protein-coding genes within plant and animal genomes. The mature miRNA causes the translational repression of the target mRNA that has imperfect complementarity to the miRNA (**Nakayashiki, 2005**).

In case of RNA interference, small RNA molecules direct the RNA-induced silencing complex that can degrade targeted mRNA molecules thereby blocking the translation into polypeptides (**Schumann et al., 2010**). All these small RNAs are derived from double stranded or hairpin RNA precursors createn by Dicer (**Fig. 7**) (**Eamens et al., 2008; Li et al., 2010**). RNA interference in fungi does not cause complete suppression of targeted gene (**Weld et al., 2006; Schumann et al., 2010**). While methods of gene knock down strategies require information of only small fragments of sequence, this can be implemented even in the organisms with less genetic information (**Weld et al., 2006; Schumann et al., 2010**). It has to be noted that efficiency of gene silencing method was not stable and constant in all fungi (**Mouyna et al., 2004**).

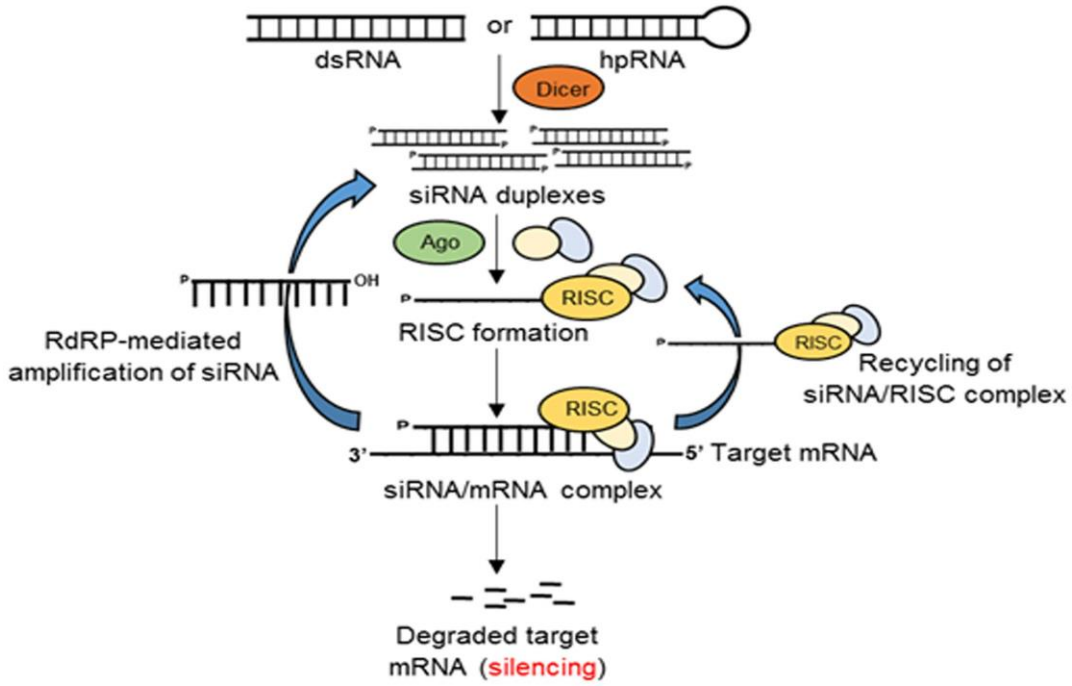


Fig 7. Schematic picture of RNA silencing mechanism in fungi (**Majumdar et al., 2017**).

Glyceraldehyde-3-phosphate dehydrogenase (GPD) is a catalyst in both glycolysis and gluconeogenesis. As a main housekeeping enzyme, its amino acid sequence demonstrates a strong structural conservation between different prokaryotic and eukaryotic organisms (**Chapman et al., 2015**). GPD is usually considered to be efficiently and constitutively expressed; for example, in *S. cerevisiaes* 2 - 5% of the poly $(A)^+$ RNA may constitute *gpd* mRNA (**Ringel et al., 2013**). On the basis of this feature, promoter of GPD-encoding genes were found to be efficient for expression of heterologous genes in several yeasts and filamentous fungi, and have been used to construct efficient transformation systems in numerous fungal species at various taxonomic positions, e.g., *Rhizomucor miehei*, *Paracoccidioides brasiliensis*, *A. nidulans* and *A. bisporus* **(Lima et al., 2003; Barbosa et al., 2004; Vastag et al., 2004; Lopes et al., 2008)**. The characterization of three individual genes (*gpd1*, *gpd2*, and *gpd3*) encoding GPD together with the use of the promoter of the *gpd1* gene for recombinant protein production in *M. circinelloides* is well describe by Wolff and Arnau (**Wolff and Arnau 2002**). Only transcription of *gpd1* was detected and the transcription was found to be highly regulated in response to carbon source (**Wolff and Arnau 2002; Larsen et al., 2004**). The promoter of *gpd1* was used in several experiments to express of homologous or heterologous genes in *M. circinelloides* (**Wolff and Arnau 2002; Larsen et al., 2004; Papp et al., 2006, 2013; Csernetics et al., 2011**).

A well-established PEG/$CaCl_2$-mediated transformation system is available for *M. circinelloides*, which is an often used as a model organism to study the carotenoid biosynthesis, dimorphism, sexual reproduction and pathogenicity (**Gooday and Adams, 1993; Papp et al., 2006, 2013; Csernetics et al., 2011; Li et al., 2011; Wang and Lin, 2012; Lee et al., 2015; Ruiz-Vázquez et al., 2015; Zhang et al., 2016**). Dominant selection markers are often not usable for Mucoromycotina fungi (**Papp et al., 2010**), thus it serves a great advantage that *M. circinelloides* MS12, a leucine and uracil auxotroph mutant *M. circinelloides* is available for the genetic modification (**Benito et al., 1992**). In addition, its genome sequence is available (**Corrochano et al., 2016**) making a good support for the characterization of genetic background of biological processes.

4. AIMS OF THE STUDY

M. circinelloides (Mucoromycotina, Mucorales) is a carotenoid producing fungus, which is a model organism of several carotenogenic studies. Carotenoids together with other terpenoids, such as ergosterol or the prenyl groups of ubiquinones and the farnesylated and geranylgeranylated proteins, are synthesized via the mevalonate-isoprenoid pathway in fungi. These metabolites play an important role in signal transduction, morphogenesis, adaptation to environmental change and protection against free radicals. Moreover, ergosterol and its biosynthesis are one of the main targets of the antifungal agents used to treat infections caused by Mucoromycotina fungi in clinics.

Till the date limited information is available about the function and regulation of the genes playing role in mevalonate-isoprenoid pathway in Mucoromycotina fungi. Characterization of these genes can serve valuable knowledge for the improvement of secondary metabolite production by Mucoromycotina fungi and may lead us to better understanding of biological processes, including morphogenesis, protection against free radicals, response to environmental change (e.g. changes in the soil composition, oxygen concentration or salinity of the environment) or pathogenesis.

In this study six genes involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides*, encoding the HMG-CoA synthase (*hmgS*), mevalonate kinase (*mvk*), diphosphomevalonate decarboxylase (*dmd*), IPP isomerase (*ipi*), FPP and GGPP synthases (*isoA* and *carG* genes, respectively) were selected for characterization. One of our goals was to improve the carotenoid production of the fungus with overexpression and silencing of the genes. Our next aim was to examine the effect of the overexpression and silencing of the genes on the ergosterol content of *M. circinelloides* and it's effect on the susceptibility to different antifungals, which may lead to identify targets for new antifungal therapy. Little is known about the efficiency of gene silencing can be achieved with different plasmid constructs in *M. circinelloides*, thus among our aims was to compare the characteristics of the mutants harbouring different plasmids for gene knockdown as well.

The following specific objectives have been addressed to present study:

1. Investigation of the transcription of selected six genes under different cultivation conditions involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides.*

Effect of cultivation conditions, such as temperature, oxygen tension, light sources, medium composition and incubation time on the transcription of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes.

2. Overexpression and silencing of selected six genes involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides* and characterization of the transformants

Development of different plasmid constructs for overexpression and silencing of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes in *M. circinelloides.* Transformation experiments with the *M. circinelloides* MS12 strain. Characterization of the transformants in detail, such as analyzes of micromorphology, carotenoid and ergosterol content, antifungal susceptibility and interaction with macrophages. Comparison of the overexpression and gene silencing efficiency achieved with the different plasmid constructs.

5. MATERIALS AND METHODS

5.1. Strains used in the experiments

During the transformation experiments the *M. circinelloides f. lusitanicus leuA*$^{-}$ and *pyrG*$^{-}$ double auxotroph mutant *M. circinelloides* MS12 strain (SZMC 12082), derived from the CBS 277.49 isolate was used (**Benito et al., 1992**). Plasmid propagation was performed in *Escherichia coli* TOP 10F^{-} (Invitrogen, genotype: F′{*lac*Iq, Tn*10*(TetR)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G).

5.2. Applied media and growth conditions

YNB minimal medium

1% D-glucose, 0.15% $(NH_4)_2SO_4$, 0.15% Na-L-glutamate, 0.05% YNB (yeast nitrogen base without amino acids, Sigma-Aldrich), supplemented with 0.05% uracil and/or 0.05% leucine (w/v) if required. For solid medium 2% (w/v) agar was added. For PEG/$CaCl_2$-mediated protoplast transformation, YNB medium with 0.8 M sorbitol containing 1% or 2% (w/v) agar were used.

Occasionally composition of the YNB medium was modified according to the experiments: the glucose concentration was increased to 2.5% - 5% (w/v) or was replaced with dihydroxyacetone (DHA), sodium-acetate (Na-ac), maltose (Mal), or trehalose (Treh) or the medium was supplemented with 1 – 3% (w/v) NaCl or 0.5 – 4 μg/ml FLU.

Yeast extract – Glucose medium (YEG)

1% D-glucose, 0.5% yeast extract, 2% agar (w/v) (pH 4.5).

Yeast extract – Peptone – Glucose broth (YPG)

2% D-glucose, 1% peptone, 0.5% yeast extract (w/v) (pH 4.5), supplemented with 0.8 M sorbitol for regeneration of protoplasts.

Luria-Bertani medium (LB)

1% NaCl, 1% tryptone, 0.5% yeast extract (w/v) (pH 7.0), supplemented with 2% (w/v) agar for making solid medium. For the selection of transformant *E. coli* strains, the medium was supplemented with ampicillin in a final concentration of 100 μg/ml.

Malt extract agar (MEA)

1% D-glucose, 0.5% yeast extract, 1% malt extract, 2% agar (w/v).

Growth conditions and strain maintenance

For cultivation of fungus 10^6 spores were inoculated onto fresh media in 150 µl final volume. Fungal strains were maintained on YNB medium (supplemented with uracil and/or leucine if necessary) at 4 °C. Their cultivation was usually done at 25 °C for four days under constant illumination. Occasionally the inoculum size (2×10^2 – 10^6 spore/plate or flask), the temperature (between 15 and 37 °C), the light source (daylight (Sylvania Daylight F8W/T5/154), warm white (Osram Warm White L8W/32-930) light source and dark), oxygen tension (aerobic – anaerobic conditions) and growth time (from 4 hours to 4 days) was varied according to the experiments. For later use, the mycelium collected from the culture medium was stored frozen at -20 ºC (after distilled water wash).

When the effect of different cultivation conditions on the gene transcription was investigated the *M. circinelloides* MS12 strain was cultivated on solid YNB plates supplemented with leucine and uracil and grown for 4 days under continuous daylight illumination at 25 °C. The inoculum size was 10^6 sporangiospores/plate (control conditions). Occasionally, the cultivation was performed in 30 ml YNB liquid medium; the inoculum size was 10^4 sporangiospores/ml. To investigation of the effect of illumination, the fungal strain was cultivated in dark, under continuous daylight or warm white light source or under continuous dark followed by 10 sec of illumination and 10 min in dark. Daylight and warm white bulbs differing in the color temperature of the emitted light (3000 K and 6500 K, respectively), the light color of a lamp depends on its spectral power distribution in the visible range; white light of the warm white source contains more components in the yellow-red wavelength range, while white light of the daylight source contains more components in the blue range. Temperature dependence of the gene transcription was tested by cultivating the fungal strain on solid YNB media at 15, 20, 25, 30 and 35 °C. To examine the effect of different carbon sources, glucose was replaced with Treh, Mal, DHA and Na-ac in a final concentration of 1% (w/v) in YNB. To test the effect of the different salt and glucose concentrations, NaCl were added to solid YNB in a final concentration of 1, 2 and 3% (w/v) or glucose of 1, 2.5 and 5% (w/v), respectively. Our research group previously analyzed the susceptibility of *M. circinelloides* MS12 against different statins (such as fluvastatin (FLU), atorvastatin, rosuvastatin, simvastatin (SIM) and pravastatin) and FLU showed the most prominent inhibition effect on the fungal

growth (**unpublished data**). To test the effect of FLU on the gene transcription, strains were grown in 30 ml liquid YNB containing FLU in final concentrations of 0.5, 1, 2 and 4 μg/ml for 4 days with shaking (150 rpm) at 25 °C. Cultivation under anaerobic condition was performed in 30 ml liquid YNB in a BBL GasPak Anaerobic System (Becton Dickinson) at 25 °C.

5.3. Primers used in this study

PCR primers were designed for the amplification of genes and gene fragments, sequencing and real-time PCR. The primers used in this study are shown in **Table 2**.

Table 2. Primers used in the experiments. Primer names, sequences, usage and the expected size of amplicons are also shown in the table. The recognition site of restriction endonucleases is shown in underlined and additional nucleotides upstream from the recognition sites are shown in normal letters

Name of primer	Sequence 5'- 3'	Fragmet size (bp)	Used for
hmgS F	GCC TCA TCT AAA GCC TTC GT	3141	amplification of the *M. circinelloides hmgS, mvk* and *dmd* genes together with their regulatory regions
hmgS R	AAA GAC CGG CTG GAA GAT GT		
mvk F	GCA ATG GTG ACC TCA ACC TCA A	2688	
mvk R	CGT AGG TCA AGA AAC CAT CCG A		
dmd F	CTA TGC CGT GCA ATG CGA GAT T	2679	
dmd R	GGA GGA GTG TGA AGC CTG TAA		
hmgS gpd F	gcgATCGAT ATG ACT GTC CCC AAC TAC AAC A	1560	amplification of the coding region of *M. circinelloides hmgS, mvk* and *dmd* genes
hmgS gpd R	tatGCGGCCGC CTA ATC CTT ACG CTT GTA GAA A		
mvk gpd F	attGTCGAC ATG ACT GCA ACT GAA CAA A	1364	
mvk gpd R	cgcGAATTC TTA GTT TTG AAA GTA TTG TTC C		
dmd gpd F	cgcATCGAT ATG AAG ACT GTT ACC TGT ACT GCT CCC	1289	
dmd gpd R	taaGCGGCCGC CTA TGC AAG ACG CTT GGG AA		
hmgSas F	tcgATCGAT TGC GTT ATT CAG TCA GAG ATG	810	amplification of gene fragments for construction of plasmids for gene silencing (*as* constructs)
hmgSas R	tatCTCGAG TGC ACG AGC AAA GGA CTT GTT		
mvkas F	tatGAGCTC CCC GTC ATC CTG ATG ATA TGC	800	
mvkas R	tcgATCGAT AAT AAA GGT AAC AGC GCA TCC		
dmdas F	tagATCGAT AGT GTC CAG AAT CGC TCG TCA	726	
dmdas R	gctCTCGAG TGG CTT AAT ACA CGA GGT CCA		
ipias F	gcgATCGAT GAT TTG AAG GAA TAT GAC GAA	887	
ipias R	tatCTCGAG GAT GAA TGG TAT CAT CGG CCT		
isoAas F	tcgATCGAT TGA TAT CAT GGA TGC TTC CAT C	843	
isoAas R	tagCTCGAG TTC TTG ACA CCG ACA GAT TCG T		
carGas F	ctgATCGAT GTG CCT GTC GCT CAC CAT ATT	858	
carGas R	aatCTCGAG TGG TGG CCT CTA CTC TGA TGG TC		
hmgS hpRNA1 F	tgtCTCGAG CCG TGC AAA ACT TGA TGG AAA A	691	amplification of gene fragments for construction of plasmids for gene silencing (*hpRNA* constructs)
hmgS hpRNA1 R	acgATCGAT GCC GGC CTA CAA TTG TGT GTT AGT CA		
hmgS hpRNA2 F	tacGCCGGC TGC ACG AGC AAA GGA CTT GTT	608	
hmgS hpRNA2 R	tcaGCGGCCGC CCG TGC AAA ACT TGA TGG AAA AG		
mvk hpRNA1 F	aggATCGAT GCC GGC GTA CCA TTT GAT CTA CAA AC	829	

mvk hpRNA1 R	tcaGCGGCCGC TGA GAC CTG ATT GAG CAG TG		
mvk hpRNA2 F	aagCTCGAG TGA GAC CTG ATT GAG CAG TG	753	
mvk hpRNA2 R	gtaGCCGGC ATA CCC GTC ATC CTG ATG ATA T		
dmd hpRNA1 F	tatATCGAT GCC GGC GTA AGT TGG CTT GTC TGA TC	756	
dmd hpRNA1 R	tatGCGGCCGC TGT CCA AAA CAG CCT TCT TCA		
dmd hpRNA2 F	gtcCTCGAG TGT CCA AAA CAG CCT TCT TCA	700	
dmd hpRNA2 R	ataGCCGGC GTC ATC AAA TAT TGG GGC AA		
ipi hpRNA1 F	tcgCTCGAG ATG GCC CCT GAT TTG AAG GA	788	
ipi hpRNA1 R	cgtATCGAT GCC GGC CTA GCA TGT CAA TAA GGT CAG		
ipi hpRNA2 F	agtGCCGGC TGC TGT CAG CAA ACA TGG CCT	731	
ipi hpRNA2 R	tatGCGGCCGC ATG GCC CCT GAT TTG AAG GA		
isoA hpRNA1 F	ttcATCGAT GCC GGC GTA AGT TGT TCT TAT CAC TGG	753	
isoA hpRNA1 R	agtGCGGCCGC GTC AAG GAT CTT GCG TTG TTC G		
isoA hpRNA2 F	cgcCTCGAG GTC AAG GAT CTT GCG TTG TTC G	693	
isoA hpRNA2 R	taaGCCGGC CTT CAA GCC TTC TTT TTG GT		
carG hpRNA1 F	tcgCTCGAG ATG CTC AAC TCA CAC AAC AGA	656	
carG hpRNA1 R	tatATCGAT GCC GGC CTG CCC GAT CGT GTT ACT C		
carG hpRNA2 F	catGCCGGC TGT TGT TGA CCA TAT CAA TGT ACT C	601	
carG hpRNA2 R	tatGCGGCCGC ATG CTC AAC TCA CAC AAC AGA		
hmgSRT F	TCC AAC ACT TGC TAC ATT CGT	112	quantitative real-time PCR
hmgSRT R	GGC AGA CAA CAT AAT CAA TAT CGT C		
mvkRT F	GAT CGC TAT TCG TTG TCG TG	122	
mvkRT R	CAA GTC CAT CGA GCA AAC AG		
dmdRT F	CGT GAT TCC AAC CAG TTC CA	139	
dmdRT R	AAG CAG CCT TCA ATT TAC CGT		
ipiRT F	ATG TGG ACC AAC ACT TGC TGC TC	176	
ipiRT R	TTG ATG CCA AGC TCA TGC TCC AG		
isoART F	ATC TCG ACT GTT ACG GTG CTC CT	119	
isoART R	CTT GCG TTG TTC GGG ATT AGC CA		
carGRT F	CAA CAT CAT CAG CCA GAA GCC CA	148	
carGRT R	ACC ACC CAA ACG CTT GAT TTC CT		
actART F	CAC TCC TTC ACT ACC ACC GCT GA	117	
actART R	GAG AGC AGA GGA TTG AGC AGC AG		
gpdP	CAT GAA GTG TGA GAC ATT GCG A	--	investigation of the presence of plasmids in transformants
gpdT	TAC ATA TCA GAG GGT TGG AAC A		
ipigpd F	ggCTCGAG ATG GCC CCT GAT TTG AAG GAA TA		
ipigpd R	ctcGCGGCCGC TTA GAA GCC TAA ACG ATG AAT GGT		
isoAgpd F	ggCTCGAG ATG GTT GCT GTC AAA TTA CA		
isoAgpd R	ctcGCGGCCGC TTA TTT AGT ACG CTT GTA AA		
carGgpd F	ggCTCGAG ATG CTC AAC TCA CAC AAC AG		
carGgpd R	ctcGCGGCCGC CTA GTC GTT GGT GGC CTC TA		
amprev1	GGC GAC ACG GAA ATG TTG AAT AC		
amprev2	CGA AAT AGA CAG ATC GCT GAG		

5.4. Plasmid constructs used in this study

M. circinelloides MS12 strain is a leucine and uracil double auxotroph mutant. The pAVB107 and pEPM901 (pEPM9) plasmids harbour the *leuA* and *pyrG* genes (encoding the α-isopropylmalate isomerase and orotidine 5'-monophosphate decarboxylase, respectively), which complement the leucine and uracil auxotrophy, were constructed previously (**Roncero et al., 1989; Benito et al., 1992; Velayos 2000; Csernetics et al., 2011**). The plasmid pPT81, which harbours the *M. circinelloides gpd1* (encoding the glyceraldehyde-3-phosphate dehydrogenase 1, EMBL Acc. No.: AJ293012) promoter and terminator regions (*gpdP* and *gpdT*) and the *pyrG* gene (in a previous study named as pPT43pyr, **Csernetics et al., 2011; Nagy et al., 2014**), and pMAT1811, provided by Victoriano Garré (Universidad de Murcia, Murcia, Spain), which harbours the *gpd1* and *zrt1* (encoding a ZIP zinc transporter protein, NCBI Acc. No.: MUCCIDRAFT_155215) promoter regions (*zrt1P*) facing to each other and the *pyrG* gene (**unpublished**), was also used in cloning experiments (**Wolff and Arnau, 2002**; **Csernetics et al., 2011**; **Trieu et al., 2017**) (**Suppl. 1**).

Different plasmids were constructed for overexpression and silencing of the six genes. For overexpression the genes were amplified together with their own promoter and terminator regions (1) or were placed under the control of the *M. circinelloides gpd1* promoter and terminator (2) (*own* and *gpd* plasmid constructs, respectively; **Fig. 8 A** and **B**):

(1) The *hmgS*, *mvk* and *dmd* genes (**Suppl. 2**, unpublished) were amplified together with their regulatory regions with the hmgS F – hmgS R, mvk F – mvk R and dmd F – dmd R primers (**Table 2**), respectively, and were ligated into pBluescript SK+ (Stratagene) or pJET1.2/blunt cloning vectors. The plasmids were digested with *Nae*I – *Cla*I, *Not*I – *Pst*I and *Not*I – *Xho*I restriction endonucleases, and the fragments harbouring the *hmgS*, *mvk* and *dmd* genes were inserted to the corresponding sites of the pEPM901 plasmid. Thus the constructed plasmids (pHMGS/own, pMVK/own and pDMD/own) harbour the *hmgS*, *mvk* or *dmd* genes together with their own regulatory regions and the *pyrG* marker gene (**Table 3, Fig. 8 A**). Plasmids, carry the *ipi* (NCBI Acc. No.: AM903092), *isoA* (NCBI Acc. No.: AJ496299) and *carG* (NCBI Acc. No.: AJ276129) genes with their own regulatory regions and *leuA* or *pyrG* marker genes - named pIPI/own, pISOA/own and pCARG/own - had been constructed previously (in that study named as pCA10, pAVB160 and pCA6, respectively, **Csernetics et al., 2011**) (**Table 3, Fig. 8 A**).

(2) The coding regions of *hmgS*, *mvk* and *dmd* genes were amplified with the hmgS gpd F – hmgS gpd R, mvk gpd F – mvk gpd R and dmd gpd F – dmd gpd R primer pairs (**Table 2**) and the PCR products were ligated into pJET1.2/blunt cloning vector. The *hmgS* and *dmd* were digested from the plasmids with *Cla*I – *Not*I restriction endonucleases and ligated into the corresponding sites of pPT81 plasmid, between the *gpd1* promoter and terminator regions. The *mvk* gene was digested from the plasmid with *Sal*I – *EcoR*I and was ligated into the *gpd1* promoter and terminator of pPT43 plasmid and then the fragment digested with *Nae*I – *Sac*I was ligated into pEPM901. The constructed pHMGS/gpd, pMVK/gpd and pDMD/gpd plasmids harbour the *hmgS*, *mvk* or *dmd* genes within the *gpd1* promoter and terminator regions and the *pyrG* marker gene (**Table 3, Fig. 8 B**). Plasmid, carry the *ipi*, *isoA* and *carG* genes under the control of the *gpd1* regulatory regions and *pyrG* or *leuA* marker genes - named pIPI/gpd, pISOA/gpd and pCARG/gpd - were constructed previously (in that study named as pPT82, pPT86 and pPT87, respectively, **Csernetics et al. 2011** and **unpublished data**) (**Table 3, Fig. 8 B**).

For gene silencing three different plasmids were constructed for all six genes: (1) the genes were inserted between the *Mucor gpd1* and *zrt1* promoter (**Fig. 8 C**); (2) a fragment of a gene was inserted between the *Mucor gpd1* promoter and terminator in inverted orientation (**Fig. 8 D**); (3) a fragment of a gene and its reverse complement together with an intron were placed under the regulation of the *gpd1* promoter and terminator (**Fig. 8 E**) (*pMAT*, *as* and *hpRNA* plasmid constructs, respectively):

(1) The pMAT1811 plasmid harbours the *gpd1* and *zrt1* promoter regions facing to each other and the *pyrG* marker gene (unpublished). The *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes were digested from pHMGS/gpd (*Xho*I – *Not*I, with reduced *Xho*I enzyme and digestion time), pMVK/gpd (*Sal*I – *Eco*RI), pDMD/gpd (*Cla*I – *Not*I), pIPI/gpd (*Xho*I – *Not*I), pISOA/gpd (*Xho*I – *Not*I) and pCARG/gpd (*Xho*I – *Not*I) plasmids, respectively, and were placed between the corresponding sites of pMAT1811, inside the *gpd1* and *zrt1* promoters, resulted in the pHGMS/pMAT, pMVK/pMAT, pDMD/pMAT, pIPI/pMAT, pISOA/pMAT and pCARG/pMAT plasmids (**Table 3, Fig. 8 C**).

(2) The amplified fragments with hmgSas F – hmgSas R, dmdas F – dmdas R, ipias F – ipias R, isoAas F – isoAas R, carGas F – carGas R primer pairs (**Table 2**) were ligated into pJET1.2/blunt cloning vector. The fragments were cut off with *Cla*I – *Xho*I restriction endonucleases and were placed into the corresponding sites of pPT81. In case of *mvk* PCR amplification was unsuccessfull, thus from pMVK/gpd a fragment digested with *Cla*I – *Not*I was ligated into the corresponding sites of pPT81. The resulted plasmids –

pHMGS/as, pMVK/as, pDMD/as, pIPI/as, pISOA/as, pCARG/as – harbours a fragment of the mevalonate-isoprenoid pathway genes between the *gpd1* promoter and terminator in inverted orientation and the *pyrG* marker gene (**Table 3, Fig. 8 D**).

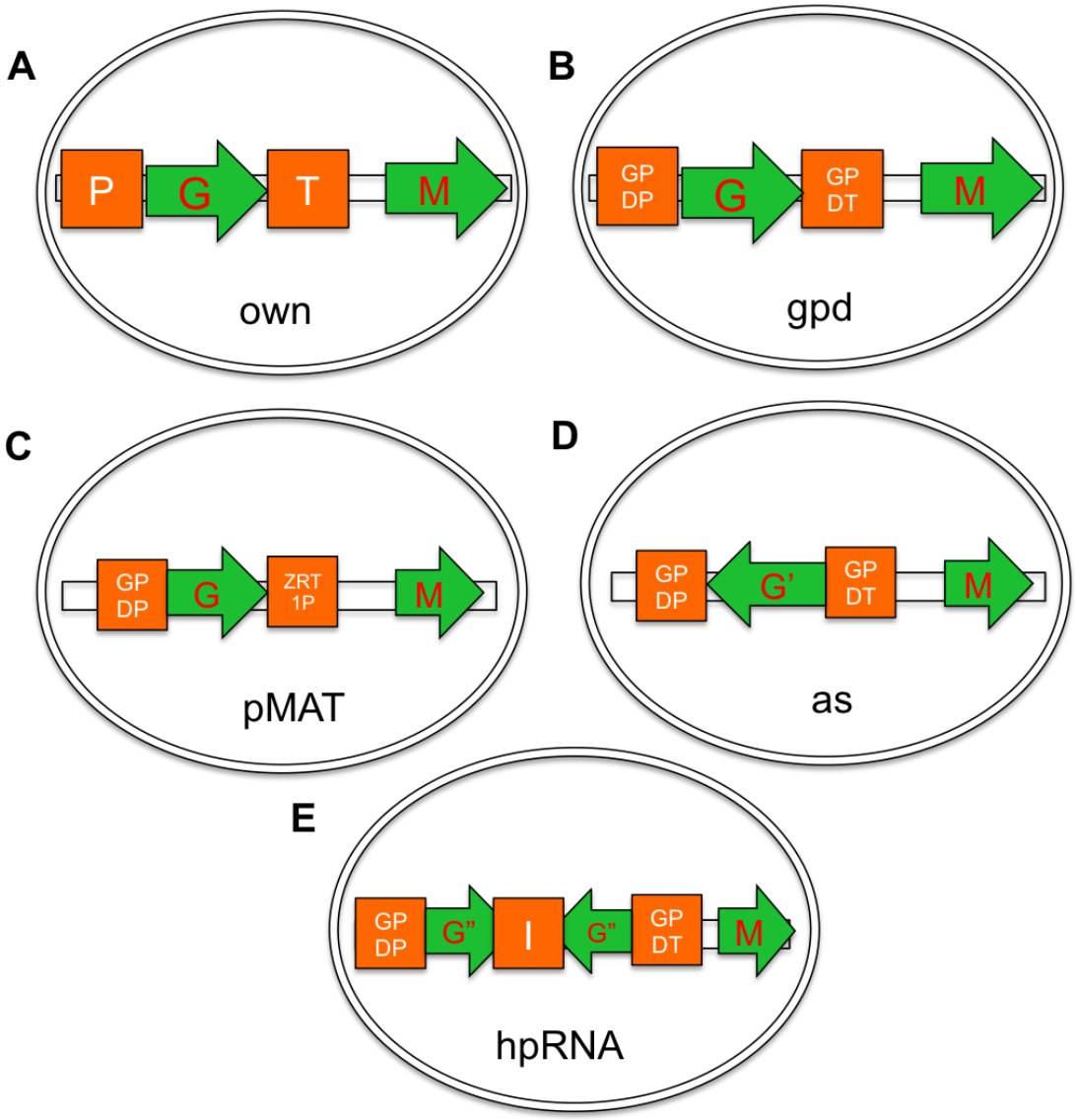


Fig 8. Schematic picture of plasmid constructions used in this study. The plasmid *own* and *gpd* were constructed for overexpression of the genes, while plasmids *pMAT*, *as* and *hpRNA* were constructed for gene silencing. The genes were amplified with their own promoter and terminator (A), or were placed under the control of *M. circinelloides gpd1* regulatory regions (B) for overexpression. For gene silencing the genes were inserted between the *Mucor gpd1* and *zrt1* promoter (C), a fragment of genes were inserted between the *M. ciricinelloides gpd1* promoter and terminator in inverted orientation (D), and a fragment of a gene and its reverse complement together with an intron were placed under the regulation of the *gpd1* promoter and terminator (E). M – marker gene (*Mucor pyrG* or *leuA*) used for selection of the transformants.

(3) To the hpRNA-mediated gene silencing two fragments were amplified for each gene with primers shown in **Table 2**: a fragment of a gene together with an intron downstream and its reverse complement without the intron. The fragments were ligated into pTZ57R/T or pJET1.2/blunt cloning vectors. The fragments together with the intron were digested from plasmids with *Cla*I – *Xho*I or *Cla*I – *Not*I restriction endonucleases and ligated into the corresponding sites of pPT81, between the *gpd1* promoter and terminator. Next, the fragments without the introns have been digested from the plasmids with *Not*I – *Nae*I or *Xho*I – *Nae*I restriction endonucleases and ligated into the corresponding sites of pPT81. The resulted plasmids – pHMGS/hpRNA, pMVK/hpRNA, pDMD/hpRNA, pIPI/hpRNA, pISOA/hpRNA and pCARG/hpRNA – carry a fragment of a gene and its reverse complement together with an intron under the regulation of the *gpd1* promoter and terminator and the *pyrG* marker gene (**Table 3, Fig. 8 E**).

5.5. Experimental methods

5.5.1. Extraction of genomic DNA from *M. circinelloides*

For DNA purification GeneElute^{TM} Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) was used following the instructions of the manufacturer. Occasionally, the total DNA was purified with home-made solutions. In that case mycelium of *M. circinelloides* was disrupted in liquid nitrogen with mortar and pestle and 2.5 ml lyses buffer (50 mM Tris (pH 8.0), 20 mM EDTA, 1% sodium lauroyl sarcosinate (w/v)) was added for each gram of mycelium powder. After mixing with 5 µl of 20 µg/ml RNase A (Sigma) the mixture was incubated at 65 °C for 30 min. After cooling down to room temperature, the mixture was centrifuged (8,000 × g, 10 min, 4 °C) and an equal volume of PCI (phenol:chloroform:isoamyl alcohol mixed in 25:24:1 ratio (v/v)) was added to the supernatant. The mixture was gently shaken (45 rpm) for 2 - 3 hours at 4 °C. After centrifugation (8,000 × g, 10 min, 4 °C) the aqeous phase was washed with CI (chloroform:isoamyl alcohol mixed in 24:1 ratio (v/v)). The aqeous phase was collected in a fresh tube and the DNA was precipitated with 96% (v/v) ethanol at -20 °C overnight. After centrifugation (13,000 × g, 20 min, room temperature) the DNA was washed with 70% (v/v) ethanol and was dried under vacuum. The precipitate was dissolved in 500 µl nuclease-free distilled water.

Table 3. Plasmids used in this study and their characteristics.

Plasmid	Gene cassette	Experiment	Marker gene	Reference
pAVB107	-	Control	*leuA*	Velayos et al. (2000)
pEPM901	-	Control	*pyrG*	Benito et al. (1992)
pPT81 (pPT43pyr)	*gpd1* promoter and terminator	Vector constructions	*pyrG*	Csernetics et al. (2011)
pPT43	*gpd1* promoter and terminator	Vector constructions	-	Csernetics et al. (2011)
pHMGS/own	Genes amplified with own promoter and terminator	Overexpression	*pyrG*	This study
pMVK/own			*pyrG*	This study
pDMD/own			*pyrG*	This study
pIPI/own			*pyrG*	Csernetics et al. (2011)
pISOA/own			*leuA*	Csernetics et al. (2011)
pCARG/own			*pyrG*	Csernetics et al. (2011)
pHMGS/gpd	Genes under the control of *gpd1* promoter and terminator	Overexpression	*pyrG*	This study
pMVK/gpd			*pyrG*	This study
pDMD/gpd			*pyrG*	This study
pIPI/gpd			*pyrG*	Csernetics et al. (2011)
pISOA/gpd			*leuA*	Csernetics et al. (2011)
pCARG/gpd			*leuA*	Csernetics et al. (2011)
pHMGS/MAT	Fragment of gene under the control of *gpd1* and *zrt1* promoter facing to each other	Gene silencing	*pyrG*	This study
pMVK/MAT				
pDMD/MAT				
pIPI/MAT				
pISOA/MAT				
pCARG/MAT				
pHMGS/as	Fragment of gene under control of *gpd1* promoter and terminator in inverted orientation	Gene silencing	*pyrG*	This study
pMVK/as				
pDMD/as				
pIPI/as				
pISOA/as				
pCARG/as				
pHMGS/hpRNA	Fragment of gene and its reverse complement together with an intron under the regulation of the *gpd1* promoter and terminator	Gene silencing	*pyrG*	This study
pMVK/hpRNA				
pDMD/hpRNA				
pIPI/hpRNA				
pISOA/hpRNA				
pCARG/hpRNA				

5.5.2. Agarose gel electrophoresis and isolation of DNA from agarose gel

For agarose gel electrophoresis 0.8 – 2% (w/v) agarose (depending on the size of the expected fragments) was dissolved in TAE buffer (40 mM Tris-acetic acid (pH 7.6), 1 mM Na_2EDTA). The separation was performed in TAE buffer with 80-110 V for 1 - 4 hour. For visualization of nucleic acid under UV-light 0.5 µg/ml ethidium bromide (from 10 mg/ml stock solution, dissolved in distilled water, Sigma) was used in the experiments.

The sample buffer was 1 × DNA Loading Dye (in final concentration, Thermo Scientific), to determine the size of the bands GeneRuler 1 kb DNA Ladder (Thermo Scientific) was applied.

The DNA was cut off from the agarose gel with sterile scalp under UV light and DNA was obtained using the GenElute Minus EtBr Spin Column Kit (Sigma-Aldrich) or Zymoclean Large fragment DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions.

5.5.3. Applied PCR techniques and reaction conditions

For cloning PCR reactions were carried out in a T3 Thermocycler (Biometra). The primers were synthesized with Integrated DNA Technologies (IDT and Bio-Science-Hungary). The applied primers are shown in **Table 2**.

To amplify genes or gene fragments from genomic DNA *Phusion High Fidelity DNA Polymerase* (Thermo Scientific) was used according to the manufacturer's recommendations. The reactions were prepared in a final volume of 25 µl as follows:

50 – 100 ng genomic DNA
0.4 – 0.4 µM specific primer
0.2 mM dNTP mix (Thermo Scientific)
1× *Phusion HF* or *GC* buffer
0.6 U *Phusion High Fidelity DNA Polymerase*

Amplification conditions were as follows: 98 °C 3 min, [35 cycles: 98 °C 30 sec, 58 – 72 °C 30 sec, 72 °C 10-100 sec] and 72 °C 10 min, cooling to 4 °C. The annealing temperature and extension time was modified according to the primers and length of the expected amplicons.

5.5.4. Restriction digestion and ligation

Restriction digestions and ligations were performed according to the commonly used methods and following the manufacturer's instructions with optimization to the particular experimental conditions (**Sambrook et al., 1989**). DNA fragments were ligated into pJET1.2/blunt (CloneJET PCR Cloning Kit, Thermo Scientific) and pTZ57R/T (Thermo Scientific) cloning vectors following the manuals.

5.5.5. Determination of sequences

Sequencing of positive clones was performed with LGC Genomics (Berlin, Germany). Sequences were analyzed with BioEdit Sequence Alignment Editor, NCBI-BLAST (National Center for Biotechnology Information - Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Expasy Bioinformatics Resource Portal (http://www.expasy.ch).

5.5.6. Preparation and transformation of competent *E. coli* cells and plasmid DNA purification

200 µl of *E. coli* TOP10F^{-} suspension was inoculated into LB broth from glycerol stock and were incubated for 16 hours at 37 °C. After that, fresh 30 ml LB medium was inoculated with 1 ml of the 16 hours cultures and the *E. coli* was grown with shaking at 200 rpm at 37 °C up to OD_{660} = 0.6. The culture was centrifuged (2160 × g, 10 min, 4 °C) and then the cells were washed with 15 ml 100 mM ice-cold $CaCl_2$ solution. After centrifugation (2160 × g, 10 min, 4 °C), the cells were resuspended in 30 ml 100 mM ice-cold $CaCl_2$ solution and incubated for 1 hour at 4 °C. After centrifugation (2160 × g, 10 min, 4 °C) and washing with 100 mM ice-cold $CaCl_2$ the sedimented cells were resuspended in 1/20 volume of cold, 20% (v/v) glycerol containing 100 mM $CaCl_2$. Competent cells were dispensed into 100 µl aliquots and stored at -70 °C until later use.

For *E. coli* transformation 100 µl of frozen competent cells were thawed on ice and 20 µl of ligation mixture and 80 µl TCM buffer (10 mM Tris (pH 7.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$) were added to the cells. The mixture was incubated for 30 min on ice. Heat shock was performed on 37 °C for 3.5 min and subsequently the mixture was incubated for 10 min at room temperature. *E. coli* cells were finally plated onto 100 µg/ml ampicillin containing LB medium (50 mg/ml ampicillin sodium salt stock solution dissolved in distilled water, Sigma). For blue-white screening of positive clones 20 – 20 µl of X-Gal (20 mg/ml stock solution dissolved in dimethylformamide, Thermo Scientific) and IPTG (isopropyl-β-D-thiogalactopyranoside, 20 mg/ml stock solution dissolved in water, Thermo Scientific) were added to the media. Petri dishes were incubated for 16 hours at 37 °C.

For plasmid DNA purification from *E. coli* Mini Plus Plasmid DNA Extraction System (Viogene) and Midi Plus Ultrapure Plasmid DNA Extraction System (Viogene) were used following the instructions of the manufacturer.

5.5.7. Total RNA purification from *M. circinelloides* and cDNA synthesis

For RNA extraction E.Z.N.A.® Fungal RNA Kit (Omega Bio-tek) and E.Z.N.A.® Total RNA Kit II (Omega Bio-tek) were used following the instructions of the manufacturer. RNA samples were treated with DNase I (Thermo Scientific) as follows:

3 μg RNA
1 × reaction buffer with $MgCl_2$
3 U DNase
the final volume was set with diethyl pyrocarbonate-treated water.

The reaction was carried out at 37 °C for 30 min and then quenched with 3 μl 50 mM EDTA at 65 °C for 10 min. Each sample was checked with PCR or qPCR for DNA contamination. Reverse transcription was performed with RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) or with the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). For the reaction, oligo $(dT)_{18}$ and random hexamer primers were used according to the manufacturer's instructions.

5.5.8. Quantitative real-time PCR

The qPCR reactions were prepared using Maxima SYBR Green qPCR Master Mix (2×) (Thermo Scientific) and was set on 96 well plates with 20 μl final volume as follows:

200 – 500 ng DNA or cDNA
0.4 μM – 0.4 μM specific primer (**Table 2**)
1× Maxima SYBR Green qPCR Master Mix

The reactions were performed with C1000 Thermal Cycler with CFX96 Real-Time System (Bio-Rad) detecting system. The results were evaluated using the $2^{-\Delta\Delta Ct}$ method (**Livak and Schmittgen, 2001**).

Amplification conditions were as follows: 95 °C 3 min, [40 cycle: 95 °C 15 sec, 60 °C 30 sec, 72 °C 30 sec]. Melting curve analyzis was performed between 65 °C – 95 °C with a 0.5 °C increment.

5.5.9. Protoplast formation and PEG/$CaCl_2$-mediated transformation of *M. circinelloides*

For protoplast formation *Mucor* spores harvested from cultures grown for 4 days, were inoculated in small drops onto cellophane sheets placed on YEG or YNB medium supplemented with uracil and leucine, and grown at 25 °C for 16 hours (**Nagy et al., 1994; Papp et al., 2006; Csernetics et al., 2011**). Young colonies were transferred into protoplast forming solution (10 mM sodium-phosphate buffer, pH 6.4; 0.8 M sorbitol; 1.5% (w/v) home-made snail gut enzyme; 100 mM sodium phosphate buffer was prepared with mixing of 25 mM Na_2HPO_4 and 75 mM NaH_2PO_4) and incubated at 25 °C for 3 hours with continuous gentle shaking (60 rpm). Protoplasts were separated from mycelia with filtration through three sheets of gauze, collected with centrifugation (2162 × g, 15 min, 4 °C) and washed once with 5 ml sorbitol − MOPS − calcium chloride buffer (SMC: 0.8 M sorbitol; 50 mM $CaCl_2$; 10 mM 3-(N morpholino)propanesulfonic acid (MOPS)). Finally it was resuspended in 250 µl SMC buffer.

The modified PEG-mediated transformation described by van-Heeswijck and Roncero was applied for transformation of protoplasts (**van-Heeswijck and Roncero, 1984; Csernetics et al., 2011**). To the resuspended protoplasts 5-10 µg of purified plasmid DNA was added together with 20 µl of PEG – sorbitol – MOPS – calcium chloride buffer (PMC: 40% (w/v) PEG 4000, 10 mM MOPS, 0.6 M sorbitol, 50 mM $CaCl_2$). The samples were incubated on ice for half an hour. After addition of 2.5 ml of PMC buffer, the samples were incubated for 20 min at room temperature. Before centrifugation (2162 × g, 15 min, 4 °C) 20 ml SMC buffer was added to the protoplasts. To promote the regeneration of protoplasts, YPG medium containing 0.8 M sorbitol was added to the sedimented cells and incubated for 30 min at 25 °C. After centrifugation (2162 × g, 15 min, 4 °C), cells were washed with SMC buffer, then resuspended into 1 – 2 ml of SMC buffer and 1% (w/v) agar containing YNB selective medium and pored onto YNB selective medium. The plates were incubated at 25 °C until 4 – 7 days. Transformants were selected on the basis of auxotrophy complementation on YNB solid media supplemented with leucine or uracil, as required.

5.5.10. Microscopy and examination of mitotic stability of the mutants

AxioCam ERc 5s (Carl Zeiss) AxioLab (Carl Zeiss) equipped fluorescence microscope was used for microscopic observations and recordings and their evaluation was performed by using ZEN 2011 software (Carl Zeiss). To determine the number of

germinating spores, hyphae branching and cytoplasmic effusions, we used YNB broth and YNB solid media. Cellophane discs were placed on the surface of solid media then inoculated by dropping of 10^4 – 10^6 sporangiospores, after that incubated at 25 °C for 4 – 24 hours. Annexin V-FITC Apoptosis Detection Kit (Sigma) was used following the instruction of the manufacturer to differentiate apoptotic and necrotic germlings (in four and eight hours old cultures in YNB broth). For the detection of propidium iodide staining, filter15 (excitation PB 546/12 nm, emission LP 590 nm, Carl Zeiss) was used and filter set 9 for the annexin V-FITC staining (excitation BP 450-490 nm, emission LP 515, Carl Zeiss).

To determine the CFU, 2×10^2 sporangiospores were spread onto selective YNB medium (pH 3.5). For the mitotic stability investigation the harvested spores were inoculated onto MEA medium and cultivated for 4 days at 25 °C. The sporangiospores were collected and fresh MEA medium was inoculated. This step was repeated up to twelve time (cycles), and then collected spores were spread onto selective YNB medium.

5.5.11. Determination of the carotenoid and ergosterol content in *M. circinelloides*

Fungal carotenoids were extracted as described earlier (**Papp et al., 2006; Csernetics et al., 2011**). The mycelium was disrupted in liquid nitrogen with mortar and pestle and 500 µl acetone was added to 500 mg mycelium powder. After vigorous vortexing followed by centrifugation (3000 × g, 1 min, room temperature), the upper phase was collected in fresh tubes. The extraction was performed until the mycelium powder has becomes colorless. The extract was combined with an equal volume of petroleum ether (40 – 70 °C). To facilitate the separation 2 ml distilled water was added and then centrifuged (2162 × g, 8 min, 4 °C). The petroleum ether fraction was collected in a fresh tube and dried under nitrogen. Dried samples were dissolved in 10 ml petroleum ether (40 – 70 °C) and their total carotenoid content was determined by using spectrophotometer at 450 nm according to Rodriguez-Amaya and Kimura (**Rodriguez-Amaya and Kimura**, **2004**). The samples were dried again and redissolved in tetrahydrofuran supplemented with butylated hydroxytoluene (100 µg/ml) directly before the HPLC analyzis. Determination of the carotenoid composition of the samples was performed with a modular Shimadzu low-pressure gradient HPLC system, as described earlier (**Csernetics et al., 2011**).

Ergosterol extraction was performed using the method described by Alcazar Fuoli and coworker with some modifications (**Alcazar Fuoli et al., 2008**). The fresh mycelium

dried overnight at 80 °C was disrupted and the weight was determined. Three ml 25% alcoholic (methanol:ethanol 3:2, v/v) KOH solution was added to the mycelium powder and the samples were vortexed vigorously for 3 min. Samples were incubated at 85 °C for 1 hour, followed by extraction with a mixture of 1 ml distilled water and 3 ml hexane. After vigorous vortexing for 3 min, extracts were centrifuged (1900 × g, 10 min, at 4 °C). The upper layer (liquid phase) was transferred to a clean plastic tube and the liquid phase was evaporated under nitrogen gas. Dried samples were redissolved in 1 ml methanol for HPLC analyzis. For HPLC a modular Shimadzu system equipped with a two channel UV/VIS detector was used, and 50 µl sample was subjected on a Prodigy C18 (4.6×250 mm, ODS 5 µm) column (Phenomenex). Isocratic separation was performed with H_2O/methanol (2:98, v/v) as mobile phase at a flow rate of 1.2 ml/min. The detection wavelengths were 210 and 280 nm; ergosterol standard was purchased from Sigma (**Nagy et al., 2014**).

5.5.12. Antifungal susceptibility test

Antifungal susceptibility tests were performed following the instructions of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 (2008) protocol with minor modifications. Sensitivity of the fungal strains to AmB, ITR and CLO (Sigma-Aldrich, powder, dissolved in DMSO), FLU (Lescol, Novartis, capsule, dissolved in methanol) and SIM (Vasilip, Egis, tablet, dissolved in methanol) was examined in a 96-well microtiter plate assay.

Isolates were grown on selective YNB medium for 5 days at 25 °C. Sporangiospores were harvested with distilled water and concentration was determined with Bürker-chamber counting; selective YNB broth was used to set the concentration to 1×10^5 spore/ml.

Before dilution, SIM was activated from its lacton pro-drug form with ethanol-NaOH hydrolysis (15% v/v ethanol, 0,25% (v/v) NaOH, the final volume was set with methanol) at 60 °C for 1 hour (**Lorenz and Parks, 1990**; **Nyilasi et al., 2014**). Ten step dilution of the agents was performed in methanol or DMSO following the instructions of the CLSI M38-A2 method, while the final concentration was set with selective YNB media. Ranges of the final concentration of agents in two-fold dilutions were as follows: AmB: 0.03125 – 16 µg/ml, ITR: 0.125 – 128 µg/ml, CLO: 0.0625 – 32 µg/ml, FLU and SIM: 0.125 – 64 µg/ml.

100 – 100 µl of medium containing antifungal agent (in double concentration) and medium containing 1×10^5 spore/ml spores (1×10^4 spores/well in final concentration) were

subjected into the wells. Growth in antifungal agent-free medium supplemented with solvent was considered as 100% of growth, while uninoculated medium was used as the background for the calibration and contamination control. Plates were incubated for 48 hours at 25 °C and the optical density of the fungal cultures was measured at 620 nm with a SPECTROstar Nano (BMG Labtech) plate reader in well-scanning mode. Minimum inhibitory concentration was considered as the lowest concentration of the agent, which result in equal or above 90% growth inhibition (MIC_{90}) compared to the untreated control. The experiments were performed in biological triplicates and technical duplicates.

5.5.13. Phagocytosis assay

Phagocytosis assay was performed by Dr. Árpád Csernetics in Germany (Hans Knöll Institute, Jena, Germany), and the candidate was involved in the data evaluation. Growth maintenance of murine alveolar MH-S macrophages (ATCC: CRL-2019) was carried out by Hans-Martin Dahse (Hans Knöll Institute, Jena, Germany) as described previously (**Kraibooj et al., 2014**). For phagocytosis assay, macrophages were plated in 24 well plates (NUNC 142475, Thermo Scientific) at a density of 2×10^5 cells/well and allowed to adhere overnight at 37 °C.

For phagocytosis assay the *Mucor* spores were collected from 5 – 7 days old cultures with 1×PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH7.4)) washed with 10 ml 1× PBS and filtrated throw cell strainer (40 µm pore size, Thermo Scientific). After washing three times with 1× PBS spores were collected with centrifugation (2160 × g, 8 min, 4 °C) and stained with 5 ml 0.1 mg/ml Fluorescein isothiocyanate (FITC, Sigma-Aldrich) dissolved in carbonate buffer (3 ml 0.1 M Na_2CO_3, 7 ml 0.1 M $NaHCO_3$ (pH 9.5)) for 30 min at 37 °C with gentle shaking (70 rpm) in dark. Spores were collected with centrifugation (2160 × g, 8 min, 4 °C), washed three times with 1× PBS, and the spore concentration was determined with Thoma chamber. Spores were washed with RPMI-1640 medium (12-167F, Lonza), supplemented with 10% (v/v) heat inactivated fetal bovine serum (ATCC-30-2020), 1% ultraglutamine I (w/v) (17-605E/U1, Lonza), 550 µl/l (50 mg/ml stock solution) gentamicin sulphate (17-518Z, Lonza), 0.05 mM 2-mercaptoethanol (31350-010, Life Technologies) and 1% (w/v) sodium-pyruvate solution (BE13-115E, Lonza)) and 10^6 spores were resuspended in 1 ml pre-warmed RPMI-1640, supplemented with 0.02 g/l uracil. The medium of MH-S macrophages was discarded and medium containing the FITC-labelled spores was added to the macrophages at multiplicity of infection 5. Synchronization was performed with

centrifugation (100 × g, 5 min, 21 °C) and the co-incubation was carried out in a humidified CO_2 incubator at 37 °C for 3 hours. After the incubation, the phagocytosis was stopped with ice-cold 1× PBS for 2 – 5 min. The non-phagocytosed spores were counter-stained with 0.1 mg/ml calcofluor white (CFW, Sigma-Aldrich) in 1× PBS for 15 min at room temperature. Wells were washed three times with 1× PBS followed by fixation with 3.7% (v/v, in 1× PBS) formaldehyde at room temperature for 15 min. Microscopic images were taken with IN Cell Analyzer 2200 (GE Healthcare Life Sciences). Ten pictures on different areas of the well were taken from each wells; and three mutants from each were analyzed in three biological and two technical replicates. The calculation of phagocytic indexes (PI) was performed for each pictures, using the following formula:

PI = (percentage of macrophages containing at least one spore) × (total number of spores inside macrophages / number of macrophages containing at least one spore)

5.5.14. Statistical analyzes

Data are presented as mean ± SD for experiment performed in three biological and two technical replicates. Differences between the groups were assessed by one-way ANOVA and *t test* unpaired using GraphPad Prism version 5.01 and Microsoft excel software package for Windows and MacBook. A probability (*P*) value less than (*) ≤ 0.05, (**) ≤ 0.01, (***) ≤ 0.001 and (****) ≤ 0.0001 were considered as statistically significant.

6. RESULTS AND DISCUSSION

6.1. Transcription of six genes under different cultivation conditions involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides*

In our study one of the aims was to characterize the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes play role in the *M. circinelloides* mevalonate-isoprenoid pathway (**Fig. 4**). First of all, the transcription of the genes under different cultivation conditions was analyzed. The experiments were carried out in two biological and two technical parallels. The *Mucor actA*, encoding actin in *M. circinelloides* was used as reference gene. The reaction conditions were optimized with purified *M. circinelloides* MS12 genomic DNA and 60 °C was found as an optimal annealing temperature at which all PCR products reached the threshold level with equal Ct values (**Fig. 9**). Beside, the results of melting curve analyzis (data not shown) also verified that no any aspecific PCR product has been amplified, moreover, together with the previous results of the research group can be concluded that the *M. circinelloides* MS12 genome contains the examined genes in one copy (**unpublished data**).

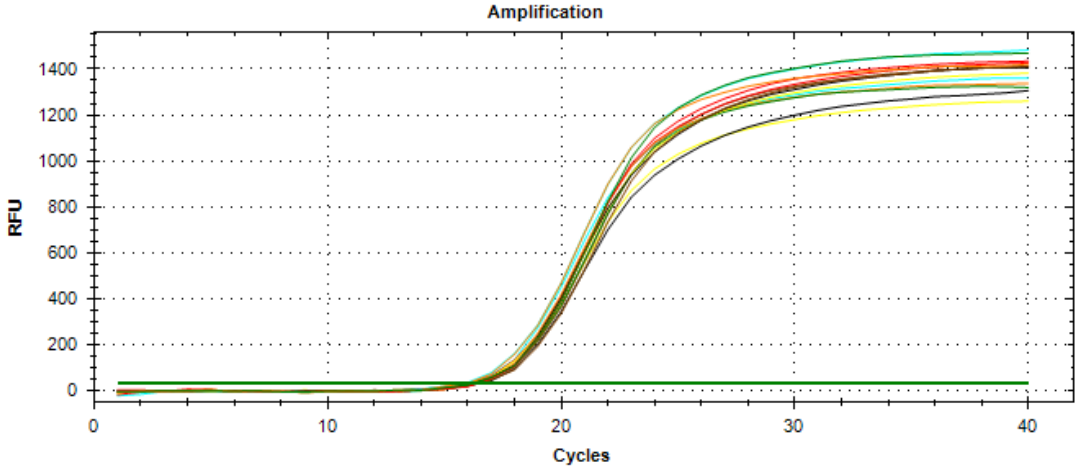


Fig 9. Optimization of the quantitative real-time PCR reaction conditions. The amplification was performed from *M. circinelloides* MS12 DNA with primers designed for qPCR and shown in **Table 2**. The reaction curves intersect the threshold line at the same Ct value: *hmgS* - yellow, *mvk* - black, *dmd* - blue, *ipi* - brown, *isoA* - red, *carG* - orange and *actA* - green.

6.1.1. Comparison of relative transcription level of selected genes

The transcription of the six selected genes was compared to each other. In the experiment *M. ciricinelloides* was cultivated in liquid and on solid YNB media supplemented with uracil and leucine up to four days. In all cases, the isoprenoid pathway genes (*ipi*, *isoA* and *carG*) showed higher transcription level than mevalonate pathway

genes (*hmgS*, *mvk* and *dmd*). Moreover, we found that *mvk* showed the lowest, while *ipi* displayed highest transcription level in comparison to each other at both conditions (**Fig. 10**).

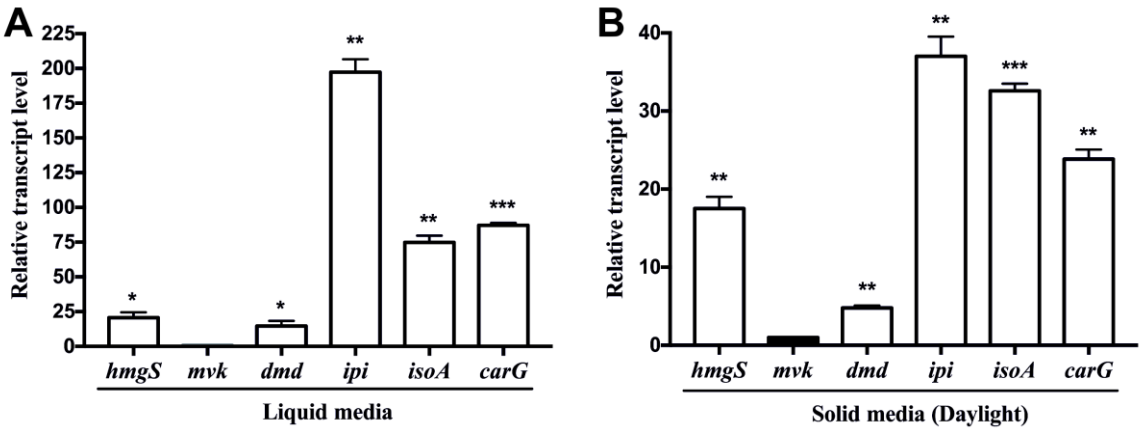


Fig 10. Comparison of the relative transcription levels of the six *M. circinelloides* mevalonate-isoprenoid pathway genes to each other: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase) data compared with *mvk* (mevalonate kinase).

6.1.2. Effect of environmental conditions on the transcription of selected *M. circinelloides* mevalonate-isoprenoid pathway genes

Effect of different environmental conditions (such as temperature, light source, incubation time and oxygen tension) on the transcription of the *hmgS, mvk, dmd, ipi, isoA* and *carG* genes was analyzed. Temperature dependence of the gene transcription was tested by cultivating the *M. circinelloides* MS12 on solid YNB media supplemented with leucine and uracil at 15 °C, 20 °C, 25 °C, 30 °C and 35 °C. In general, the fungus is cultivated on the optimal 25 °C in our experiments. It has been published that an optimum growth temperature range of 25 – 29 °C, while minimum growth temperature range of -3 to -4°C for *Mucor* spp. (**Sautour et al., 2002; Gougouli et al., 2011**). The 35 °C growth temperature significantly increased the transcription of *hmgs, mvk, dmd* and *ipi* genes in comparison with the control condition (25 °C). Temperature, lower than the optimum (15 °C and 20 °C) also affected the transcription of the genes, the both increased the transcription of the isoprenoid pathway genes (*ipi, isoA* and *carG*) (**Fig. 11**).

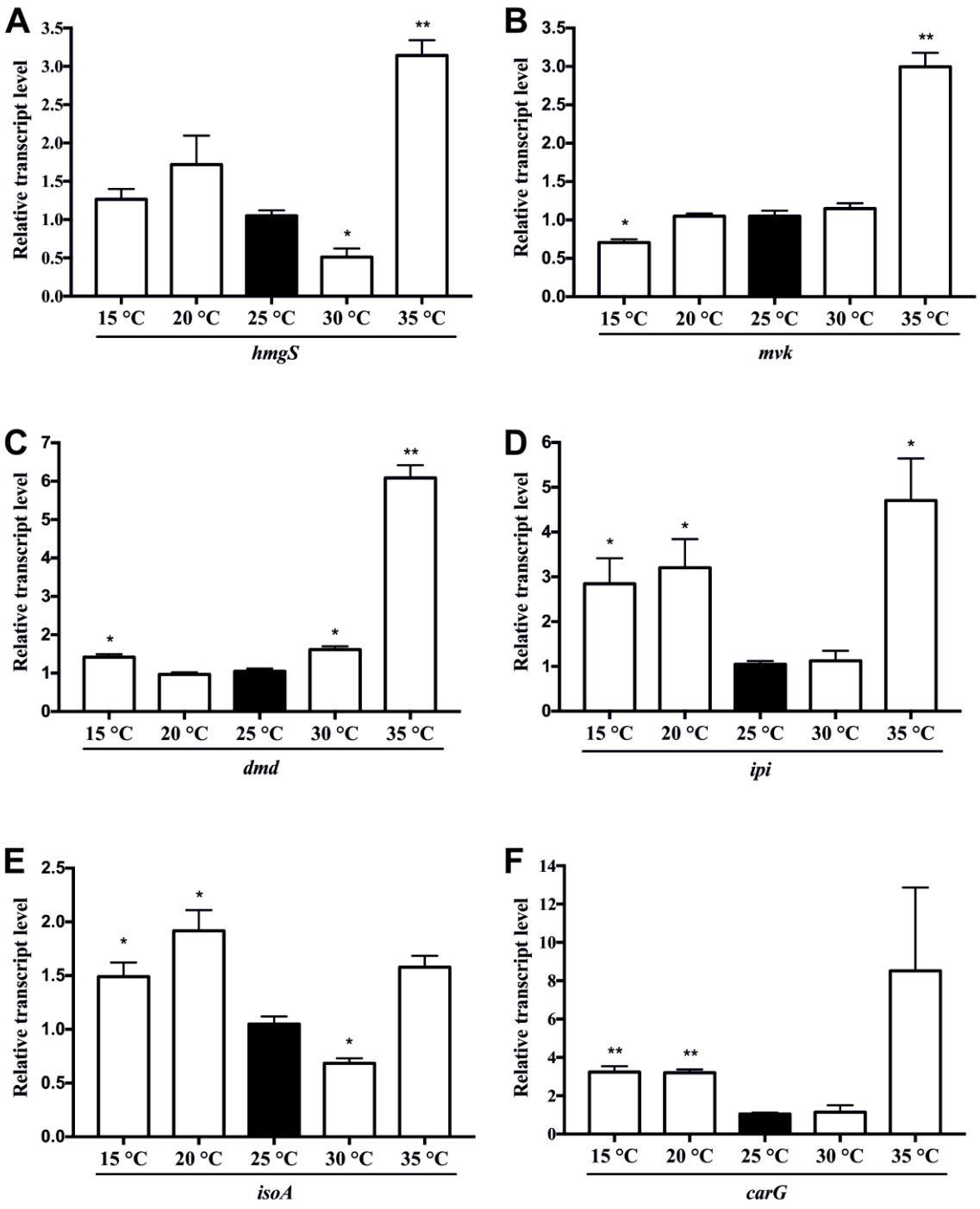


Fig 11. Effect of temperature (15 °C, 20 °C, 25 °C, 30 °C and 35 °C) on the transcription of the *M. circinelloides* mevalonate-isoprenoid genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

The cultivation temperature has a strong effect on production of primary and secondary metabolites, such as ergosterol and carotenoid (**Mosqueda-Cano and Gutierrez-Corona, 1995; Nagy et al., 2012; Papp et al., 2013**). When the cultivation temperature was increased from 28 °C to 40 °C the carotenoid content of *M. rouxii* was

increased three times (**Mosqueda-Cano and Gutierrez-Corona, 1995**). Also increased carotenoid production and β-carotene accumulation was observed on 20 °C and 35 °C in comparison with cultivation of *M. circinelloides* on 25 °C (**Papp et al., 2013**). In our experiments *hmgS, mvk, dmd* and *ipi* genes showed higher transcription level when the fungus was cultivated on 35 °C in comparison with 25 °C. This may happen that at higher temperature can accumulate more precursors for β-carotene accumulation, which may protect the cells against the increased level of endogenous reactive oxygen species (**Papp et al., 2013**).

Studies have shown that temperature can also influence the ergosterol content of the fungal plasma membrane in *Penicillium roqueforti* (**Li et al., 2009**). In psychrophilic yeast *Xanthophyllomyces dendrorhous* 50% increment in overall carotenoid content was observed at low temperature with increased levels of astaxanthin (**Ducrey Sanpietro and Kula, 1998**). Similarly, in case of *Dunaliella* decreasing the cultivation temperature from 34 °C to 17 °C resulted 7.5-fold increase in the carotenoid content (**Orset and Young, 1999**). The *M. circinelloides ipi, isoA* and *carG* genes showed higher transcription at 15 °C and 20 °C than on 25 °C in our study, which can be also due to increased ergosterol and carotenoid accumulation. Similar result was found in case of *hmgR1* gene, encoding a HMG-CoA reductase in *M. circinelloides*: the transcription of *hmgR1* increased, when the fungus was cultivated at 15 °C and 20 °C than on 25 °C (**Nagy et al., 2014**).

Light is one of the essential environmental factors that can regulate several biological processes, including growth, tropism and morphogenesis in fungi (**Schumacher et al., 2017**). Effect of illumination on the transcription of the *M. circinelloides* MS12 mevalonate-isoprenoid pathway genes was analyzed. The fungus was cultivated up to four days under daylight light source, in dark, and in continuous dark with exposure to 10 sec light followed by 10 min dark. The genes *ipi* and *carG* showed significantly lower transcription when the fungus was cultivated in continuous dark in comparison with cultures exposed continuously. The short light exposure (light induction) significantly increased the transcription of *mvk, dmd, isoA* and *carG* genes in comparison with cultivation in continuous light. When the dark and light induction was compared with each other we found a significant increase in the transcription level of *ipi* and *carG* (**Fig. 12**).

To investigate whether the different light sources may affect the gene transcription, the impact of different white light sources (warm white and daylight) on the transcription of the six genes was examined (daylight was used in all other experiments). Only the transcription of the *isoA* and *carG* showed significant difference in the case of the two light

sources. The *carG* showed higher transcription level, when the strain was cultivated under daylight, than under warm white source; while the *isoA* showed higher transcription level, when the *M. circinelloides* MS12 was cultivated under warm white light source (**Fig. 13**).

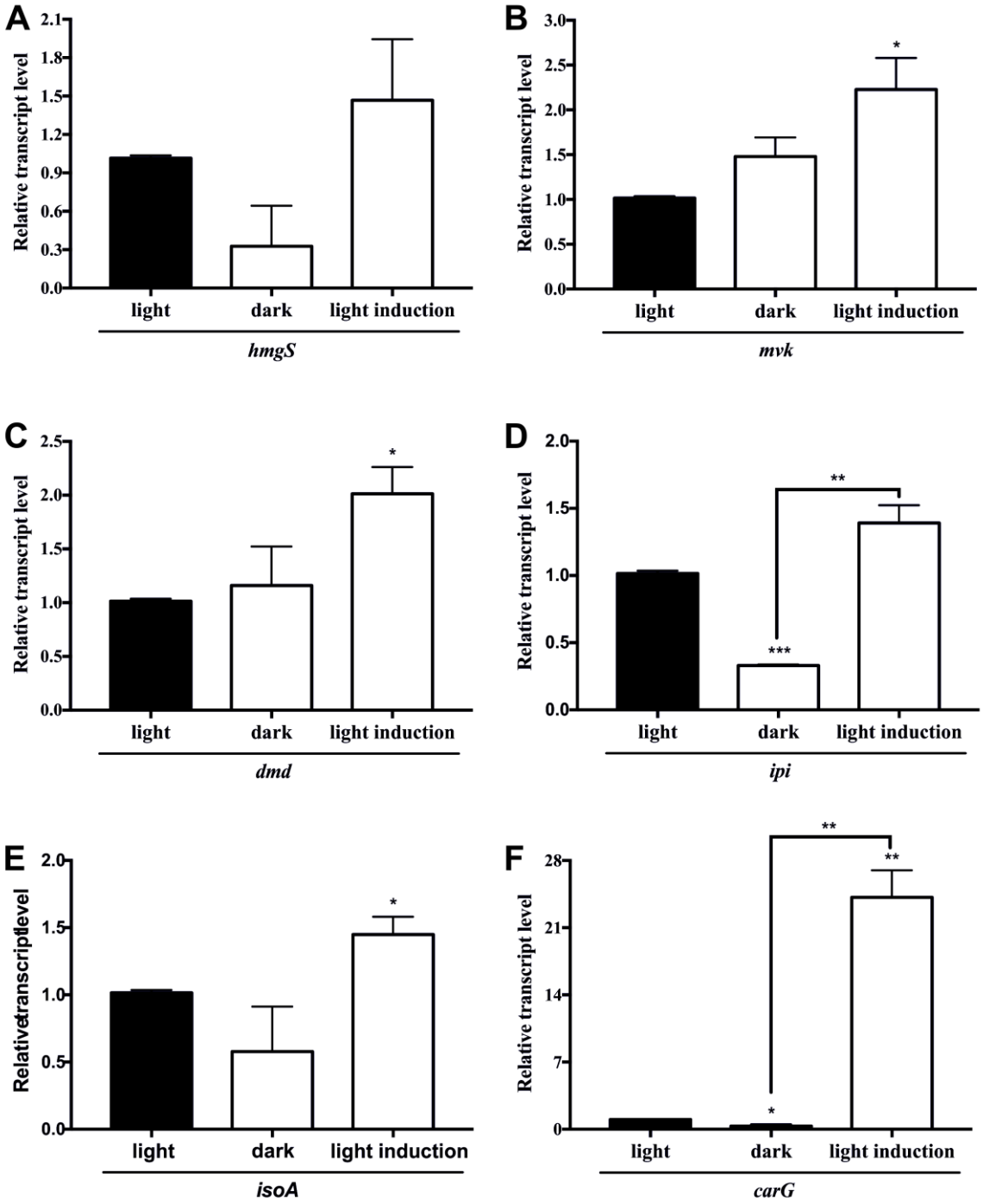


Fig 12. Effect of light (light, dark and dark with following 10 sec light and 10 min dark (light induction)) on the transcription of *M. circinelloides* mevalonate-isoprenoid genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

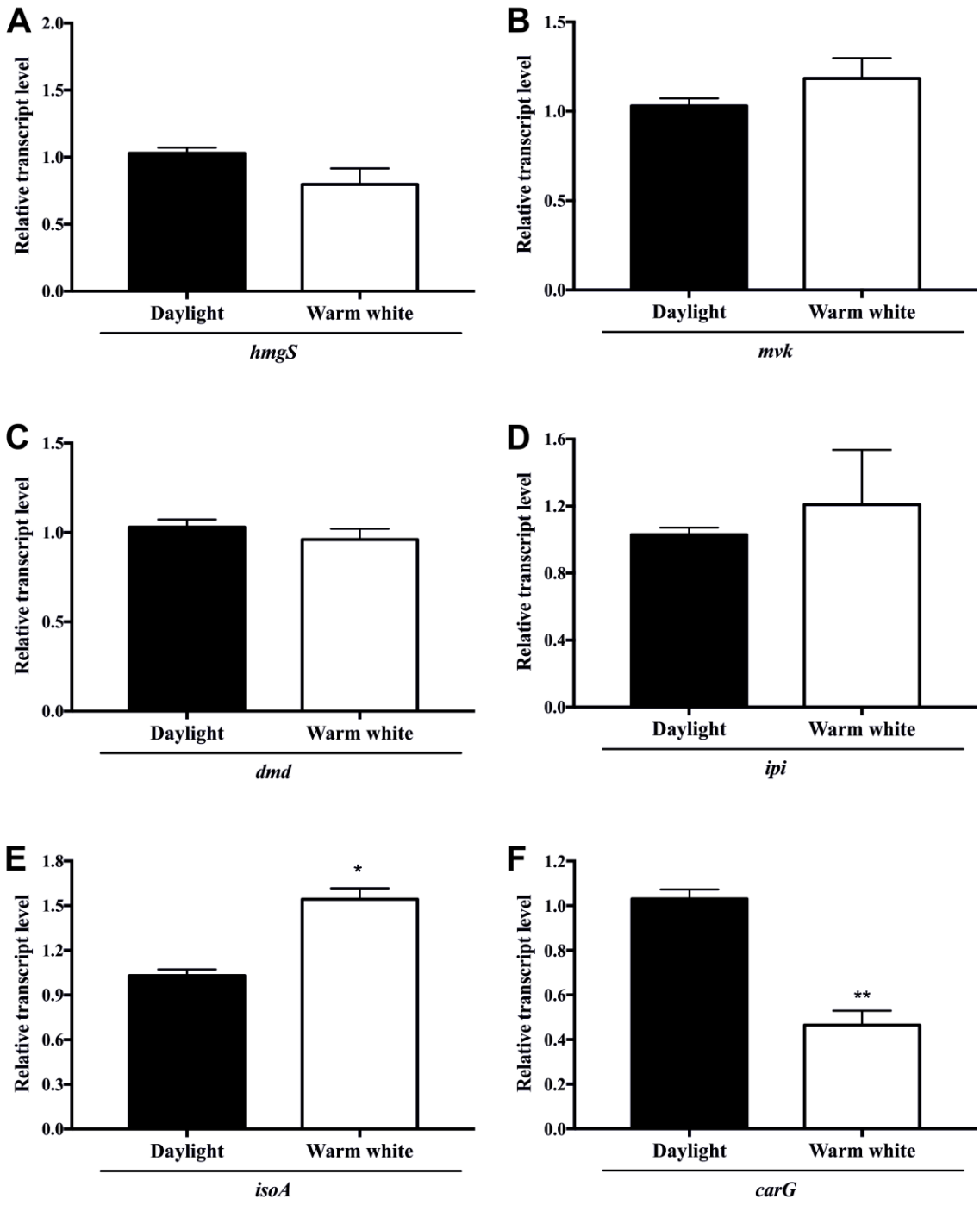


Fig 13. Effect of different light sources (daylight and warm white) on the transcription of *M. circinelloides* mevalonate-isoprenoid genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

The genes involved in the carotenoid biosynthesis had been shown to be positively photo-regulated not only in fungi, but also in bacteria and tomato plant (**Sandmann 1994; Velayos et al., 2000a**). *M. rouxii* accumulated about ten times more ß-carotene when grown continuously in the presence of light than corresponding cultures grown in the dark

(**Mosqueda-Cano and Gutierrez-Corona 1995**). The transcription of the *carB* gene (encoding the phytoene dehydrogenase) in *M. circinelloides* reached up to 150-fold higher level in light than in dark (**Velayos et al., 2000a**). The promoter region, common for both the *carB* and *carRP* (encoding the phytoene synthase/lycopene cyclase) genes contains several APE (al-3 proximal element)-like sequences that are involved in light regulation of gene expression (**Velayos et al., 2000b**). Genes encoding GGPP synthase have been isolated from several fungi: *N. crassa*, *Nigrospora sphaerica* and *M. circinelloides*, while in *G. fujikuroi* two genes which are thought to be involved in different branches of the isoprenoid pathways, have been isolated (**Tudzynski and Hölter, 1998; Iturriaga et al., 2000**). At least in *N. crassa* and *M. circinelloides*, it has been shown that light induces the expression of gene encoding GGPP synthase (**Morelli et al., 1993; Velayos et al., 2003, 2004**). However, this photocarotenogenic response is not universal in other fungi such as *B. trispora*, light has no effect on the carotenoid production (**Sutter, 1970**; **Linden et al., 1997**). In our study significant increase was observed in the transcription of *mvk, dmd, isoA* and *carG* genes with short light induction in comparison with cultivation in continuous light.

The transcription of *carG* after blue light irradiation showed that the expression of this gene is up-regulated by blue light, as happens with the other structural genes (*carB* and *carRP*) involved in carotenogenesis in *M. circinelloides*, which presume the similar regulation of these genes (**Velayos et al., 2003**). Navarro and co-workers reported that *M. circinelloides* responds to blue light by activating carotenoid biosynthesis due to a rapid increase in the level of transcription of structural genes for carotenogenesis (**Navarro et al., 2001**). Mucoromycotina fungi were incubated in dark and under different types of light sources, cultures of certain fungi such as those of *Amylomyces rouxii*, *M. circinelloides* and *Mucor mucedo*, incubated in light with higher color temperature (daylight) were able to survive at 37 °C, which proved to be lethal if they were growing in dark or in light with lower color temperature (warm white) (**Nagy et al., 2012**). In our study cultivation of the *M. circinelloides* under daylight light source, which contains more components in the blue range, resulted significantly higher transcription of *carG* in comparison with warm white light source. At the same time interestingly, the *isoA* showed higher transcription level, when the *M. circinelloides* was cultivated under warm white light source (**Fig. 13**).

After spore inoculation, the morphology of *M. circinelloides* sporangiospores significantly changes: four hours after the inoculation the germ tubes are developed, while hyphal branches appear at about 8 hours old cultures. The transcription of the six

mevalonate-isoprenoid biosynthesis genes was analyzed after different cultivation time (4, 8, 24, 48 and 96 hours old cultures). The mevalonate pathway genes (*hmgS*, *mvk* and *dmd*) showed the highest transcription level at four hours after inoculation, which decreased by the time. The highest transcription levels of *isoA* at eight hours, while of *carG* at 96 hours postinoculation were observed (**Fig. 14**). These results suggest that precursors of all isoprenoids are synthesized and accumulated in germlings, while those that are responsible for carotenoid production are present mainly in 96 hours old mycelia.

Effect of cultivation time on the transcription of *M. circinelloides hmgR1, hmgR2* and *hmgR3* genes was analyzed. During the cultivation period, the transcription level of *hmgR2* and *hmgR3* showed similar patterns and both reached high level at four hours postinoculation. More over transcription of *hmgR2* showed the highest transcription level in eight hours cultures, while *hmgR1* and *hmgR3* reached their maximum at 48 hours after the inoculation (**Nagy et al., 2014**).

Morphological dimorphism is a characteristic feature of *M. circinelloides*. In the absence of oxygen and/or presence of high hexose concentration in the medium, filamentous growth of the fungus switches to a yeast-like form. Effect of oxygen tension on the transcription of the mevalonate-isoprenoid genes was analyzed under aerobic, anaerobic and anaerobic followed by one hour aerobic incubation condition. The transcription of six genes increased under anaerobic compare to aerobic conditions. Anaerobic condition followed by one hour aerobic exposure increased the transcription of *dmd* and significantly decreased the transcription of *carG* gene compared to cultures under continuous anaerobic cultivation, while no any significant change could be observed in the case of *hmgS*, *mvk*, *ipi* and *isoA* genes (**Fig. 15**).

Productions of isoprenoids are highly regulated by oxygen tension. Ergosterol and carotenoid biosynthesis are oxygen dependent processes (**Mantzouridou et al., 2002**; **Cˇertík et al., 2005; Iigusa et al., 2005; Galea and Brown, 2009**). Studies have shown that carotenoid biosynthesis is stimulated by aeration in *Rhodotorula* spp. (**Simova et al., 2004**). The synthesis of astaxanthin was significantly increased when *Phaffia* strains were cultivated in media with optimum amount of oxygen (**Cˇertík et al. 2005**). Biosynthesis of ergosterol has high oxygen request and administrative impact of low-oxygen conditions on the transcription of the *hmgR* gene has also been demonstrated in *S. cerevisiae*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Schizosaccharomyces pombe* (**Bien and Espenshade, 2010**). In spite of the increased transcription level of mevalonate pathway gene *hmgR3*, the whole average ergosterol content radically decreased in the anaerobically

grown cells (0.2 mg/g [dry weight]) compared to that of the aerobically grown mycelium (5.5 mg/g [dry weight]) (**Nagy et al., 2014**). Similar situation was previously observed in *Mucor genevensis* (**Gordon, et al., 1970**), which had <0.3 and 3 mg/g [dry weight] ergosterol content during anaerobic and aerobic growth, respectively.

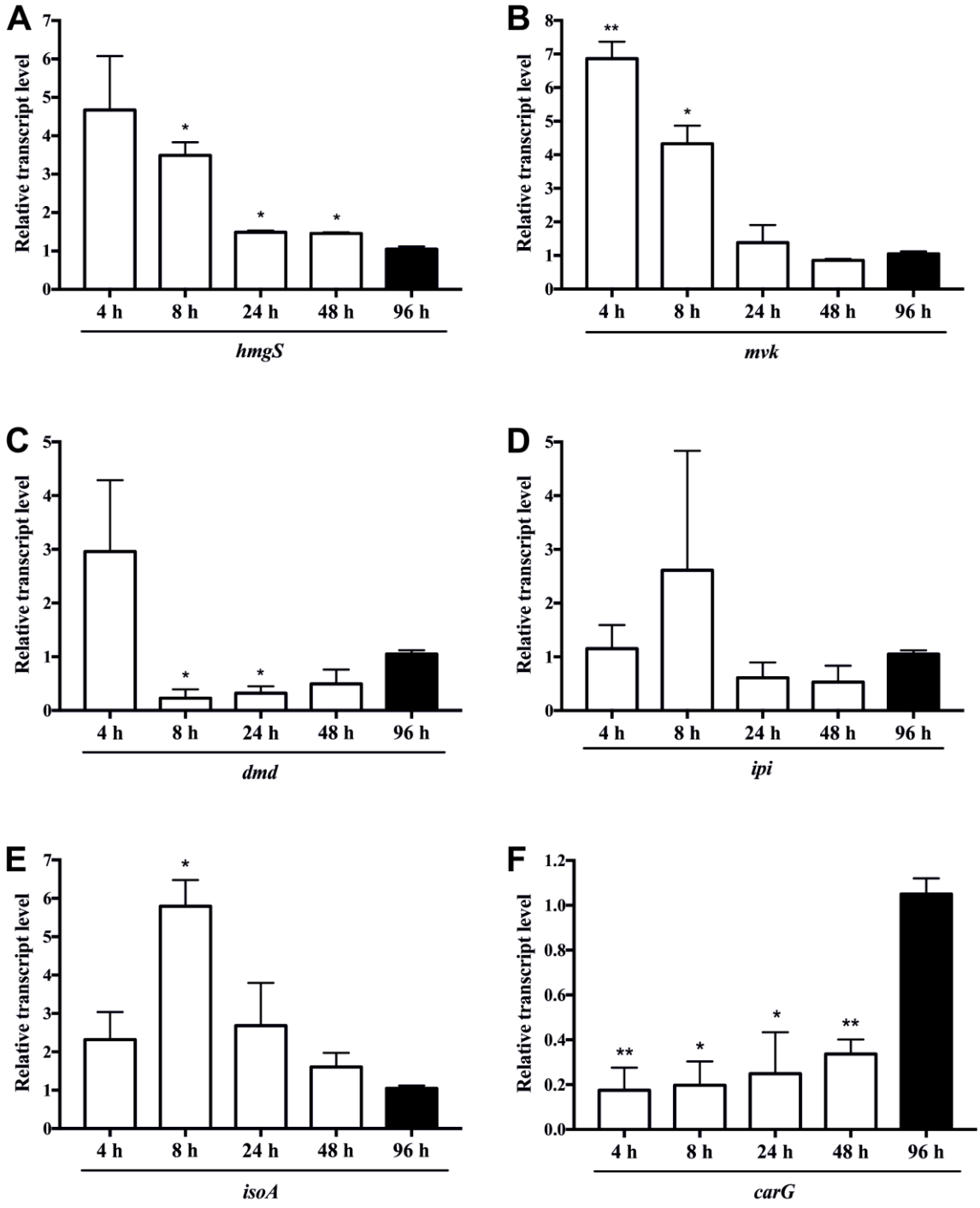


Fig 14. Kinetics of transcription of the *M. circinelloides* mevalonate-isoprenoid pathway genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase). Gene transcription was analyzed in 4 h, 8 h, 24 h, 48 h and 96 h old cultures and all data are compared to 96 hour cultures.

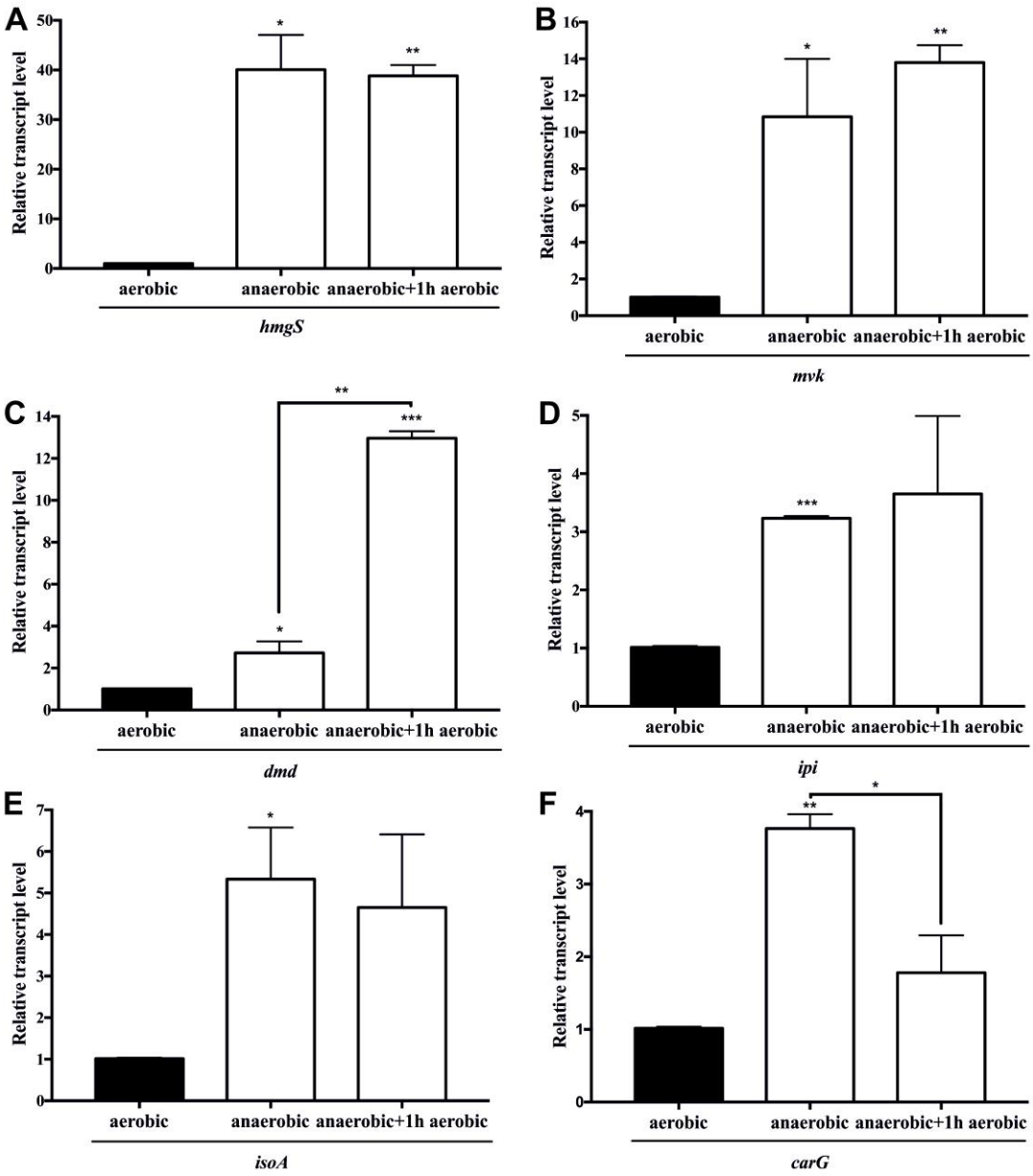


Fig 15. Effect of oxygen tension (aerobic, anaerobic and anaerobic followed by 1 hour aerobic exposure) on the transcription of *M. circinelloides* mevalonate-isoprenoid pathway genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

6.1.3. Effect of medium composition on the transcription of the *M. circinelloides* six mevalonate-isoprenoid pathway genes

Next, effect of the composition of the cultivation medium (such as different carbon sources, glucose concentration, addition of sodium chloride) on the transcription of the *hmgS, mvk, dmd, ipi, isoA* and *carG* genes was investigated in *M. circinelloides*. To determine the effect of medium composition on the transcription of the genes, *M.*

circinelloides was grown on minimal YNB medium supplemented with leucine and uracil and complete MEA medium. We found that relative transcription level of *mvk* and *isoA* significantly decreased, whereas *carG* significantly increased on MEA in comparison to YNB, while no any significant changes were observed in the case of the other genes (**Fig. 16**). The exact composition of MEA medium, as it contains yeast- and malt extract, is not known, thus the effect of several compounds (e.g. salts, carbon sources) may result in difference in transcription levels in comparison with the defined YNB.

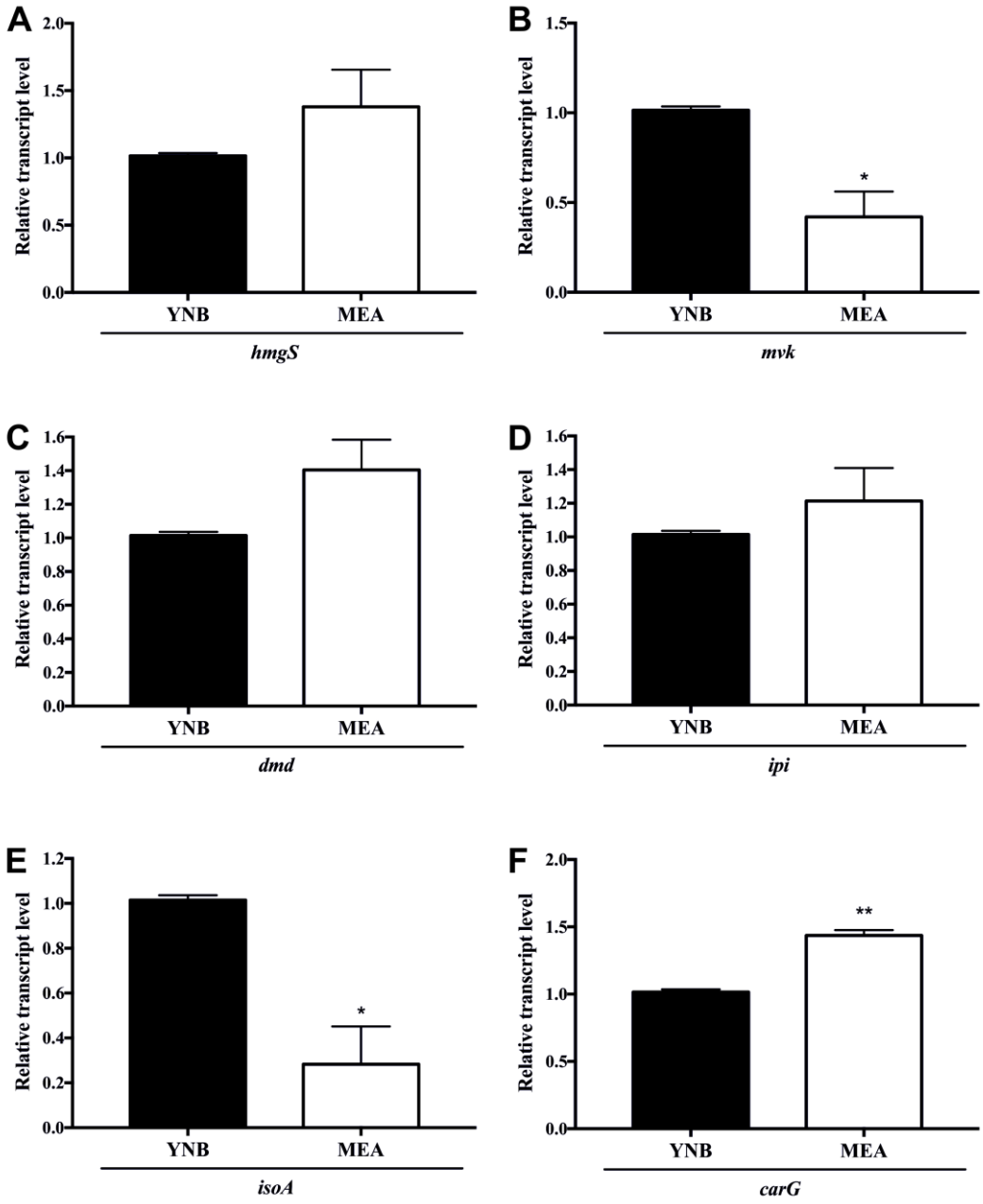


Fig 16. Effect of the minimal (YNB) and complete (MEA) media on the transcription of the *M. circinelloides* mevalonate-isoprenoid biosynthesis pathway genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

Glucose is the most widely utilizable carbon source, and it is known, that glucose affect several biochemical processes. To investigate the effect of glucose concentration on the gene transcription, glucose was added to YNB medium in a final concentration of 1%, 2.5% and 5% (w/v) and cultivation was carried out for four days under daylight light source at 25 °C. Significant decreases in the transcript levels of the *ipi* and *isoA* on both concentrations, in the case of *hmgS* on 5% (w/v) glucose only, and in case of *carG* at 2.5% (w/v) glucose was observed (**Fig. 17**). Effect of the glucose concentration on the six gene transcription was also investigated at eight hours after inoculation; in that case the *M. circinelloides* MS12 was cultivated in YNB broth. In this case cultivation in 2.5% and 5% (w/v) glucose containing media resulted decrease in the transcription of all six genes in comparison with 1% (w/v) glucose (**Fig. 18**). Similar decrease was observed in the transcription of *ipi*, *isoA* and *carG*, when *M. circinelloides* MS12 was cultivated on 2.5% and 5% (w/v) glucose containing media in comparison with 1% (w/v) glucose after 96 hours incubation (**Csernetics et al., 2011**). The results suggesting that increased glucose concentration affect the production of metabolites synthesized via the mevalonate-isoprenoid pathway in *M. circinelloides*.

It had been shown that carotenoid biosynthesis is repressed by glucose in *X. dendrorhous*, moreover low carotenoid accumulation was detected, when high glucose concentration was used in the medium (**Marcoleta et al., 2011**). Besides, it was recently reported that the catabolic repressor Mig1 contribute to the regulation of carotenoid accumulation in *X. dendrorhous* (**Alcaíno et al., 2016**). Possible glucose-dependent regulation of *ERG10A* and *ERG10B* genes was studied by Werner and coworkers in *X. dendrorhous* (**Werner et al., 2016**). The transcription level of *ERG10A* (encodes a thiolase involved in the mevalonate pathway) did not show any significant changes after the addition of glucose. In contrast, transcription of *ERG10B* (thiolase involved in the β-oxidation of fatty acids) reduced approximately 120-fold when glucose was added to the culture compared to the untreated control (**Werner et al., 2016**). However, this decrease was only temporary as the transcription levels normalized to control levels when the glucose in the media was consumed. Similarly, in our study, on 2.5% and 5% (w/v) glucose more prominent decrease was detected in the transcription of all six genes after 8 hours cultivation than after 96 hours, moreover no significant decrease was observed in the latter case in the transcription of *mvk* and *dmd* genes in comparison with 1% (w/v) glucose, which can be due to glucose consumption (**Fig. 17** and **Fig. 18**).

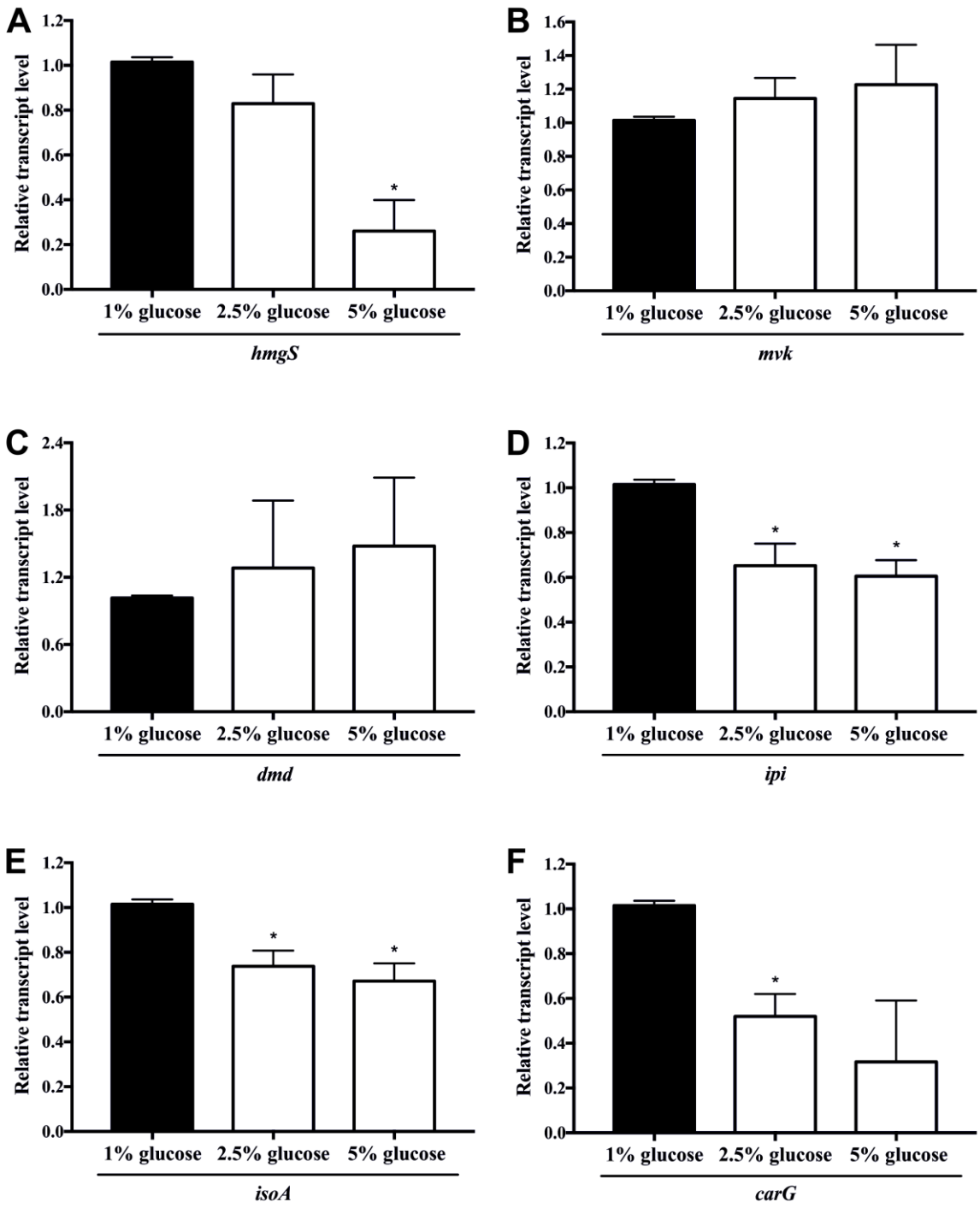


Fig 17. Effect of the glucose concentration on the transcription of the *M. circinelloides* mevalonate-isoprenoid genes after four days incubation: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

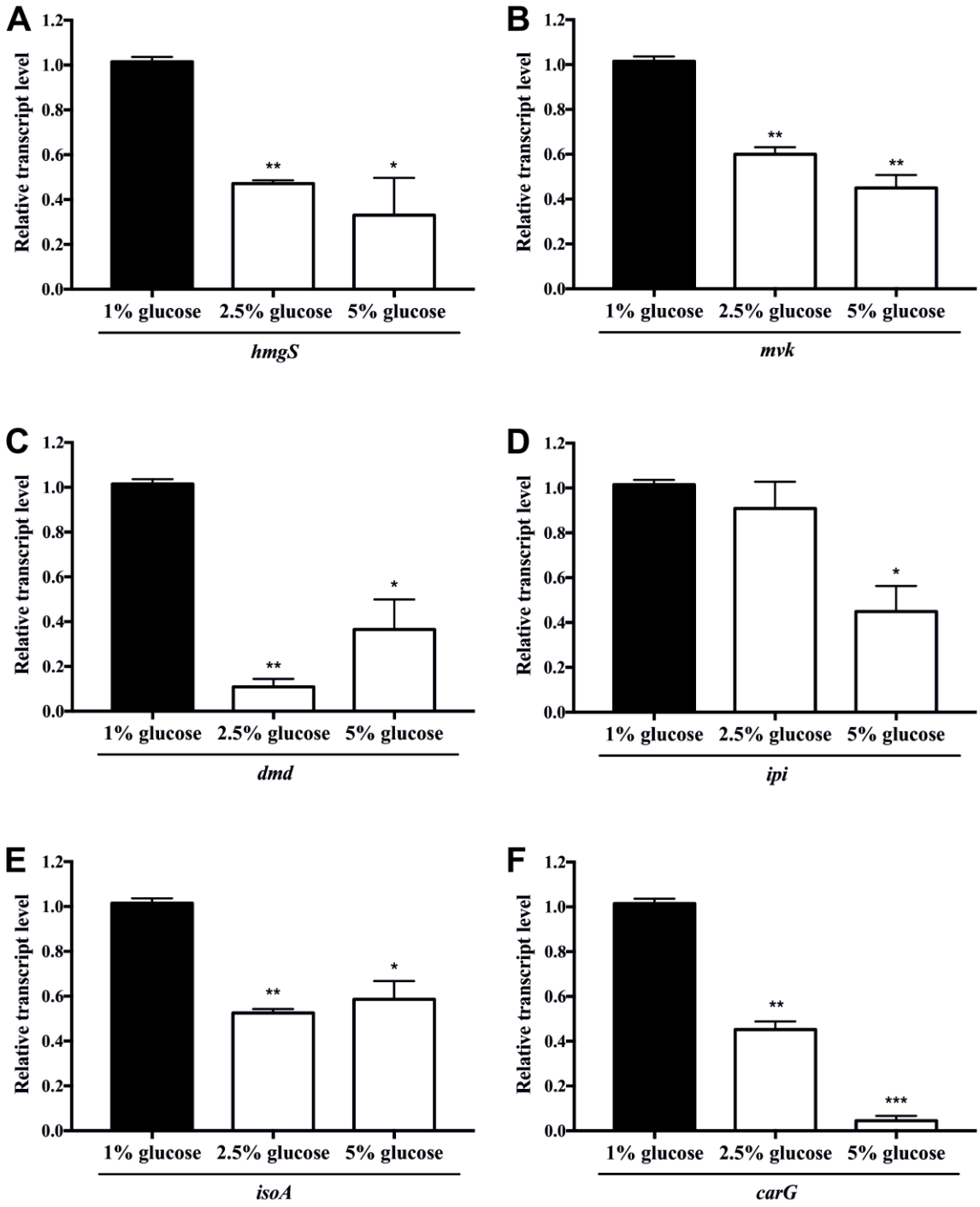


Fig 18. Effect of the glucose concentration on the transcription of the *M. circinelloides* mevalonate-isoprenoid genes after eight hours cultivation: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

In order to examine the effect of different carbon sources on the transcription of the six mevalonate-isoprenoid pathway genes, glucose was replaced by dihydroxyacetone (DHA), maltose (Mal), sodium acetate (Na-ac) or trehalose (Treh) in YNB medium at a

final concentration 1% (w/v). The relative transcription levels normalized to glucose are shown in **Fig. 19**.

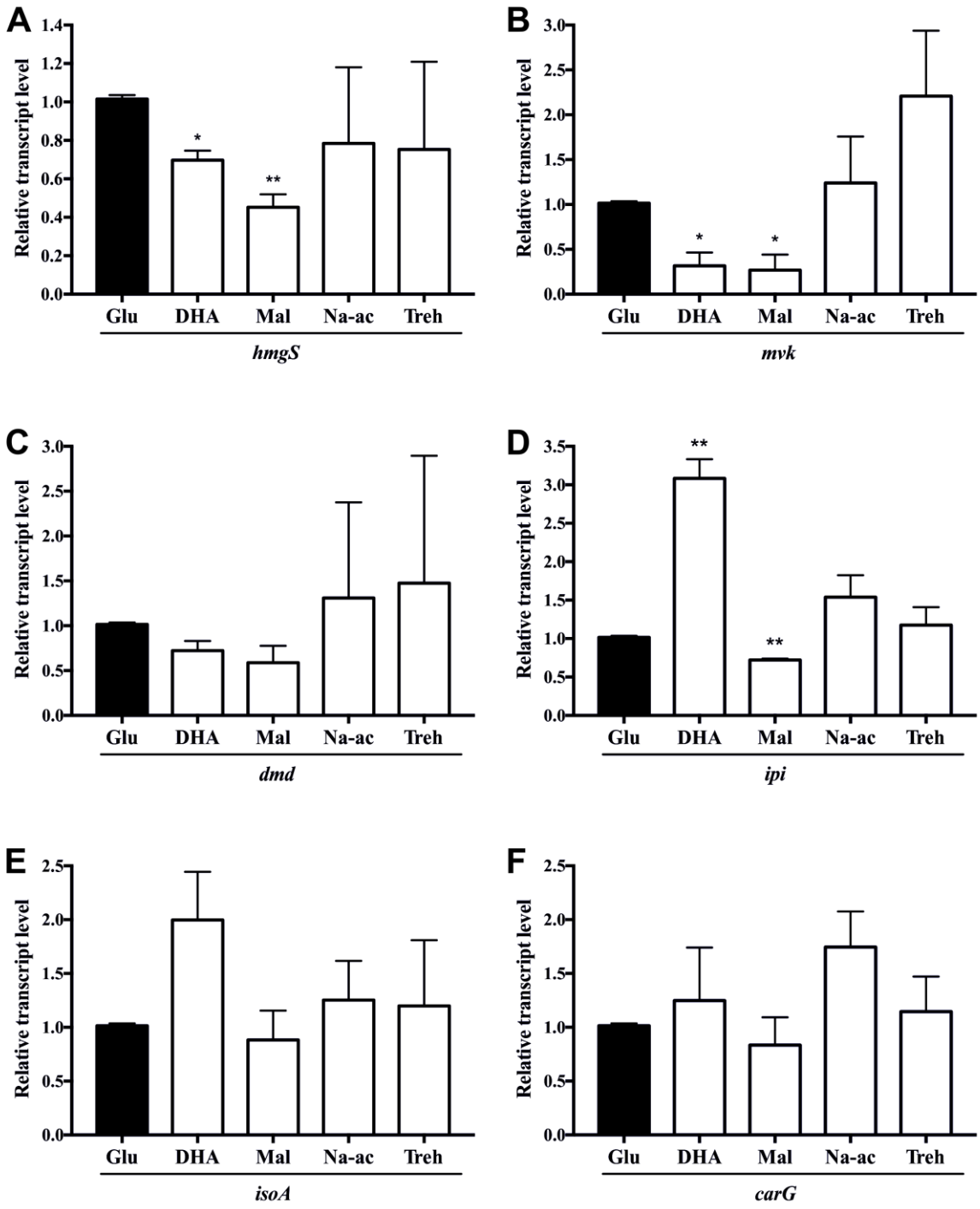


Fig 19. Effect of the different carbon sources on *M. circinelloides* mevalonate-isoprenoid biosynthesis pathway gene transcription: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase). Dihydroxyacetone (DHA), maltose (Mal), sodium acetate (Na-ac), or trehalose (Treh) in a final concentration 1% (w/v) was used in YNB media.

The carbon sources were selected on the basis of previous results of our research group, in which effect of several carbon sources on the carotenoid production of *M. circinelloides* was analyzed (**Nagy et al., 2012; Papp et al., 2013**). DHA and Mal resulted in significant change in the transcription of *hmgS*, *mvk* and *ipi* genes in comparison with cultivation on glucose containing medium. DHA and Mal significantly reduced the transcription level of *hmgS* and *mvk*, while in the case of *ipi* Mal reduced and DHA increased it in comparison with cultivation of the *M. circinelloides* MS12 on glucose containing YNB. Treh and DHA increased the total carotenoid content of several Mucoromycotina fungi, besides, increased the canthaxanthin content in *M. circinelloides* mutants harbouring a bacterial β-carotene ketolase encoding *crtW* gene (**Nagy et al., 2012**; **Papp et al., 2013**). Interestingly, Treh had no any significant effect on the transcription of the investigated genes, while DHA reduced the transcription of *hmgS* and *mvk*, but significantly increased that of *ipi*. The latter is responsible for formation of DMAPP, a building block of all isoprenoids. DHA take part in the glycolysis and thus, may provide precursors for the mevalonate pathway (**Nagy et al., 2014**). The *hmgR1* and *hmgR3* genes of *M. circinelloides* (encoding HMG-CoA reductases) displayed increased transcription level on Na-ac and DHA containing medium in comparison with glucose, moreover, relative transcription level of all three *hmgR* genes on Mal and Treh decreased in comparison with glucose (**Nagy et al., 2014**). Acetate, as carbon source showed affect on terpenoid biosynthesis in *Blakesleea* and *Phycomyces* (**Kuzina and Cerdá-Olmedo, 2007**).

Effect of the salt stress on the transcription of *M. circinelloides* six mevalonate-isoprenoid pathway genes was also analyzed. In this experiment NaCl was added to the YNB media in 1%, 2% and 3% (w/v) final concentration. Interestingly, in our study NaCl did not affected significantly the gene transcriptions; addition of 2 or 3% (w/v) NaCl to the media slightly, but not significantly decreased the transcription of the *hmgS*, but 2 % (w/v) NaCl significantly increased the transcription of the *carG* (**Fig. 20**). The *carG* gene is responsible for the formation of GGPP a precursor of gernylgeranylated proteins. This protein prenylation is a posttranslational modification and essential for the function by over approximately 100 proteins in the eukaryotic cell (**Terry et al., 2006; DeGraw et al., 2012**). In the presence of NaCl modification of the quantity and the composition of sterols in the cell membrane is an important factor of the adaptation to the environmental osmotic changes (**Rep et al., 2000; Yancey, 2005**). In previous studies, the *hmgR* found to be essential for the adaptation of certain fungi to the changing salinity of the environment (**Bidle et al., 2007; Vaupotič et al., 2008; Nagy et al., 2014**). The three *hmgR* genes of *M.*

circinelloides showed increase transcription level in the presence of NaCl (**Nagy et al., 2014**). HMG-CoA reductase of the extremely halotolerant *Hortaea werneckii*, HwHmg2, is up-regulated under hypo-saline and extremely hyper-saline conditions, while activity of HwHmg1 is constant under different growth conditions (**Vaupotic and Plemenitas, 2007**).

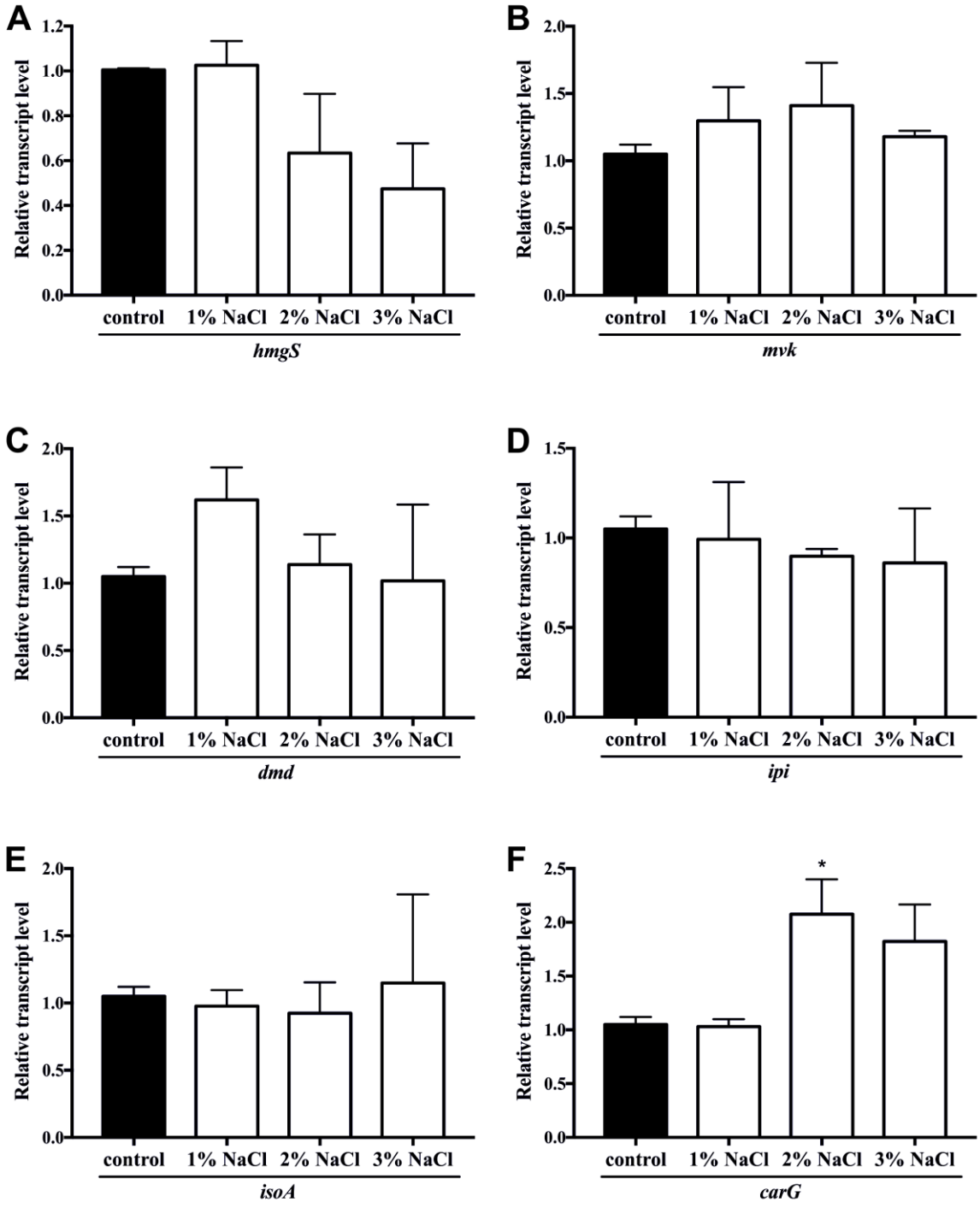


Fig 20. Effect of different concentration of sodium chloride (NaCl) on the transcription of *M. circinelloides* mevalonate-isoprenoid genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

6.1.4 Effect of statin treatment on the transcription of the *M. circinelloides* six mevalonate-isoprenoid pathway genes

Statins are cholesterol-lowering drugs and competitive inhibitors of HMG-CoA reductases and they induce apoptosis-like process in organisms (**Qi et al., 2013**). Previously our research group investigated on the susceptibility of *M. circinelloides* against different statins, such as atorvastatin, fluvastatin, rosuvastatin, simvastatin and pravastatin in range 0.125 – 64 μg/ml. Based on the results FLU was selected to investigate on the effect of statins on the transcription of the *M. circinelloides* mevalonate-isoprenoid six genes. The fungus was cultivated in liquid YNB medium containing FLU in 0.5, 1, 2 and 4 μg/ml concentration up to four days at 25 °C. Addition of FLU to the medium significantly increased the transcription levels of all six genes in comparison with cultivation in FLU-free medium. At the same time 4 μg/ml FLU concentration (which is the MIC_{90} to the fungus determined in that experiment) resulted significant decrease in the transcription levels in comparison to treatment with 2 μg/ml FLU (**Fig. 21**).

In eukaryotic organisms, statins inhibit the class I HMG-CoA reductases; as an outcome, it can inhibit the formation of mevalonate from HMG-CoA. Mevalonate is a precursor for other non-steroidal isoprenoid compounds as well, such as FPP and GGPP, which take part in the protein prenylation (**Stancu and and Sima, 2001**). It was reported that lovastatin treatment cause decreased growth, inhibition of sporangiospore germination and apoptosis-like cell death in *Mucor* species (**Roze and Linz, 1998; Nagy et al., 2014**). Transcription of the *hmgR* genes did not showed significant change in the presence of statins, at the same time overexpression of the *hmgR2* and *hmgR3* genes led to decreased susceptibility to FLU, atorvastatin and rosuvastatin in *M. circinelloides* (**Nagy et al., 2014**). Statins are also able to inhibit the growth of bacteria, although proper mechanisms are not understood. Some studies have attributed the antimicrobial action to the promotion of apoptosis or to the hydrophobic nature of statins causing disruption of the bacterial membrane, resulting in cell death (**Bergman et al., 2011; Masadeh et al., 2012**).

In our experiments we found that, spatially in case of *mvk*, *isoA* and *carG* genes, FLU significantly induced the transcription of the genes. Increased accumulation of precursors of isoprenoid-type metabolites assume isoprenoids (such as ergosterol, prenylted proteins and carotenoids) play role in fungal survival in presence of statins.

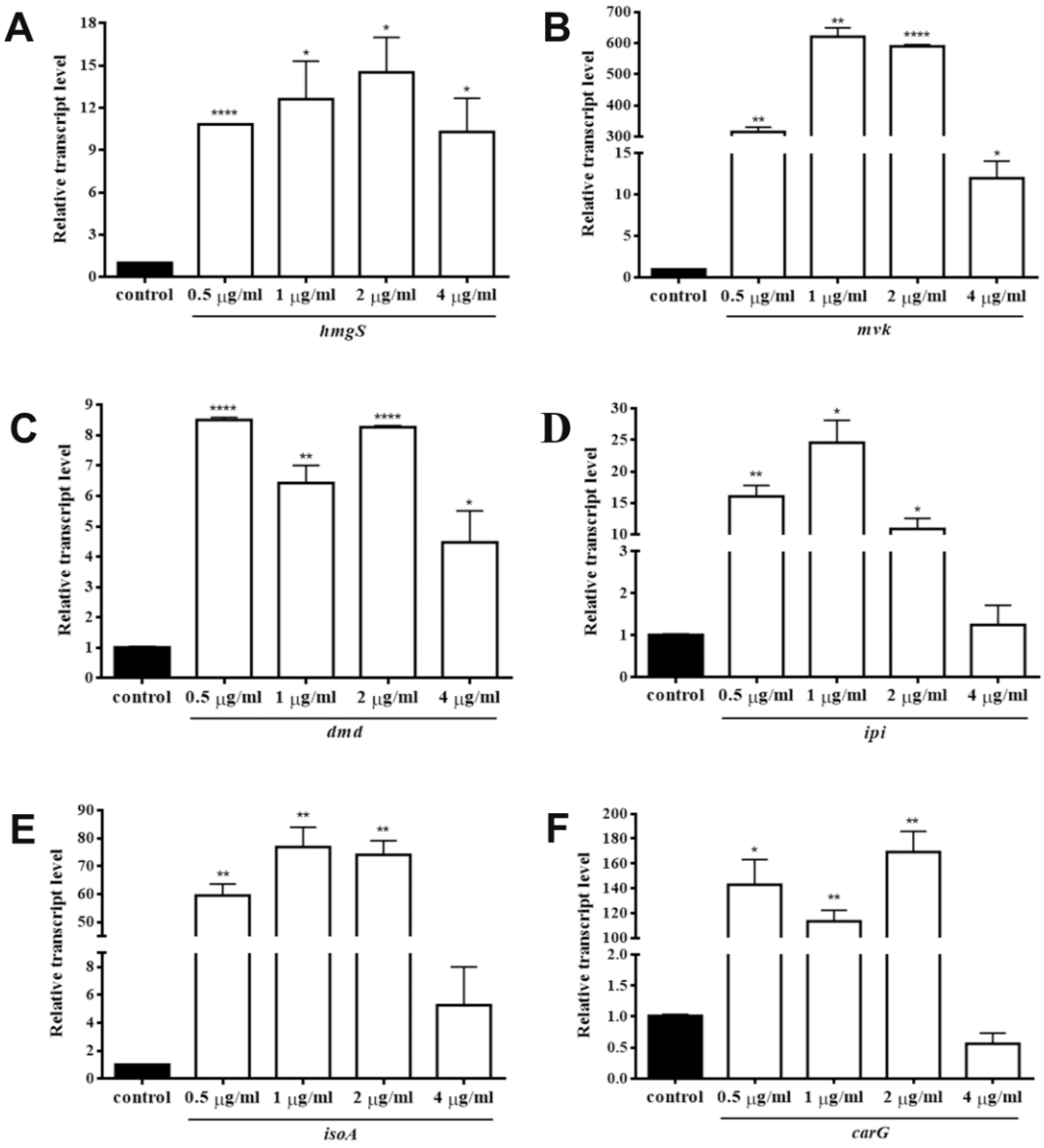


Fig 21. Effect of FLU treatment at different concentrations on the transcription of *M. circinelloides* mevalonate-isoprenoid genes: *hmgS* (HMG-CoA synthase), *mvk* (mevalonate kinase), *dmd* (diphosphomevalonate decarboxylase), *ipi* (IPP isomerase), *isoA* (FPP synthase) and *carG* (GGPP synthase).

6.2. Construction of plasmids for overexpression and silencing of *M. circinelloides* six genes involved in the mevalonate-isoprenoid biosynthesis and transformation experiments

Plasmids were constructed for overexpression and silencing of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes in *M. circinelloides.* No well-developed transformation system was available for targeted gene disruption in *M. circinelloides* when the experiments started (the CRISPR-Cas9 system was developed to *M. circinelloides* by our research group in

2017, **Nagy et al., 2017**), moreover, metabolites synthesized via the mevalonate-isoprenoid pathway play role in several biological processes and disruption of biosynthesis genes could be lethal for the fungus. Overexpression and silencing are commonly used techniques for functional characterization of genes in fungi, also in Mucoromycotina fungi (**Moralejo et al., 2002; Kadotani et al., 2003; Lombraña et al., 2004; de Haro et al., 2009; Csernetics et al., 2011; Nicolas et al., 2015; Zhang et al., 2017**). The efficiency of overexpression highly depends on the applied promoter. The *M. circinelloides gpd1* promoter is well characterized: it is strongly expressing and can be induced with increased glucose concentration (**Wolf and Arnau, 2002**), moreover it has been used successfully in several studies (**Hirano et al., 2000; Lima et al., 2003; Lopes et al., 2008; Csernetics et al., 2011, 2015**, **Papp et al., 2013**).

For overexpression the genes were amplified together with their own promoter and terminator regions or were placed under the control of the *M. circinelloides gpd1* promoter and terminator (*own* and *gpd* constructs, **Fig. 8 A-B**, **Table 3**, **chapter 5.4. Plasmid constructs used in this study**). For gene silencing three different plasmids were constructed for all six genes: (1) the genes were inserted between the *Mucor gpd1* and *zrt1* promoter regions (*pMAT* constructs, **Fig. 8 C, Table 3**); (2) a fragment of a gene was inserted between the *Mucor gpd1* promoter and terminator in inverted orientation (*as* constructs, **Fig. 8 D, Table 3**); (3) a fragment of a gene and its reverse complement together with an intron were placed under the regulation of the *gpd1* promoter and terminator (*hpRNA* constructs, **Fig. 8 E, Table 3**). The *Mucor pyrG* and *leuA* genes were used as selection markers, which complement the uracil and the leucine auxotrophy, respectively. *M. circinelloides* MS12 a *leuA*$^{-}$, *pyrG*$^{-}$ mutant derived from the *M. circinelloides* CBS 277.49 was used in all transformation experiments (**Benito et al., 1992**).

Successful PEG/$CaCl_2$-mediated transformation of *M. ciricinelloides* protoplasts with the plasmids was carried out. Transformation with pAVB107 and pEPM901 plasmids (harbouring the *leuA* and *pyrG* genes, respectively) was also performed and beside the *M. circinelloides* MS12 those transformants were used as control. The mutants were selected based on auxotrophy complementation on selective YNB medium. The transformation frequency was 1 – 15 colony/10^5 protoplasts/5 - 10 μg plasmid DNA and lower number of transformants could have been isolated in case of transformation with the *hpRNA* plasmids (1 – 5 colony/transformation). The presence of plasmid DNA in mutants was analyzed and verified by PCR with primers shown in **Table 2** (data not shown). Four transformants were

inoculated for further investigation from each layout. Examination of the transformants was started after 3 – 5 consecutive cultivation cycles on selective YNB medium.

6.3. Characterization of the mevalonate-isoprenoid biosynthesis pathway mutant *M. circinelloides* strains

6.3.1 Examination of plasmid copy number and relative transcription level change in the mutants

The plasmid copy number was investigated in the mutants with quantitve real-time PCR and found as 0.3 – 10 copy/genom. No difference was observed in copy number between the mutants harbouring different plasmid constructs, but fluctuation was verified within the consecutive cultivation cycles (data not shown); this fluctuation can be due to unequal distribution of the plasmids in spores. Previously, our research group observed similar fluctuation in the plasmid copy number when *ipi*, *isoA* and *carG* genes were overexpressed or the *crtS* and *crtR* genes of *X. dendrorhous* were expressed in *M. circinelloides* (**Csernetics et al., 2011, 2015**);

Next, change in the relative transcription level of the overexpressed and silenced genes were analyzed. We observed an increase in the relative transcription level of the ovexpressed genes that was more prominent in case of the mevalonate-isoprenoid pathway mutants harbouring the genes in extra copies under the control of the *gpd1* promoter and terminator (**Fig. 22**). Similar increase was observed in a previous study of our research group, in those transformants harbouring the *ipi*, *isoA* and *carG* genes under the control of the *gpd1* regulatory sequences, showed higher transcription level than those with the native regulatory regions (**Csernetics et al., 2011**). The *gpd1* promoter was used several times for expression of genes with high efficiency in *Rhizomucor miehei* (**Vastag et al., 2004**), *P. brasiliensis* (**Barbosa et al., 2004**), *A. nidulans* (**Lima et al., 2003**) and *A. bisporus* (**Lopes et al., 2008**). Silencing with the *hpRNA* and *as* plasmid constructs resulted in significant decrease in the relative transcription level in most of the transformants, from which the most prominent decrease was observed in the case of using the *hpRNA* plasmids (**Fig. 22**). It has to be mentioned that high deviation in the relative transcription levels was observed in some of the transformants (e.g. in case of MS12+pDMD/hpRNA and MS12+pISOA/hpRNA) which caused by high transcription level detected in one out of four analyzed mutants. In most cases with *pMAT* plasmid constructs increase was observed

in the relative transcription levels, thus with *as* and *hpRNA* constructs higher silencing efficiency could reach in case of the investigated genes. The variance in the transcription levels could be due to the mitotic instability of the plasmids, thus fluctuation in the plasmid copy number. Moreover, in the case of silencing, a post transcriptional gene silencing was carried out and decrease in the relative transcription cannot be detected necessarily. In *M. circinelloides* a plasmid similar to *hpRNA* construct of our study was used to silence the *carB* (encoding phytoene dehydrogenase) gene constructed by inserting an inverted-repeat sequence corresponding to the *carB* gene under the control of the *M. circinelloides gpd1* and own promoter. More albino transformants was isolated using plasmid with *gpd1* promoter, which could trigger silencing of the *carB* gene in over 85% of the transformants (**de Haro et al., 2009**). Our aim was also to compare the efficiency of overexpression and silencing using different plasmids in *M. circinelloides*, which may serve valuable information for future studied. On the basis of the results of the present study we are concluding that overexpression and silencing was the most effective using the *gpd* and *hpRNA* plasmid constructs, respectively.

The mitotic stability of the transformants was also analyzed with 12 consecutive cultivation cycles on MEA medium, followed by inoculation onto selective YNB medium. Transformants of MS12+pHMG/hpRNA, MS12+pDMD/hpRNA and MS12+pIPI/hpRNA were less stable, which were unable to grow on YNB after 4^{th} - 7^{th} cycle of cultivation on MEA medium. Moreover, overexpression and silencing of the *isoA* (MS12+pISOA/gpd and MS12+pISOA/as) also resulted reduction in mitotic stability. These results presume, that silencing of the *hmgS, dmd, ipi* (precursors of all isoprenoids) and modification of the *isoA* (precursor of ergosterol and functional group of farnesylated proteins) have significant effect to the fungus.

Previously it was shown that transformants of *M. circinelloides* and closely related species (i.e. *Rhizopus oryzae*) carrying autonomously replicating vectors are often unstable and the copy number of the circular plasmid remains low (**Anaya and Roncero 1991; Ibrahim and Skory 2006; Mertens et al. 2006**). In contrast with these results, we found that *M. circinelloides* transformants are mitotically stable (except MS12+pHMG/hpRNA, MS12+pDMD/hpRNA and MS12+pIPI/hpRNA). In a previous study transformants harbouring plasmid for overexpression of the *ipi*, *isoA* and *carG* genes were mitotically stable after ten consecutive cultivation cycles on minimal (YNB) and complete (MEA) media (**Csernetics et al., 2011**). Cultivation on complete medium did not resulted in a decrease in the plasmid copy number, however, fluctuations were observed generally,

irrespectively of that the transferred gene was homologous or heterologous (**Csernetics et al., 2011; 2015**).

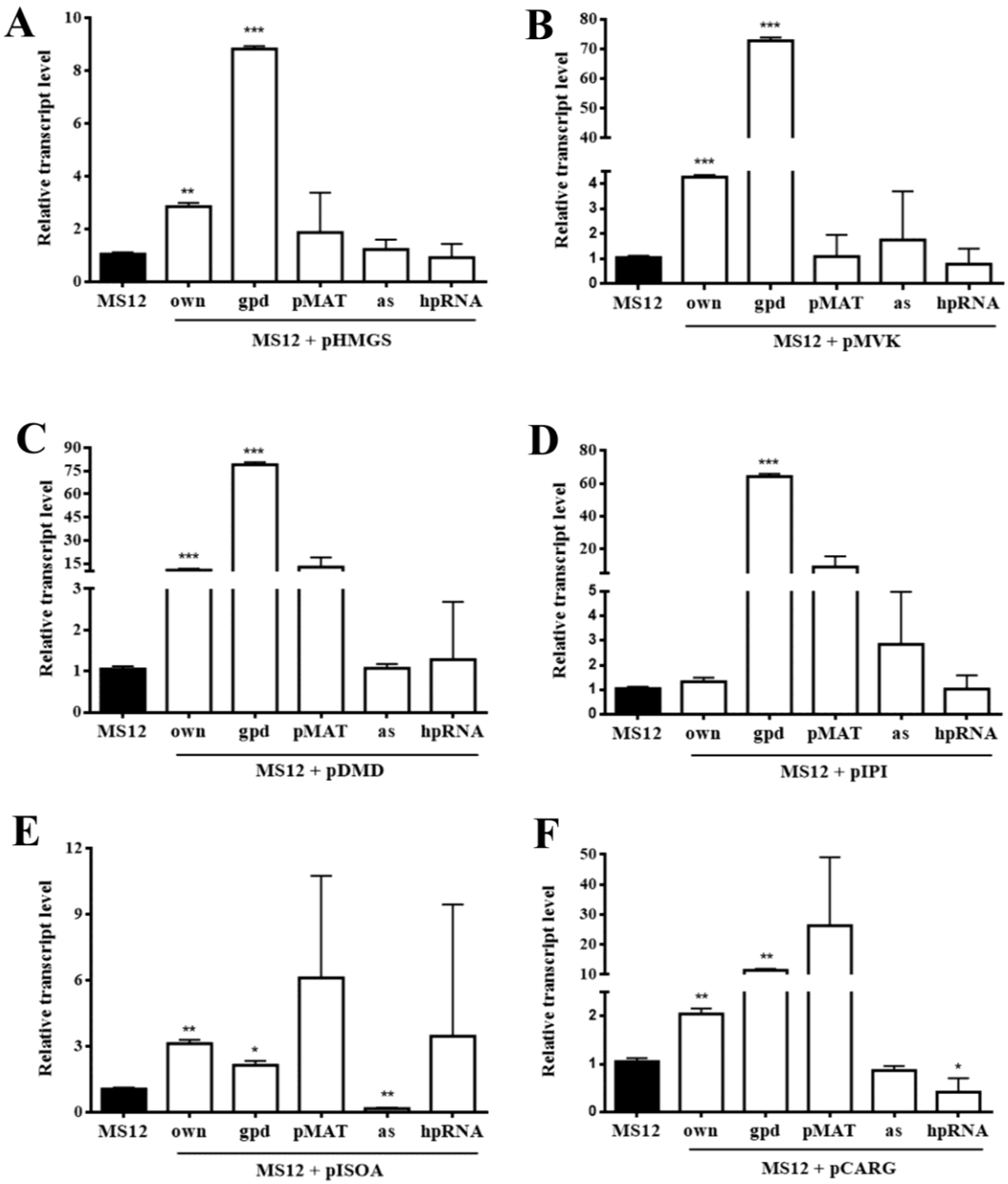


Fig 22. Relative transcription level of transformants harbouring the plasmids for overexpression (*own* and *gpd*) and silencing (*pMAT*, *as* and *hpRNA*) of the six mevalonate-isoprenoid genes in *M. circinelloides* (plasmid details shown in **Table 3**).

6.3.2 Investigation of macro- and micromorphology of the transformants

The colony forming unit (CFU) gives information about the plasmid distribution and viability of spores, which was studied with inoculation of 2×10^2 spores onto selective

YNB medium (pH 3.2). All transformants showed significant reduction in CFU compared to the *M. circinelloides* MS12 (**Fig. 23**), also those harbouring the pAVB107 and pEPM901 plasmids (**Fig. 23 A**). The reduction in CFU could be due to unequal distribution of plasmids and it does not depend on the plasmid sequence or the marker gene. This observation was verified in case of MS12+pAVB107 and MS12+pEPM901, in which the leucine and uracil auxotrophy was complemented and CFU of those transformants also reduced significantly (**Fig. 23 A**). When transformants were compared with those harbouring plasmids with marker gene only, we found that mutants in which silencing was achieved with *hpRNA* plasmid (MS12+pHMGS/hpRNA, MS12+pDMD/hpRNA and MS12+pISOA/hpRNA) showed significant reduction in CFU (**Fig. 23 B, D and F**). It seems that silencing of *hmgS, dmd* and *isoA* with *hpRNA* plasmids result in significant decrease in the viability of spores.

Spore germination was investigated with two different incubation time and media: at four hours and eight hours postinoculation in liquid media and on solid media (on cellophane discs); the examination was carried out with bright field microscope. All *M. circinelloides* mutants in the mevalonate-isoprenoid pathway showed significant decrease in number of germinating spores in comparison with *M. circinelloides* MS12. Similar decrease was also observed in MS12+pEPM901 strain harbours the plasmid with only the marker gene (data not shown), which presume that decrease in number of germinating spores was caused mainly by unequal distribution of plasmids and not the modification of the mevalonate-isoprenoid pathway.

The colony diameter of the strains was determined at 24 hours intervals up to four days, with inoculation of 10^5 spores on YNB selective medium (**Fig. 24 A-G**). No any significant changes were found between *M. circinelloides* MS12 and MS12+pAVB107 and MS12+pEPM901 strains in colony diameter (**Fig. 24 G**). In general, silencing with the *hpRNA* plasmids resulted decrease in the colony diameter in case of all six genes (but not at all intervals) in comparison with the *M. circinelloides* MS12 (**Fig. 24 A-G**). It has to be mentioned that in case of MS12+pHMGS/as, MS12+pDMD/as, MS12+pIPI/as and MS12+pCARG/as transformants also significant reduction in colony diameter was observed in comparison with *M. circinelloides* MS12 (**Fig. 24 A-G**), presuming that the observed changes was the effect of the silencing of the mevalonate-isoprenoid biosynthesis pathway genes of *M. circinelloides* and not the fluctuation in plasmid copy number.

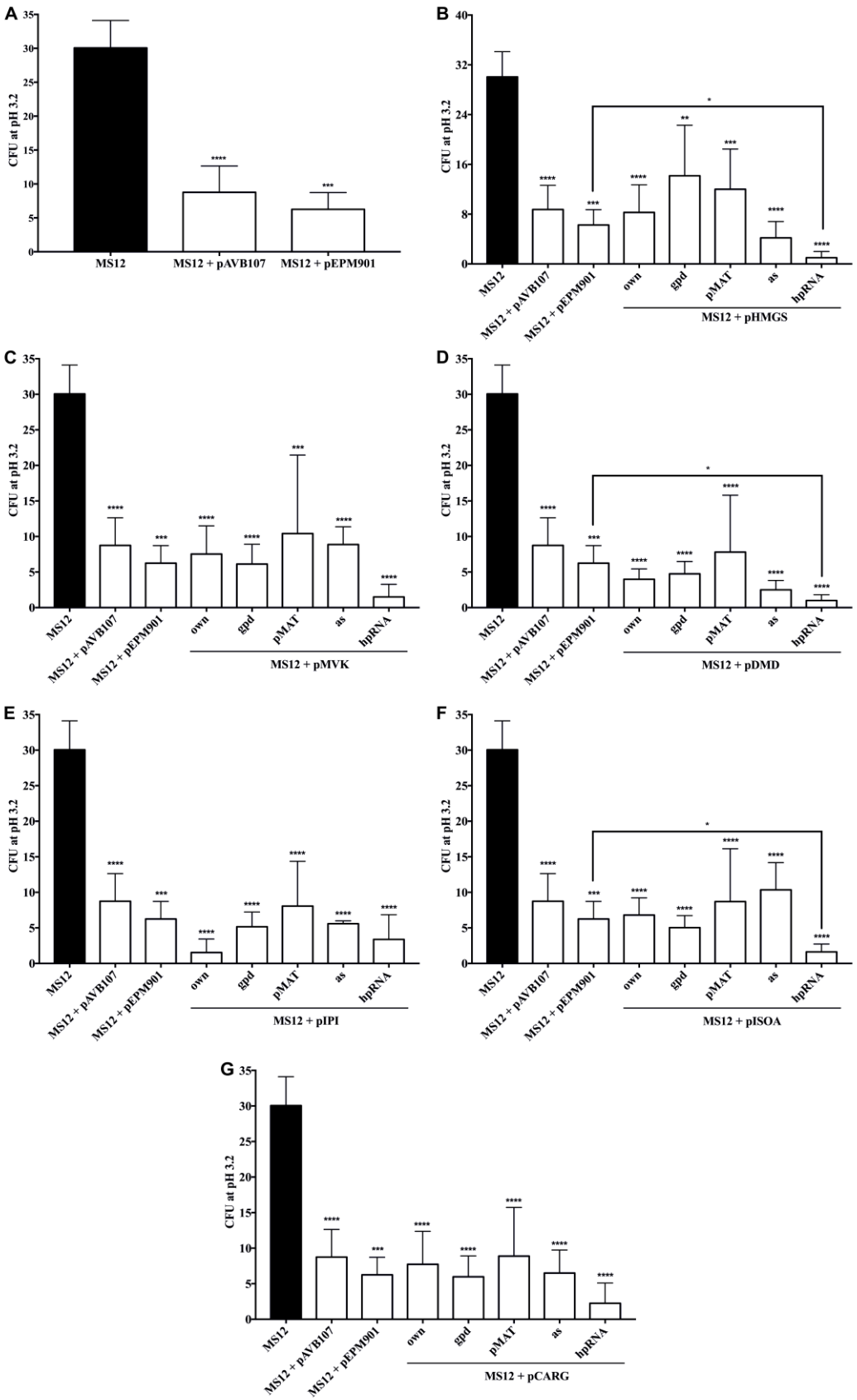


Fig 23. Percentage of colony forming units (CFU) of the *M. circinelloides* MS12, MS12+pAVB107 and MS12+pEPM901 (**A**) and mutants in the mevalonate-isoprenoid pathway (**B-G**) on YNB selective medium.

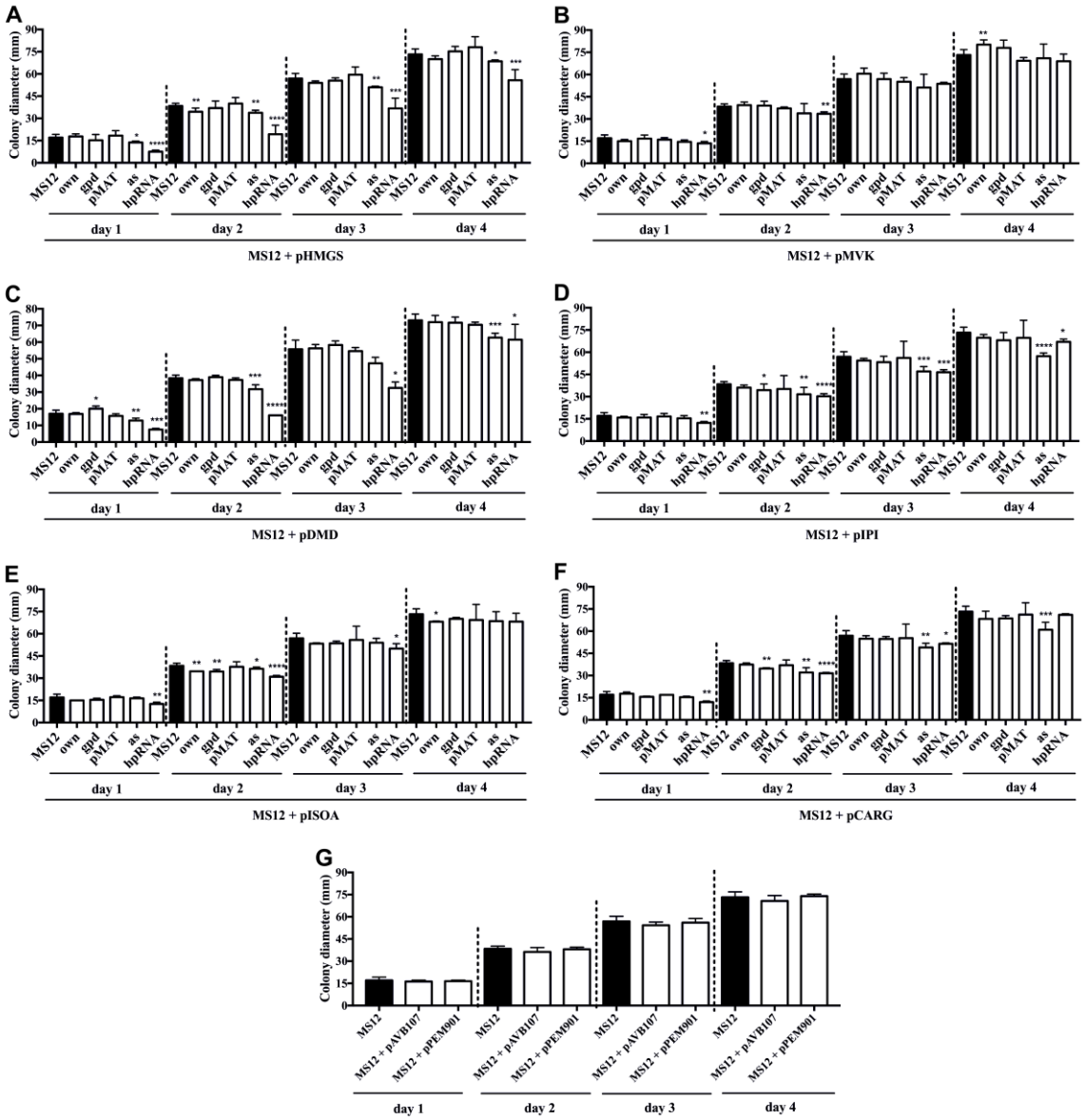


Fig 24. Colony diameter of the *M. circinelloides* strains mutant in mevalonate-isoprenoid pathway **(A-F)** and MS12+pAVB107 and MS12+pEPM901 strains **(G)**, which was determined at 24 hours intervals up to four days.

Micromorphology of the transformants (including hyphae branching and cytoplasmic effusion) was investigated under bright field microscope (**Fig. 25 A-L**). We found that MS12+pDMD/hpRNA mutants need more time for the growth. Moreover, in most of the transformants increased number of hyphal branches and cytoplasmic effusion was observed in four days old cultures. To quantify, the strains were inoculated onto cellophane discs after taht hyphal branches and cytoplasmic effusions were counted in 24 hours old cultures.

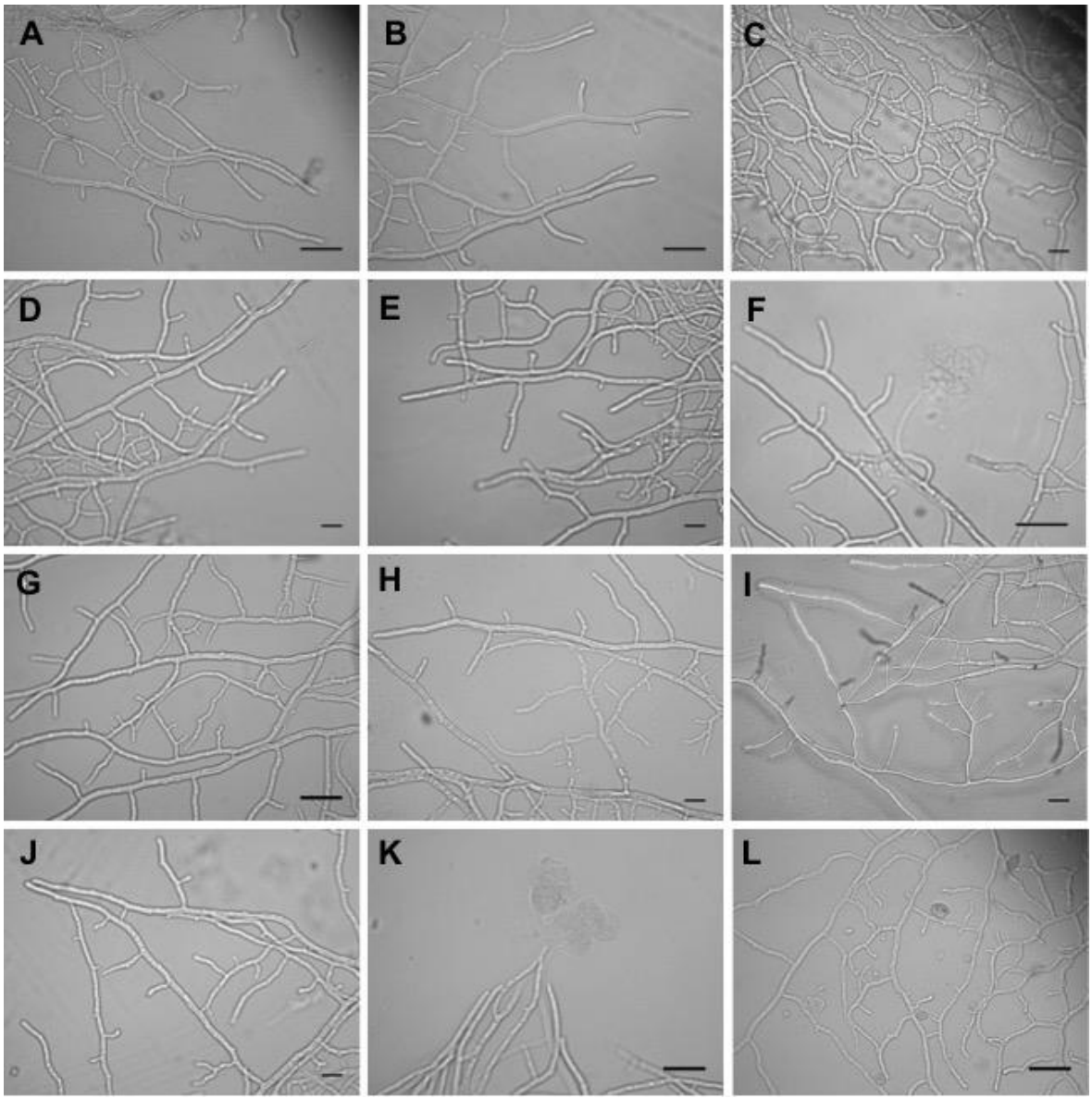


Fig 25. Micromorphology of selected transformants observed under bright field microscope: (A) MS12, (B) MS12+pEPM901, (C) MS12+pHMGS/own, (D) MS12+pMVK/own, (E) MS12+pDMD/AS, (F) MS12+pDMD/hpRNA, (G) MS12+pIPI/gpd, (H) MS12+pISOA/gpd, (I) MS12+pISOA/hpRNA, (J) MS12+pCARG/gpd, (K) MS12+pCARG/as and (L) MS12+pCARG/hpRNA, scale bars for each picture are 100 μm.

The branches were counted on hyphae originates from one germ tube and we found that mutant strains showed increase in hyphae branching in comparison to *M. circinelloides* MS12 (**Fig. 26**). In case of MS12+pAVB107 no difference was observed in comparison with *M. circinelloides* MS12, while in number of hyphae branches significant increase was found at MS12+pEPM901 (**Fig. 26**). It seems that complementation of the uracil auxotrophy has effect on the micromorphology of *M. circinelloides*: it increase the number of hyphae branches, while, interestingly, in case of mutants harbouring plasmids with the *leuA* marker gene (i.e. MS12+pAVB107, MS12+pISOA/gpd and

MS12+pCARG/gpd) this phenomenon could not be observed (except MS12+pISOA/own, in which slight increase was observed in number of hyphae branches in comparison with MS12).

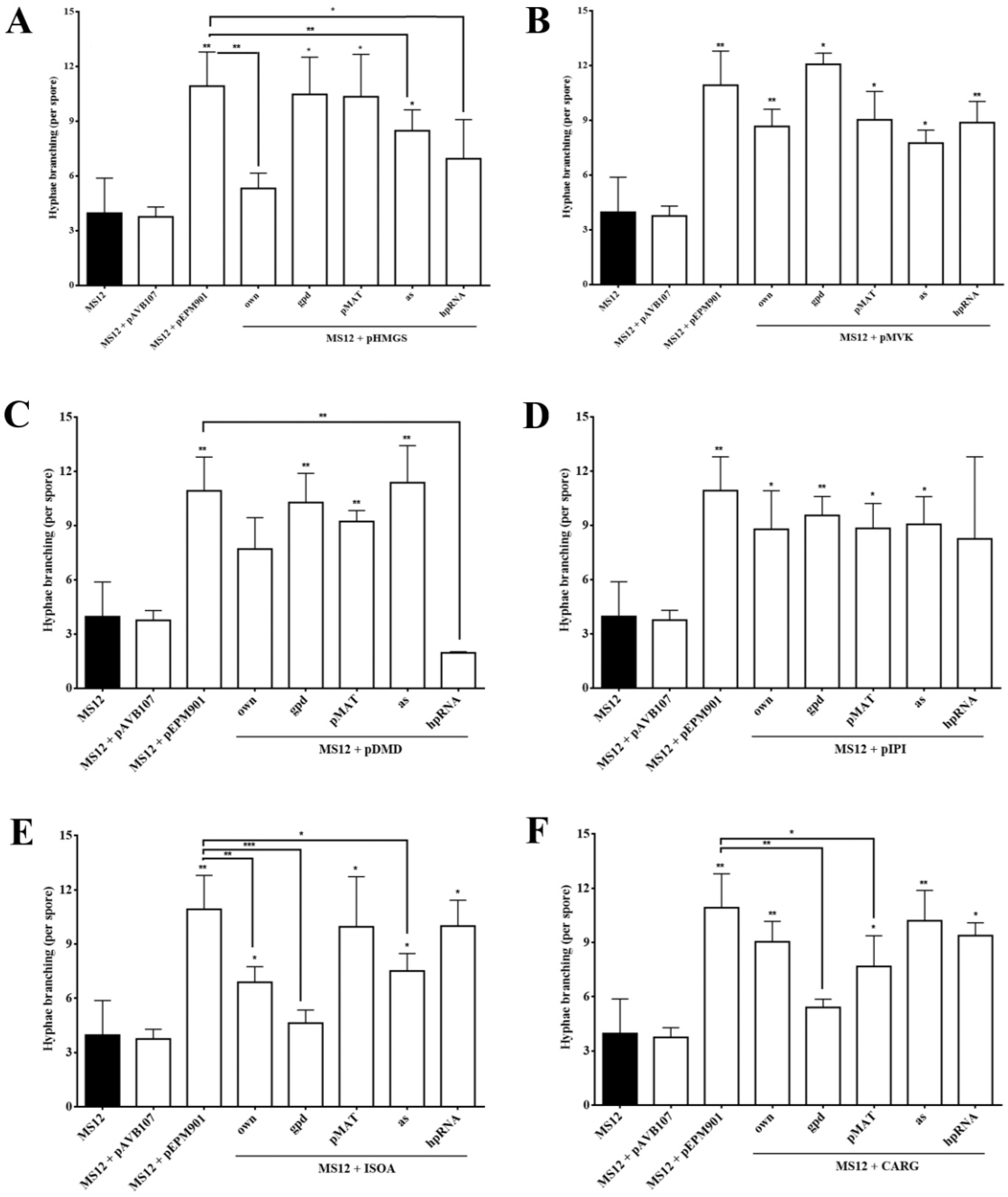


Fig 26. Number of hyphae branching analyzed at per microscopic area of *M. circinelloides* strains mutant in mevalonate-isoprenoid pathway (data are representing with comparison to *M. circinelloides* MS12 and MS12+pPEPM901).

The hyphal branching counted in the mutants was also compared to MS12+pEPM901. In this comparison, taking into account the above mentioned observations, mainly overexpression of the *hmgS* with *own* plasmid and silencing of the *dmd* and *isoA* with *hpRNA* and *as* plasmids, respectively, resulted decrease in number of hyphae branching (**Fig. 26**). It has to be noted that in four days old cultures an increase number in hyphal branching was observed, but in those cultures counting was not performed due to technical difficulties. Together with the results of colony diameter measurments it seems that silencing of the genes, mainly those responsible for formation of IPP (*dmd*) and precursors of ergosterol and prenyl groups of proteins (*isoA*) have the major effect in colony growth. IPP is the binding block of all isoprenoids, and FPP is the precursor of ergosterol. Sterol-rich membrane domains define prospective growth sites in fission yeasts (**Makushok et al., 2016**). Ergosterol is an important component of fungal cell membrane and decrease in the ergosterol content may result in abnormal hyphal branches in filamentous fungi (**Lin and Momany, 2004; Alvarez et al., 2007; Abe et al., 2009**).

In four days old mycelia increased number of cytoplasmic effusion was observed in comparison with the *M. circinelloides* MS12, which was quantified in 24 hours old mycelia. Ergosterol is playing role in maintenance of fungal membrane intergrity; cytoplasmic effusion presume fungal cell membrane integrity damage, which can be caused by decrease in the ergosterol content (**Alvarez et al., 2007; Abe et al., 2009**). In case of young mycelia no significant increase was observed in the number of cytoplasmic effusions of the transformants in comparison with *M. circinelloides* MS12 or MS12+pPEPM901 (**Fig 27**).

Apoptosis is an important process in multicellular organisms (**Collins et al., 1992**). This normally occurs during the progression of a cell and it is associated with maintenance of cell homeostasis, removal of damaged cells, response to infectious agents, and differentiation, as well as in the acclimatize responses of cells to biotic and abiotic stresses (**Danial and Korsmeyer, 2004; Green, 2005**). Apoptotic and necrotic cells of mutants harbouring the *hpRNA* plasmids for gene silencing were quantified after four and eight hours postinoculation. In case of MS12+pMVK/hpRNA, MS12+pDMD/hpRNA, MS12+pIPI/hpRNA and MS12+pCARG/hpRNA significant increase in the number of apoptotic cells was observed after four hours, however significant increment of necrotic cells was quantified only in MS12+pHMGS/hpRNA and MS12+pCARG/hpRNA compared to *M. circinelloides* MS12. While, after eight hours postinoculation,

significantly increased number of apoptotic cells was observed in the transformants of MS12+pMVK/hpRNA, MS12+pDMD/hpRNA, MS12+pIPI/hpRNA and MS12+pCARG/hpRNA, parallel with necrotic cells were observed in MS12+pHMGS/hpRNA compared to *M. circinelloides* MS12 (data not shown).

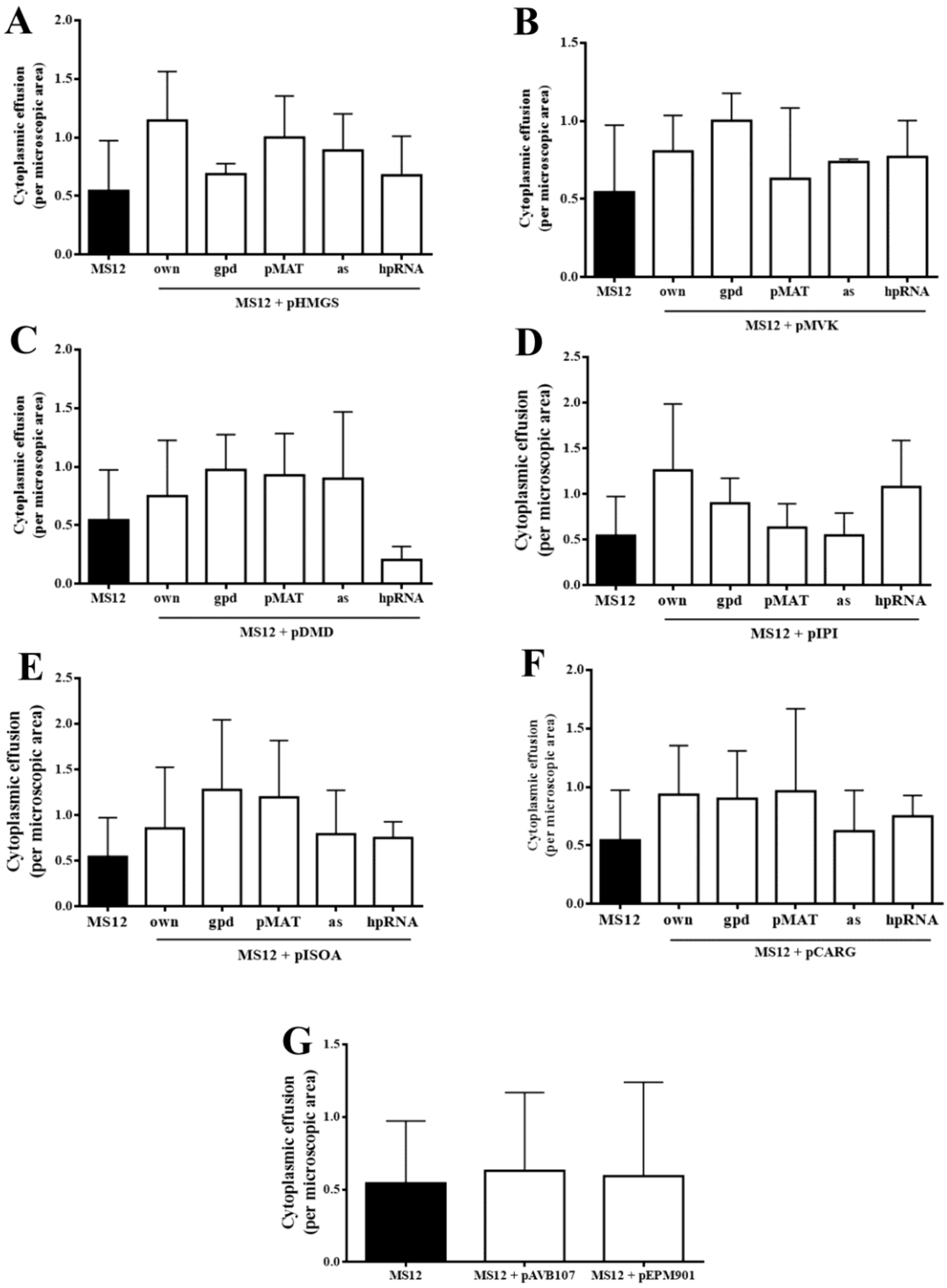


Fig 27. Number of cytoplasmic effusions at per microscopic area in *M. circinelloides* MS12 mutant of mevalonate-isoprenoid pathway (A-F), MS12 + pAVB107 and MS12 + pEPM901 (G).

6.4. Carotenoid and ergosterol content of the *M. circinelloides* strains mutant in mevalonate-isoprenoid pathway

Carotenoids are secondary metabolites, which are synthesised via the mevalonate-isoprenoid pathway in fungi. Carotenoid content of the mevalonate-isoprenoid biosynthesis pathway mutant *M. circinelloides* strains was analyzed. Overexpression of the genes resulted in increase in the carotenoid content, which was most prominent in transformants harbouring the plasmids with genes placed under the control of the *gpd1* promoter and terminator. The most significant increase was observed in case of overexpressing of the *carG*, followed by *ipi* and *mvk* genes, while silencing of *hmgS*, *mvk* and *dmd* (MS12+pHMGS/hpRNA, MS12+pMVK/hpRNA and MS12+pDMD/as) resulted in significant decrease in carotenoid production in comparison with the *M. circinelloides* MS12. Presumably by silencing the early mevalonate-isoprenoid pathway genes the precursors of carotenoid biosynthesis decreased, resulting in less accumulation of carotenoids. Interestingly, silencing of *isoA* gene resulted slight increase in the carotenoid content in comparison with the *M. circinelloides* MS12, while silencing of the *ipi* and *carG* had no any significant effect on the carotenoid production (**Fig. 28**). In strains MS12+pAVB107 and MS12+pEPM901 no significant difference was found in the carotenoid content in comparison with *M. circinelloides* MS12 (**Fig. 28 G**). The carotenoid composition of several mutants was investigated with HPLC and was found that overexpression or silencing of the genes do not affect significantly the carotenoid composition, thus β-carotene remains the main carotenoid compound and γ-carotene, lycopene, zeaxanthin and β-cryptoxanthin production in low amount could be also detected (data not shown).

In our study, overexpression of the *mvk* resulted significant increase in the carotenoid content. Mevalonate kinase encoded by the *mvk* gene is responsible for the formation of mevalonate-5P, a middle step in the mevalonate pathway. In comparison of the transcription of the investigated six genes in *M. circinelloides*, *mvk* showed the lowest transcription level (**Fig. 10**). With overexpression of the gene and presumably with increased availability of the precursor resulted significant increase in the accumulation of carotenoids. Overexpression of the *ipi* and *carG* genes, similarly to the previous study of our research group, increased the carotenoid production in *M. circinelloides,* moreover the increment was more prominent in case when the genes were placed under the control of the *gpd1* promoter (**Csernetics et al., 2011**). For example, in that study three times higher

carotenoid content than that of the original strain was found when expression of the *ipi* gene was driven by the *gpd1* promoter (**Csernetics et al., 2011**). In our study less difference was observed, i.e. overexpression of the *ipi* gene with own promoter resulted 646 μg/g dry weight carotenoid content, while in case when the gene was placed under the control of the *gpd1* promoter resulted 920 μg/g dry weight in compare to the control *M. circinelloides* MS12, 426 μg/g dry weight (in Csernetics and coworkers study it was 674 μg/g dry weight and 1177 μg/g dry weight, in compare to the control *M. circinelloides* MS12, 399 μg/g dry weight respectively, **Csernetics et al., 2011**). This variation in the carotenoid production between the two studies can be due to differences in the plasmid copy numbers. Overexpression of the *carG* resulted the most significant increase in the carotenoid production of *M. circinelloides* in both studies. In our study when the expression of *carG* was driven by its own promoter, 665 μg/g dry weight carotenoid content was determined, while in the case of *gpd1* promoter 1045 μg/g dry weight. All these findings demonstrate that formation of mevalonate-5P, DMAPP and GGPP are a rate-limiting steps in the carotenoid biosynthesis in *M. circinelloides.*

Beta-carotene accumulation was achieved by heterologous expression of the *X. dendrorhous* phytoene desaturase and phytoene synthase/lycopene cyclase encoding genes in the non-carotenoid producing *S. cerevisiae* and it was enhanced by the overexpression of the *S. cerevisiae BTS1* gene, encoding the geranylgeranyl pyrophosphate synthase (**Ukibe et al., 2009**). The intermediate enzyme of mevalonate-isoprenoid biosynthesis pathway, such as IPP isomerase, has been presumed to be a key regulatory enzyme in the isoprenoid pathway, and the effect of its (over) expression on carotenoid biosynthesis has been studied in detail in carotenoid producing mutant *E. coli* (**Lee and Schmidt-Dannert, 2002; Das et al., 2007**). *E. coli* engineered with heterologous cDNAs of IPP isomerases from *Haematococcus pluvialis* and *X. dendrorhous* resulted enhanced carotenoid accumulation (**Misawa and Shimada, 1997; Albrecht et al., 1999**). Indeed, the introduction of *Enterococcus faecalis mvaE* and *mvaS* genes, encoding the acetoacetyl-CoA synthase/HMG-CoA reductase and HMG-CoA synthase, respectively, into *E. coli* has been reported to improved direct production of mevalonate and substantially improve the productivity of carotenoids or sesquiterpenes that are synthesized from DMAPP (**Middleton, 1972; Tabata et al., 2004; Anthony et al., 2009; Yang et al., 2012**). In *N. crassa* increased accumulation of the HMG-CoA, led to a several-fold improvement in lycopene and neurosporaxanthin production (**Wang and Keasling 2002**). Overexpression of the *hmgS*, responsible for the formation of HMG-CoA in *M. circinelloides*, also led to a

significant increase in the carotenoid accumulation in our study. Overexpression of genes involved in the early steps in isoprenoid biosynthesis pathway, such as the *hmgR2* and *hmgR3* genes also led to carotenoid overaccumulation in *M. circinelloides* (**Nagy et al., 2014**).

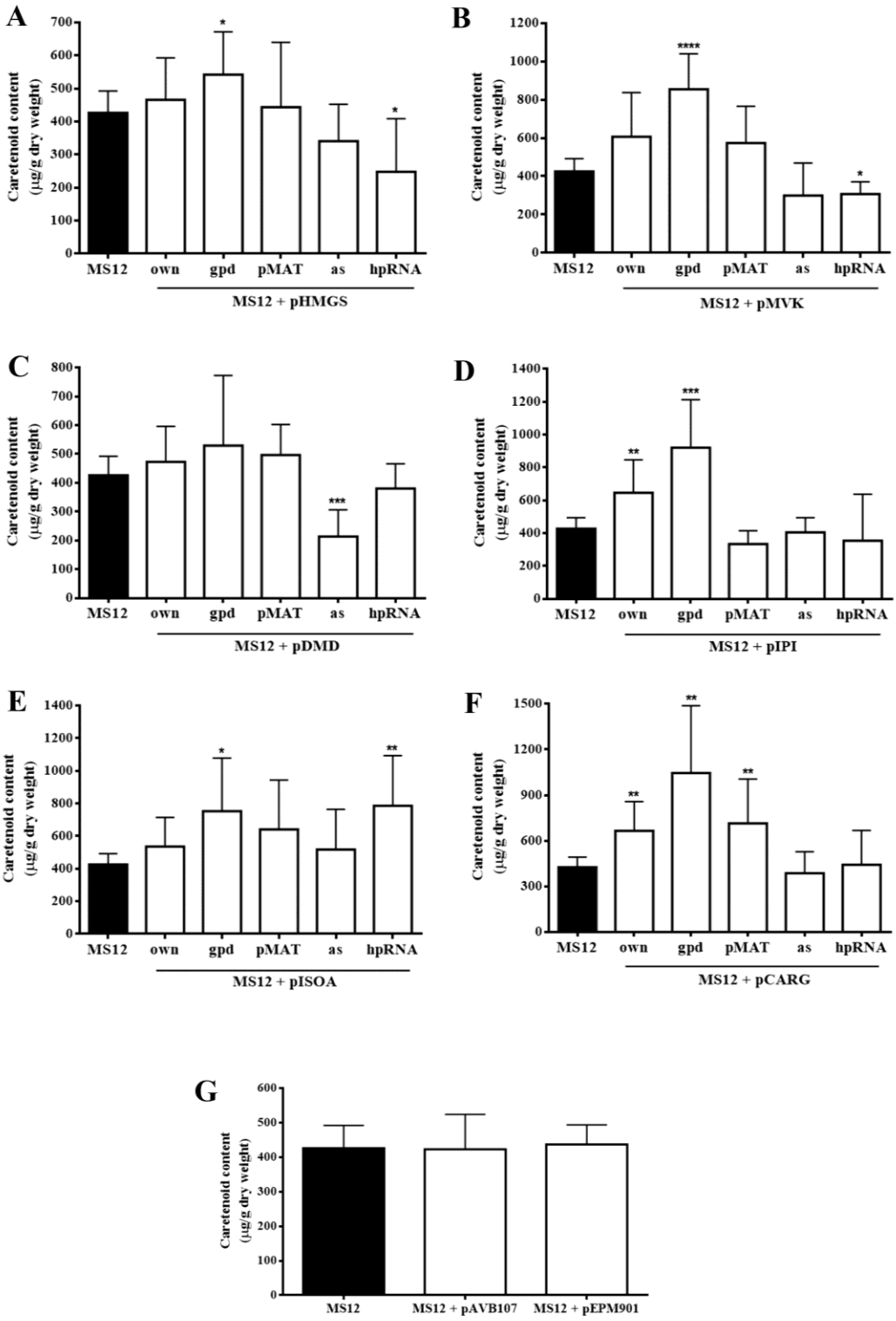


Fig 28. Carotenoid content of the *M. circinelloides* mutants in mevalonate-isoprenoid biosynthesis pathway. Data represent the carotenoid content in µg/g dry weight of mycelia.

Today the Mucoromycotina *B. trispora* is still used for industrial β-carotene production. It has to be mentioned that the total carotenoid content of the wild-type *B. trispora* is 260 – 300 μg/g dry weight, which is similar to *M. circinelloides* MS12 (**Mehta et al., 2003**). For industrial carotenoid production mixed culture of different mating types of *B. trispora* and optimized fermentation media are used (**Mehta et al., 2003**). However, transformation systems are not available for genetic modification to improve carotenoid production in *B. trispora*, at the same time efficient transformation systems are available to *M. circinelloides* and with optimization of the fermentation conditions its carotenoid production could be increased more.

Ergosterol content of the *M. circinelloides* strains mutant in the mevalonate-isoprenoid pathway was also analyzed by HPLC. Interestingly, overexpression of the genes did not resulted in significant increment in ergosterol content, however overexpression of the *mvk*, *ipi* and *isoA* decreased the ergosterol content. Silencing of all six genes with *hpRNA* plasmids significantly decreased the content of ergosterol in comparison to the untransformed *M. circinelloides* MS12. No any significant change in the ergosterol content was found between MS12, MS12+pAVB107 and MS12+pEPM901 (**Fig. 29**).

Nagy and coworkers reported that overexpression of HMG-CoA reductase encoding genes in *M. circinelloides* slightly, but not significant increased the ergosterol content in comparison to the untransformed *M. circinelloides* MS12 (**Nagy et al., 2014**). Our results are similar to those, overexpression of the six mevalonate-isoprenoid pathway genes in *M. circinelloides* did not resulted significant ergosterol overaccumulation. At the same time Yasmin and coworkers described that overexpression of *hmg1* gene, encoding a HMG-CoA reducatse increased the ergosterol content in *A. fumigatus* (**Yasmin et al., 2011**). In *Phaffia rhodozyma* it had been shown that astaxanthin overproduction reduced the ergosterol content (leading to precursor accumulation, and transfer to the astaxanthin pathway) compared with the wild type (**Miao et al., 2011**). Similarly, in our study overexpression of the *mvk*, *ipi* and *isoA* genes, as well as silencing of the *isoA* led to increase in the carotenoid production and reduction in ergosterol accumulation. In case of our study knock down of all six genes by *hpRNA* plasmids resulted significant reduction in ergosterol content in *M. circinelloides* with range 40 – 54% (**Fig. 29**).

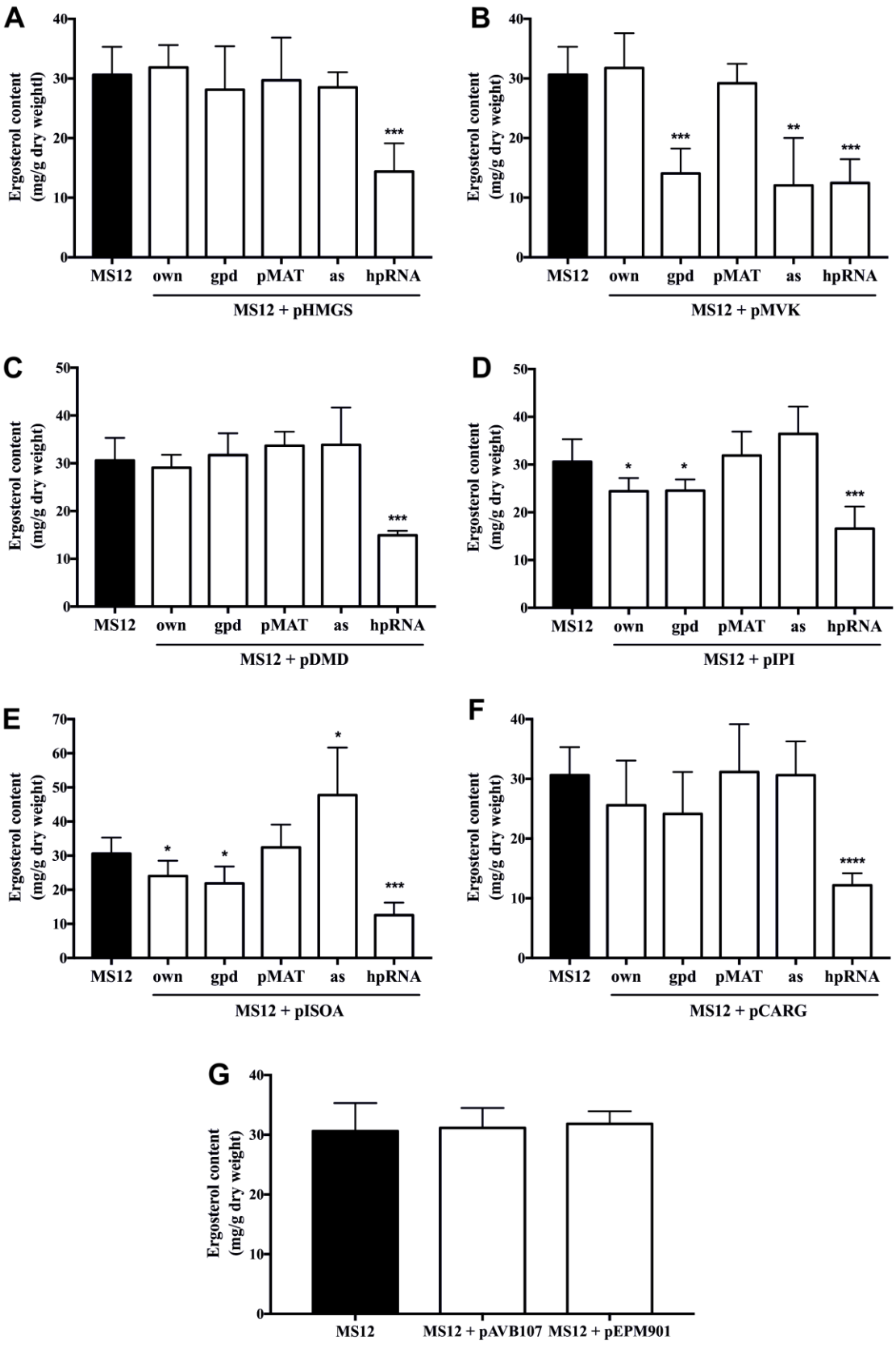


Fig 29. Ergosterol content of the *M. circinelloides* mutants in mevalonate-isoprenoid biosynthesis pathway, analyzed by HPLC. Data are representing the ergosterol content in mg/g dry weight of mycelia.

6.5. Antibiotic susceptibility of the *M. circinelloides* strains mutant in mevalonate-isoprenoid pathway

The mortality rate associated with mucormycoses remains extremely high, while Mucoromycotina fungi shows intrinsic resistance to most of the clinically used antifungal agents, thus identification of new potential novel drug targets would be necessary (**Arendrup, 2011; Howard and Arendrup, 2011; van derLinden et al., 2011**). Although Mucorales fungi have long been considered a homogenous group in terms of susceptibility to antifungals, it is now clear that this trait differs between species of this order (**Alastruey-Izquierdo et al., 2009**). Our laboratoary previously analyzed the susceptibility of *M. circinelloides* MS12 against twenty-one antifungals (**unpublished data**). Based on these data ITR, CLO, SIM, FLU, and AmB were selected for for investigation of the susceptibility of *M. circinelloides* mutants in the mevalonate isoprenoid pathway.

In our study *in vitro* susceptibility of the mutants in mevalonate-isoprenoid biosynthesis to ITR, CLO, SIM, FLU, and AmB was determined. No any major difference in the susceptibility to AmB of the mutants was found in comparison with parental strain *M. circinelloides* MS12. Susceptibility of MS12+pIPI/own, MS12+pHMG/hpRNA and MS12+pDMD/hpRNA mutants significantly changed to ITR, the MIC_{90} in those strains was determined in 2 - 8 μg/ml (MIC_{90} >128 μg/ml in the case of *M. circinelloides* MS12 as well as MS12+pAVB107, MS12+pEPM901), moreover paradox effect was also observed at these strains, thus increasing the ITR concentration to more than 8 μg/ml did not resulted 90% growth inhibition. This phenomenon was also observed in *Candida* and *Aspergillus* species against echinocandins, but the mechanism still not clear, presumably induction of chitin synthesis is one of the possible mechanism of survival (**Eagle and Musselman, 1948; Shields et al., 2011; Stover and Cleary, 2015**). Increased susceptibility of these strains to CLO was also observed: MS12+pIPI/own, MS12+pHMG/hpRNA and MS12+pDMD/hpRNA showed susceptibility at 1 – 4, 4 – 16 and 2 – 8 μg/ml to CLO, respectively, in comparision to the 32 μg/ml in the case of *M. circinelloides* MS12. It has to be also mentioned that in case of MS12+pEPM901 (4 – 16 μg/ml) increased susceptibility to CLO was also determined, but not so prominent as in case of MS12+pIPI/own, MS12+pHMG/hpRNA and MS12+pDMD/hpRNA mutants. Difference in the susceptibility to FLU and SIM was mainly observed in those mutants, in which *mvk*, *dmd* and *ipi* were overexpressed or silenced (**Table 4**). In a previous study, overexpression of the *hmgR2* and *hmgR3* in *M. circinelloides* resulted decrease in the susceptibility to fluvastatin, atorvastatin and rosuvastatin (**Nagy et al., 2014**). Similarly,

we also observed significant decrease in the susceptibility to FLU and SIM in the case of overexpression of the *mvk* and *dmd* genes.

Table 4. Antifungal susceptibility of the mutants in mevalonate-isoprenoid biosynthesis to AmB (amphotericin B), ITR (itraconazole), CLO (clotrimazole), FLU (fluvastatin) and SIM (simvastatin) *in vitro*.

Strain	MIC_{90} (µg/ml)				
	AmB	**ITR**	**CLO**	**FLU**	**SIM**
MS12	1	>128	32	1	1
MS12 + pHMGS-OE	0.5 – 2	>128	32	1 – 2	0.5 – 2
MS12 + pMVK-OE	0.5 – 1	>128	32	1 – 4	1 – 4
MS12 + pDMD-OE	1 – 2	>128	32 - >32	**2 – 8**	1 – 4
MS12 + pIPI-OE	0.5 – 2	**2 – 8 µg/ml, paradadox effect**	**1 – 4**	0.5 – 1	**0.25 – 1**
MS12 + pISOA-OE	1 – 2	>128	32	0.5 – 2	2
MS12 + pCARG-OE	0.5 – 1	>128	32	0.5 – 2	0.5 – 4
MS12 + pHMGS-GPD	0.5	>128	8 – 16	1 – 4	0.5 – 4
MS12 + pMVK-GPD	1	>128	32	**4 – 8**	**2 – 8**
MS12 + pDMD-GPD	0.5 – 2	>128	32	**2 – 8**	**2 – 8**
MS12 + pIPI-GPD	1 – 2	>128	16 – 32	1	0.5 – 1
MS12 + pISOA-GPD	1	>128	16	2	2
MS12 + pCARG-GPD	2	>128	16 – 32	1 – 2	1 – 2
MS12 + pHMGS-AS	1	>128	16 – 32	0.5 – 1	0.5 – 1
MS12 + pMVK-AS	0.5	>128	16	1 – 2	1 – 4
MS12 + pDMD-AS	0.5 – 2	>128	8 – 32	0.5 – 2	**0.125 – 1**
MS12 + pIPI-AS	1 – 2	>128	8 – 16	0.5 – 2	1
MS12 + pISOA-AS	1	>128	32	0.5 – 1	0.5 – 1
MS12 + pCARG-AS	2	>128	16 – 32	1 – 2	1 – 2
MS12 + pHMGS-MAT	0.5	>128	16	0.5	1
MS12 + pMVK-MAT	0.5 – 1	>128	32	1	1 – 4
MS12 + pDMD-MAT	0.5	>128	32	0.5 – 1	0.5 – 2
MS12 + pIPI-MAT	2	>128	32	1	**2 – 4**
MS12 + pISOA-MAT	0.5 – 2	>128	32	1	0.5 – 1
MS12 + pCARG-MAT	0.5	>128	4 – 32	0.5 – 1	**0,5 – 8**
MS12 + pHMGS-HPRNA	0.5 – 2	**4 – 8 µg/ml, paradadox effect**	**4 – 16**	0.5 – 2	0.5 – 2
MS12 + pMVK-HPRNA	0.5 – 1	>128	8 – 16	1 – 2	0.5 – 1
MS12 + pDMD-HPRNA	0.5 – 4	**2 – 8 µg/ml, paradadox effect**	**2 – 8**	**0,125 – 1**	0.5 – 1
MS12 + pIPI-HPRNA	1	>128	8 – 16	0.5 – 4	**1 – 8**
MS12 + pISOA-HPRNA	1	>128	8	1 – 2	1
MS12 + pCARG-HPRNA	0.5 – 1	>128	16	0.5 – 2	0.5 – 1
MS12 + pAVB107	1	>128	32	1 – 2	2
MS12 + pEPM901	0.5 – 2	>128	8 – 16	0.5 – 1	1

The MIC of AmB (0.06 – 1 µg/ml), ITR (2 – >8 µg/ml) and FLU (>25 µg/ml) to some *M. circinelloides* strains was determined in previous studies (**Caramalho et al., 2015; Almyroudis et al., 2007; Drogari-Apiranthitou et al., 2012; Salas et al., 2012 Galgóczy et al., 2011**). In our experiments MIC_{90} of AmB and FLU to MS12 was determined in 1 µg/ml, while in case of ITR no MIC_{90} could be determined in the investigated concentrations. It has to be mentioned that in our experiments the antifungal susceptibility tests were performed in YNB medium to maintain the selective conditions

and not in RPMI-1640 as recommended by the CLSI M38-A2 method. Differences in the susceptibility of *M. circinelloides* MS12 to antifungal agents in YNB and RPMI-1640 media was also observed by our research group previously (**unpublished data**).

Azoles are inhibitors of the cytochrome-P450 dependent lanosterol 14-α demethylase, a key enzyme in ergosterol biosynthesis (**Vandeputte et al., 2012**). In our experiments overexpression of the *ipi* and silencing of the *hmgS* and *dmd* genes resulted increase in the susceptibility to azoles and these mutants showed reduction in ergosterol content, as well (**Fig. 29**). Moreover silencing of the *dmd* gene increased the susceptibility of *M. circinelloides* to statins, which are competitive inhibitors of the HMG-CoA reductase, **Nagy et al., 2014**). Based on all of these observations, silencing of the *dmd* gene resulted the most prominent change in the susceptibility to azoles and statins (**Table 4**).

6.6. Interaction of the mevalonate-isoprenoid pathway mutant *M. circinelloides* strains with MH-S macrophages

In our study phagocytosis assay was performed with MH-S macrophages and the mevalonate-isoprenoid pathway mutant *M. circinelloides* strains. Overexpression of the *hmgS*, *ipi* and *carG* and silencing of *mvk* and *isoA* genes (with *hpRNA* plasmids) resulted significant decrease in the phagocytic indexes (PI), while overexpression of *mvk* and *dmd*, as well as silencing of *hmgS, mvk, dmd* and *carG* genes with *pMAT* plasmid and *mvk* with *as* plasmid resulted significant increase in PI in comparison with *M. circinelloides* MS12 (**Fig. 30**). In case of MS12+pAVB107 and MS12+pEPM901 no significant change in PI was observed in comparison with *M. circinelloides* MS12 (**Fig. 30**). Up to days no any data available regarding interaction of macrophages with Mucormycotina fungi mutant in mevalonate-isoprenoids pathway.

Heuston and coworker summarized that classical mevalonate pathway or the alternative 2C-methyl-D-erythritol-4-phosphate (MEP) pathway or its intermediate 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMB-PP) pathway can activate human Vc9/Vd2 T-cells (**Heuston et al., 2012**). Begley and coworkers have previously described that both pathways, either the classical mevalonate pathway or the alternative non-mevalonate are functional in *Listeria monocytogenes* and can provide sufficient IPP for normal growth (**Begley et al., 2004**). Murine studies revealed that mutants lacking the MEP pathway were impaired in virulence relative to the parental strain (**Begley et al., 2008**). Presumably, modification of the amount of accumulated metabolites synthesized

via the mevalonate-isoprenoid pathway in *M. circinelloides* (such as ergosterol) resulted change in the PI in comparison with the untransformed *M. circinelloides* MS12, however, to uncover the backround further investigations are required.

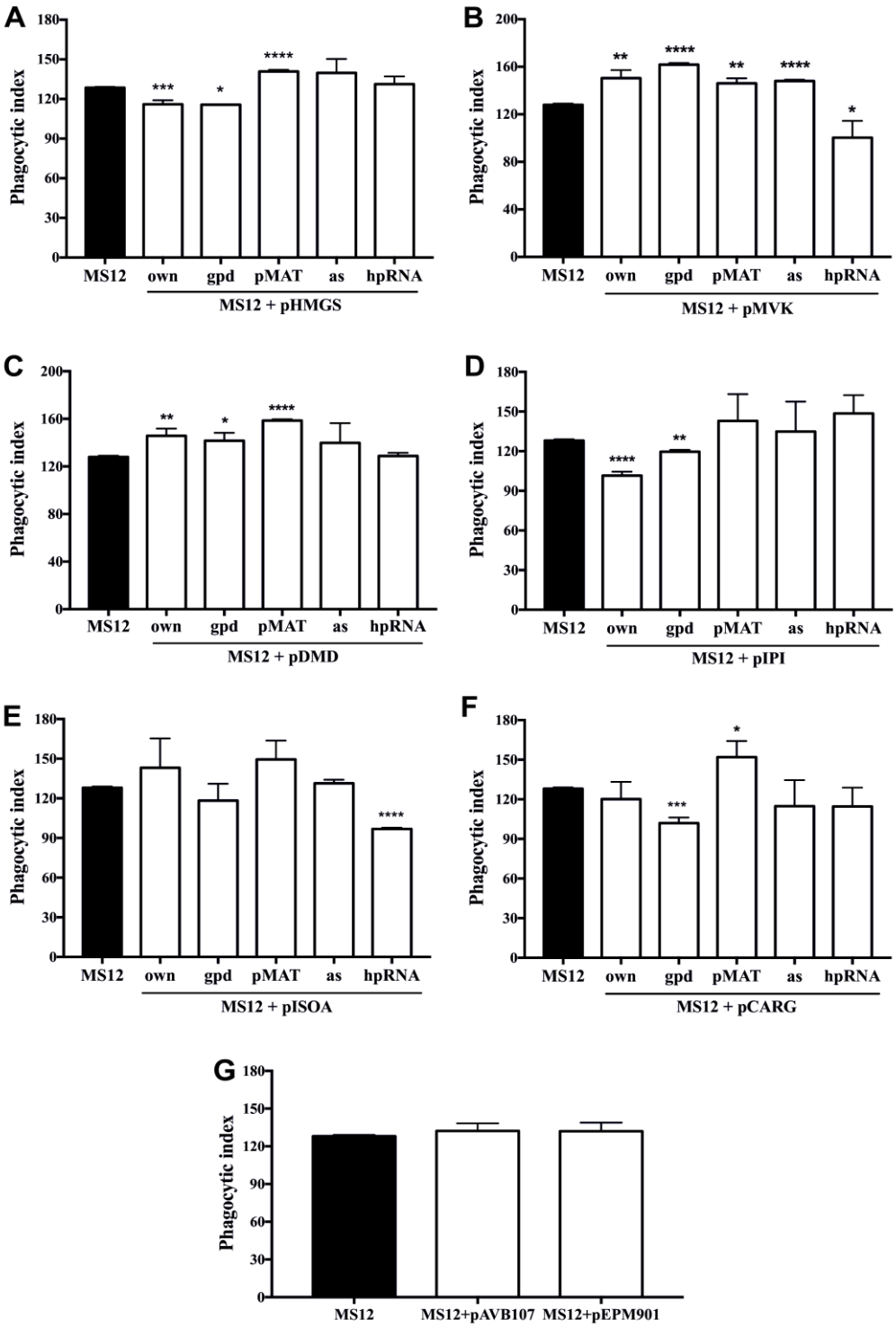


Fig 30. Phagocytic indexes of *M. circinelloides* mutants defective in mevalonate-isoprenoid biosynthesis pathway.

7. CONCLUSIONS

As a result of our research, new information was added to our knowledge regarding the mevalonate-isoprenoid biosynthesis pathway in *M. circinelliodes*. Six genes involved in that pathway (*hmgS, mvk, dmd, ipi, isoA* and *carG*) were selected for characterization and the transcription of the genes was determined under different cultivation conditions, which can contribute in further studies to modify the metabolite production of the fungus (such as carotenoids and ergosterol). Characteristics of the mutant *M. circinelloides* strains were also compared to each other and found that overexpression and silencing are the most effective with plasmids harbouring the genes under the control of the *gpd1* promoter and *hpRNA* constructs, respectively.

Carotenoid overproducing mutants were isolated with overexpression of the *mvk, ipi* and *carG* genes, which can be used in further studies to improve the β-carotenoid production of Mucoromycotina fungi. Moreover, 40 – 54% decrease in the ergosterol content was detected with silencing of all six genes with *hpRNA* plasmid and increased susceptibility to azoles and statins of mutants harbouring the *hpRNA* plasmid for silencing of *dmd* gene was determined, which may serve as potential target for new antifungal therapy in future.

8. REFERENCES

Abe A, Sujaya IN, Sone T, Asano K, Oda Y (2004) Microflora and selected metabolites of potato pulp fermented with an Indonesian starter *Ragi Tapé. Food Technol Biotechnol* 42:169–173.

Abe, F, Usui K, Hiraki T (2009) Fluconazole modulates membrane rigidity, heterogeneity, and water penetration into the plasma membrane in *Saccharomyces cerevisiae*. *Biochemistry* 48:8494–8504.

Alastruey-Izquierdo A, Castelli MV, Cuesta I, Zaragoza O, Monzon A, Mellado E, Rodríguez-Tudela JL (2009) *In vitro* activity of antifungals against zygomycetes. *Clin Microbiol Infect* 15:71–76.

Albrecht M, Misawa N, Sandmann G (1999) Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids ß-carotene and zeaxanthin. *Biotechnol Lett* 21:791–795.

Alcaíno J, Bravo N, Córdova P, Marcoleta AE, Contreras G, Barahona S, Sepúlveda D, Fernández-Lobato M, Baeza M, Cifuentes V (2016) The involvement of Mig1 from *Xanthophyllomyces dendrorhous* in catabolic repression: an active mechanism contributing to the regulation of carotenoid production. *PLoS One* 11:e0162838.

Alcazar-Fuoli L, Mellado E, Garcia-Effron G, Lopez JF, Grimalt JO, Cuenca-Estrella JM, Rodriguez-Tudela JL (2008) Ergosterol biosynthesis pathway in *Aspergillus fumigatus. Steroids* 73:339–347.

Almyroudis NG, Sutton DA, Fothergill AW, Rinaldi MG, Kusne S (2007) *In vitro* susceptibilities of 217 clinical isolates of zygomycetes to conventional and new antifungal agents. *Antimicrob Agents Chemother* 51:2587–90.

Alvarez E, Sutton DA, Cano J, Fothergill AW, Stchigel A, Rinaldi MG, Guarro J (2009) Spectrum of zygomycete species identified from clinically significant specimens in the United States. *J Clin Microbiol* 47:1650–1656.

Alvarez FJ, Douglas LM, Konopka JB (2007) Sterol rich plasma membrane domains in fungi. *Eukaryot Cell* 6:755-763.

Amey RC, Athey-Pollard A, Burns C, Mills PR, Bailey A, Foster GD (2002) PEG-mediated and *Agrobacterium*-mediated transformation in the mycopathogen *Verticillium fungicola*. *Mycol Res* 106:4–11.

Anaya N, Roncero MI (1991) Transformation of a methionine auxotrophic mutant of *Mucor circinelloides* by direct cloning of the corresponding wild type gene. *Mol Gen Genet* 230:449–455.

Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, Keasling JD (2009) Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha- 4,11-diene. *Metab Eng* 11:13–19.

Appel KF, Wolff AM, Arnau J (2004) A multicopy vector system for genetic studies in *Mucor circinelloides* and other zygomycetes. *Mol Genet Genom* 271:595-602.

Araújo JP, Hughes DP (2016) Diversity of entomopathogenic fungi: which groups conquered the insect body?. *In Advances in genetics* (Vol. 94, pp. 1-39), Academic Press.

Arcidiacono S, Kaplan DL (1992) Molecular weight distribution of chitosan isolated from *Mucor rouxii* under different culture and processing conditions. *Biotechnol Bioeng* 39:281-286.

Azami Y, Hattori A, Nishimura H, Kawaide H, Yoshimura T, Hemmi H (2014) (R)-mevalonate 3-phosphate is an intermediate of the mevalonate pathway in *Thermoplasma acidophilum*. *J Biol Chem* 289:15957–15967.

Azanza JR, Sádada B, Reis J (2015) Liposomal formulations of amphotericin B: differences according to the scientific evidence. *Rev Esp Quimioter* 206:275–281.

Balance DJ, Buxton FP, Turner G (1983) Transformation of *Aspergillus nidulans* by the orotidine-5 phosphate decarboxylase gene of *Neurospora crassa*. *Biochem Biophys Res Commun* 112:284–289.

Barbosa MS, Cunha Passos DA, Felipe MS, Jesuíno RS, Pereira M, de Almeida Soares CM (2004) The glyceraldehyde-3-phosphate dehydrogenase homologue is differentially regulated in phases of *Paracoccidioides brasiliensis*: molecular and phylogenetic analysis. *Fungal Genet Biol* 41:667-675.

Batista NT, Reberšek M, Vernier PT, Mali B, Miklavčič D (2016) Effects of high voltage nanosecond electric pulses on eukaryotic cells (*in vitro*): A systematic review. *Bioelectrochem* 110:1-12.

Beggs JD (1978) Transformation of yeast by a replicating hybrid plasmid. *Nature* 275:104–109.

Begley M, Bron PA, Heuston S, Casey PG, Englert N, Wiesner J, Jomaa H, Gahan CG, Hill C (2008) Analysis of the isoprenoid biosynthesis pathways in *Listeria*

monocytogenes reveals a role for the alternative 2-C-methyl-D-erythritol 4-phosphate pathway in murine infection. *Infect Immun* 76:5392-5401.

Begley M, Gahan CG, Kollas AK, Hintz M, Hill C, Jomaa H, Eberl M (2004) The interplay between classical and alternative isoprenoid biosynthesis controls γδ T cell bioactivity of *Listeria monocytogenes*. *FEBS Lett* 561:99-104.

Bemis LT, Denis CL (1998) Identification of Functional Regions in the Yeast Transcriptional Activator ADR1. *Mol Cell Biol* 8:2125-2131.

Benito EP, Díaz-Mínguez JM, Iturriaga EA, Campuzano V, Eslava AP (1992) Cloning and sequence analysis of the *Mucor circinelloides pyrG* gene encoding orotidine-5′-monophosphate decarboxylase and its use for homologous transformation. *Gene* 116:59–67.

Benny GL, Humber RA, Morton JB (2001) Zygomycota: Zygomycetes. In: McLaughlin DJ, McLaughlin EG, Lemke PA, eds. The Mycota VII, Systematics and evolution, Part A. Berlin: Springer-Verlag:113–146.

Benny GL, O'Donnell K (2000) *Amoebidium parasiticum* is a protozoan, not a Trichomycete. *Mycologia* 92:1133-1137.

Bergman P, Linde C, Putsep K, Pohanka A, Normark S, Henriques-Normark B, Andersson J, Bjorkhem-Bergman L (2011) Studies on the antibacterial effects of statins–*in vitro* and *in vivo*. *PLoS ONE* 6:e24394.

Bhosale P, Bernstein (2005) Microbial xanthophylls. *Appl Micobiol Biotechnol* 68:445-455.

Bidle KA, Hanson TE, Howell K, Nannen J (2007) HMG-CoA reductase is regulated by salinity at the level of transcription in *Haloferax volcanii*. *Extremophiles*11:49–55.

Bien CM, Espenshade PJ (2010) Sterol regulatory element binding proteins in fungi: hypoxic transcription factors linked to pathogenesis. *Eukaryotic Cell* 9:352–359.

Bondaryk M, Kurzatkowoski W, Staniszewska M (2013) Antifungal agent commonly used the superficial and mucosal candidiasis treatment: mode of action and resistance development. *Postepy Dermatol Alergol* 30:293-301.

Britton G, Liaaen-Jensen S, Pfander H (2009) Carotenoids volume 5: nutrition and health. Basel: Birkhäuser; Editors: Britton, George, Liaaen-Jensen, Synnove, Pfander, Hanspeter: DOI 10.1007/978-3-7643-7501-0.

Buhaescu I, Izzedine H (2007) Mevalonate pathway: A review of clinical and therapeutical implications. *Clin Biochem* 40:575–584.

Burg JS, Espenshade PJ (2011) Regulation of HMG-CoA reductase in mammals and yeast. *Prog Lipid Res* 50:403–410.

Cˇertík M, Masrnová S, Sitkey V, Minárik M, Breierová E (2005) Biotechnological production of astaxanthin. *Chem Listy* 99:237–240.

Cafaro MJ (2005) Eccrinales (Trichomycetes) are not fungi, but a clade of protists at the early divergence of animals and fungi. *Mol Phylogenet Evol* 35:21–34.

Campoy S, Perez F, Martin JF, Gutierrez S, Liras P (2003) Stable transformants of the azaphilone pigment-producing *Monascus purpureus* obtained by protoplast transformation and *Agrobacterium*-mediated DNA transfer. *Curr Genet* 43:447–452.

Caramalho R, Maurer E, Binder U, Arau´jo R, Dolatabadi S, Lass-Flo¨rl C, Lackner M (2015) Etest cannot be recommended for *in vitro* susceptibility testing of Mucorales. *Antimicrob Agents Chemother* 59:3663–3665.

Caramalho R, Tyndall JD, Monk BC, Larentis T, Lass-Flörl C, Lackner M (2017) Intrinsic short-tailed azole resistance in mucormycetes is due to an evolutionary conserved aminoacid substitution of the lanosterol 14α-demethylase. *Sci Rep* 7:15898.

Carrier RL, Milletr LA, Ahmed I (2007) The utility of cyclodextrins for enhancing oral bioavailability. *J Control Release* 123:78-99.

Carris LM, Little CR, Stiles CM (2012) Introduction to Fungi. *The Plant Health Instructor* DOI:10.1094/PHI-I-2012-0426-01.

Case ME, Schweizer M, Kushner SR, Giles NH (1979) Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. *Proc Natl Acad Sci USA* 76:5259–5263.

Centre for Disease Control (CDC) USA (last updated at December **2015)** Mucormycosis risk and prevention.

Chang W, Song H, Liu H, Liu P (2013) Current development in isoprenoid precursor biosynthesis and regulation. *Curr Opin Chem Biol* 17:571-579.

Chapman JR, Waldenström J (2015) With reference to reference genes: A systematic review of endogenous controls in gene expression studies. *PLoS One* 10:e0141853.

Chávez-Fumagalli MA, Ribeiro TG, Castilho RO Fernandes SO, Cardoso VN, Coelho CS, Mendonça DV, Soto M, Tavares CA, Faraco AA, Coelho EA, Chen M, Poulter CD (2010) Characterization of thermophilic archaeal isopentenyl phosphate kinases. *Biochem* 49:207–217.

Chayakulkeeree M, Ghannoum MA, Perfect JR (2006) Zygomycosis: the reemerging fungal infection. *Eur J Clin Microbiol Infect Dis* 25:215-229.

Cheng P, Yang Y, Wang L, He Q, Liu Y (2003) WHITE COLLAR-1, a multifunctional *neurospora* protein involved in the circadian feedback loops, light sensing, and transcription repression of *wc-2*. *J Biol Chem* 278:3801–3808.

Collins RJ, Harmon BV, Gobe GC, Kerr JFR (1992) Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int J Radiat Biol* 61:451–453.

Cornely OA, Arikan-Akdagli S, Dannaoui E, Groll AH, Lagrou K et al. (2014) ESCMID and ECMM joint clinical guidelines for the diagnosis and management of mucormycosis. *Clin Microbiol Infect* 20:5–26.

Corrochano LM, Kuo A, Marcet-Houben M, Polaino S, Salamov A, Villalobos-Escobedo JM et al (2016) Expansion of signal transduction pathways in fungi by extensive genome duplication. *Curr Biol* 26:1577-1584.

Covert SF, Kapoor P, Lee M, Briley A, Nairn CJ (2001) *Agrobacterium tumefaciens* mediated transformation of *Fusarium circinatum*. *Mycol Res* 105:259–264.

Csernetics Á, Nagy G, Iturriaga EA, Szekeres A, Eslava AP, Vágvölgyi CS, Papp T (2011) Expression of three isoprenoid biosynthesis genes and their effects on the carotenoid production of the zygomycetes *Mucor circinelloides*. *Fungal Genet Biol* 48:696–703.

Csernetics A, Tóth E, Farkas A, Nagy G, Bencsik O, Vágvölgyi C, Papp T (2015) Expression of *Xanthophyllomyces dendrorhous* cytochrome P450 hydoxylase and reductase in *Mucor circinelloides*. *World J Micobiol Biotecnol* 31:321-336.

Dandan Li, Tang Y, Lin J, Cai W (2017) Methods for genetic transformation of filamentous fungi. *Microb Cell Fact* 16:168.

Danial NN, Korsmeyer SJ, (2004) Cell death: critical control points. *Cell* 116:205–219.

Das A, Yoon SH, Lee SH, Kim JY, Oh DK, Kim SW (2007) An update on microbial carotenoid production: application of recent metabolic engineering tools. *Appl Microbiol Biotechnol* 77:505–512.

de Haro JP, Calo S, Cervantes M, Nicolás FE, Torres-Martínez S, Ruiz-Vázquez RM (2009) A single dicer gene is required for efficient gene silencing associated with two classes of small antisense RNAs in *Mucor circinelloides*. *Eukaryot Cell* 10:1486-1497.

DeGraw AJ, Hast MA, Xu J, Mullen D, Beese LS, Barany G, Distefano MD (2008) Caged protein prenyltransferase substrates, tools for understanding protein prenylation. *Chem Biol Drug Des* 72:171–181.

Dellas N, Thomas ST, Manning G, Noel JP (2013) Discovery of a metabolic alternative to the classical mevalonate pathway. *Elife* 2:e00672.

Dixon DM, Walsh TJ (1996) Antifungal agents. In: Medical Microbiology. 4th edition (ed. Baron S) Galveston (TX): University of Texas Medical Branch at Galveston.

Drogari-Apiranthitou M, Mantopoulou FD, Skiada A, Kanioura L, Grammatikou M, Vrioni G, Mitroussia-Ziouva A, Tsakris A, Petrikkos G (2012) *In vitro* antifungal susceptibility of filamentous fungi causing rare infections: synergy testing of amphotericin B, posaconazole and anidulafungin in pairs. *J Antimicrob Chemother* 67:1937–1940.

Ducrey Sanpietro LM, Kula MR (1998) Studies of astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* (Phaffia rhodozyma). Effect of inhibitors and low temperature. *Yeast* 14:1007–1016.

Dupont S, Lemetais G, Ferreira T, Cayot P, Gervais P, Beney L (2012) Ergosterol biosynthesis: a fungal pathway for life on land. *Evolution* 66:2961–2968.

Eagle H, Musselman AD (1948) The rate of bactericidal action of penicillin *in vitro* as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. *J Exp Med* 88:99.

Eamens A, Wang MB, Smith NA, Waterhouse PM (2008) RNA silencing in plants: yesterday, today, and tomorrow. *Plant physiology* 147:456-468.

Eslava AP, Alvarez MI, Delbrck M (1975) Meiosis in *Phycomyces*. *Pro Natl Acad Sci USA* 72:4076-4080.

Ferreira ME, Colombo AL, Paulsen I, Ren Q, Wortman J, Huang J, Goldman MH, Goldman GH (2005) The ergosterol biosynthesis pathway, transporter genes, and azole resistance in *Aspergillus fumigatus*. *Med Mycol* 43:S313–S319.

Fincham JRS (1989) Transformation in fungi. *Microbio Rev* 53:148–170.

Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M, Morschhäuser J (1998) Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob Agents Chemother* 42:3065–3072.

Fuller KK, Chen S, Loros JJ, Dunlap JC (2015) Development of the CRISPR/Cas9 system for targeted gene disruption in *Aspergillus fumigatus*. *Eukaryot Cell* 14:1073–1080.

Galea AM, Brown AJ (2009) The special relationship between sterols and oxygen: were sterols an adaptation to aerobic life?. *Free Radic Biol Med* 47:880–889.

Galgóczy L, Nyilasi I, Papp T, Vágvölgyi C (2011) Statins as antifungal agents. *World J Clin Infect Dis* 1:4-10.

Garre V, Nicolas FE, Torres-Martínez S, Ruiz-Vazquez RM (2014) The RNAi machinery in Mucorales: the emerging role of endogenous small RNAs. In A. Sesma, and von der Haar T. (Eds.), Fungal RNA biology (pp. 291e313). Cham: Springer International Publishing Switzerland.

Georgopapadakou NH, Bertasso A (1992) Effects of squalene epoxidase inhibitors on *Candida albicans*. *Antimicrob Agent Therap* 36:1779–1781.

Gladieux P, Ropars J, Badouin H, Branca A, Aguileta G, De Vienne DM, Rodriguez de la Vega RC, Branco S, Giraud T (2014) Fungal evolutionary genomics provides insight into the mechanisms of adaptive divergence in eukaryotes. *Mol Ecol* 23:753–773.

Gomes MZ, Lewis RE, Kontoyiannis DP (2011) Mucormycosis caused by unusual mucormycetes, non-*rhizopus*, *Mucor*, and *Lichtheimia* species. *Clin Microbiol Rev* 24:411–445.

Gonzalez-Hernandez GA, Herrera-Estrella L, Rocha-Ramirez V, Roncero MIG, Gutierrez-Corona JF (1997) Biolistic transformation of *Mucor circinelloides*. *Mycological research* 101:953-956.

Gooday GW, Adams DJ (1993) Sex hormones and fungi. *Advances in Microbial Physiology* 34:69-145.

Gordon PA, Stewart PR, Clark-Walker DG (1970) Fatty acid and sterol composition of *Mucor genevensis* in relation to dimorphism and anaerobic growth. *J Bacteriol* 107:114–120.

Gougouli M, Kalantzi K, Beletsiotis E, Koutsoumanis KP (2011) Development and application of predictive models for fungal growth as tools to improve quality control in yogurt production. *Food Microbiol* 28:1453-1462.

Green DR (2005) Apoptotic pathways: ten minutes to dead. *Cell* 121:671–674.

Greenberg RN, Mullane K, van Burik JA, Raad I, Abzug MJ et al (2006) Posaconazole as salvage therapy for zygomycosis. *Antimicrob Agents Chemother* 50:126–133.

Grochowski LL, Xu H, White RH (2006) *Methanocaldococcus jannaschii* uses a modified mevalonate pathway for biosynthesis of isopentenyl diphosphate. *J Bacteriol* 188:3192–3198.

Gutierrez A, Lopez-Garcia S, Garre V (2011) High reliability transformation of the basal fungus *Mucor circinelloides* by electroporation. *J Micribiol Method* 84:442-446.

Gyetvai Á, Emri T, Takács K, Dergez T, Fekete A, Pesti M, Lenkey B (2006) Lovastatin possesses a fungistatic effect against *Candida albicans*, but does not trigger apoptosis in this opportunistic human pathogen. *FEMS Yeast Res* 6:1140-1148.

Harms H, Schlosser D, Wick LY (2011) Untapped potential: exploiting fungi in bioremediation of harzadous chemical. *Nat Rev Microbiol* 9177-9192.

Harrison CJ, Bohm AA, Nelson HCM (1994) Crystal structure of the DNA binding domain of the heat shock transcription factor. *Science* 263:224-227.

He Q, Liu Y (2005) Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. *Genes Dev* 19:2888–2899.

Hemmerlin A, Harwood JL, Bach TJ (2012) A raison d'être for two distinct pathways in the early steps of plant isoprenoid biosynthesis? *Prog Lipid Res* 51:95-148.

Heuston S, Begley M, Gahan CGM, Hill C (2012) Isoprenoid biosynthesis in bacterial pathogens. *Microbiology* 158:1389–1401.

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF et al (2007) A higher-level phylogenetic classification of the fungi. *Mycol Res* 111:509–547.

Hinnen A, Hicks JB, Fink GR (1978) Transformation of yeast. *Proc Natl Acad Sci USA* 75:1929–1933.

Hirano T, Sato T, Yaegashi K, Enei H (2000) Efficient transformation of the edible basidiomycete *Lentinus edodes* with a vector using a glyceraldehyde-3-phosphate dehydrogenase promoter to hygromycin B resistance. *Mol Gen Genet* 263:1047–1052.

Hoffmann K, Voigt K, Kirk PM (2011) Mortierellomycotina subphyl. nov., based on multigene genealogies. *Mycotaxon* 115:353–363.

Hood E (2004) RNAi: What's all the noise about gene silencing?. *Environ Health Perspect* 112:A224–A229.

Howard SJ, Arendrup MC (2011) Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection. *Med Mycol* 49:S90–S95.

Hussein G, Sankawa U, Goto H, Matsumoto K, Watanabe H (2006) Astaxanthin, a carotenoid with potential in human health and nutrition. *J Nat Prod* 69:443–449.

Ibrahim AS, Skory CD (2006) Genetic manipulation of Zygomycetes in Medical mycology: cellular and molecular techniques. (ed. Kavanagh, K.):305–326.

Ibrahim AS, Spellberg B Edwards J (2008) Iron acquisition: A novel prospective on mucormycosis pathogenesis and treatment. *Curr Opin Infect Dis* 21:620-625.

Ibrahim AS, Spellberg B, Walsh TJ, Kontoyiannis DP (2012) Pathogenesis of mucormycosis. *Cin Infect Dis* 54:16-22.

Idnurm A (2011) Sex determination in the first-described sexual fungus. *Eukaryot cell* 10:1485-1491.

Iigusa H, Yoshida Y, Hasunuma K (2005) Oxygen and hydrogen peroxide enhance light-induced carotenoid synthesis in *Neurospora crassa*. *FEBS Lett* 579:4012–4016.

Istvan ES, Deisenhofer J (2001) Structural mechanism for statin inhibition of HMG-CoA reductase. *Science* 292:1160–64.

Iturriaga EA, Velayos A, Eslava AP (2000) The structure and function of the genes involved in the biosynthesis of carotenoids in the Mucorales. *Biotechnol Bioprocess Eng* 5:263–274.

James TY, Kerry O (2004) Zygomycota. Microscopic 'Pin' or 'Sugar' Molds. Version 21 December 2004 (complete) *http://tolweb.org/Zygomycota/20518/2004.12. 12.21 in* The Tree of Life Web Project, *http://tolweb.org/.*

Joseph HT, Hollomon D, Loeffler RS, Kelly SL (1995) Cross-resistance to polyene and azole drugs in *Cryptococcus neoformans*. *Antimicrob Agents Chemother* 39:1526–1539.

Joseph-Horne T, Hollomon DW (1997) Molecular mechanism of azole resistance in fungi. *FEMS Microbiol Lett* 149:141-149.

Jung MK, Ovechkina Y, Prigozhina N, Oakley CE, Oakley BR (2000) The use of -D-glucanase as a substitute for Novozym 234 in immunofluorescence and protoplasting. *Fungal Genet Newslett* 47:65–66.

Kadotani N, Nakayashiki H, Tosa Y, Mayama S (2003) RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. *Mol Plant Microb Interact* 16:769-776.

Karimi K, and Zamini A (2013) *Mucor indicus*: biology and industrial application perspective: a review *Biotechnol Adv* 31:466-481.

Kelly SL, Lamb DC, Taylor M, Corran AJ, Baldwin BC, Powderly WG (1994) Resistance to amphotericin B associated with defective sterol D 8r7 isomerase in a

Cryptococcus neoformans strain from an AIDS patient. *FEMS Microbiol Lett* 122:39–42.

Kraibooj K, Park HR, Dahse HM, Skerka C, Voigt K, Figge MT (2014) Virulent strain of *Lichtheimia corymbifera* shows increased phagocytosis by macrophages as revealed by automated microscopy image analysis. *Mycoses* 57:56–66.

Kuwano T, Shirataki C, Itoh Y (2008) Comparison between polyethylene glycol- and polyethylenimine-mediated transformation of *Aspergillus nidulans*. *Curr Genet* 54:95–103.

Kuzina V, Cerda-Olmedo E (2007) Ubiquinone and carotene production in the Mucorales *Blakeslea* and *Phycomyces*. *Appl Microbiol Biotechnol* 76:991–999.

Kwon-Chung KJ (2012) Taxonomy of fungi causing mucormycosis and entomophthoramycosis (zygomycosis) and nomenclature of the disease: molecular mycologic perspectives. *Clin Infect Dis* 54:8-15.

Lamers PP, Janssen M, De Vos RC, Bino RJ, Wijels RH (2008) Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell factory applications. *Trends Biotechnol* 26:631–638.

Larsen GG, Appel KF, Wolff A (2004) Characterisation of the *Mucor circinelloides* regulated promoter gpd1P. *Curr Genet* 45:225–234.

Lee PC, Schmidt-Dannert C (2002) Metabolic engineering towards biotechnological production of carotenoids in microorganisms. *Appl Microbiol Biotechnol* 60:1–11.

Lee SC, Li A, Calo S, Inoue M, Tonthat NK, Bain JM, Louw J, Shinohara ML, Erwig LP, Schumacher MA, Ko DC, Heitman J (2015) Calcineurin orchestrates dimorphic transitions, antifungal drug responses and host-pathogen interactions of the pathogenic mucoralean fungus *Mucor circinelloides*. *Mol Microbiol* 97:844–865.

Lemke A, Kiderlen AF, Kayser O (2005) Amphotericin B. *Appl Microbiol Biotechnol* 68:151-62.

Lepak AJ, Andes DR (2015) Antifungal Pharmacokinetics and Pharmacodynamics. *Cold Spring Harb Perspect Med* 5:a019653.

Li CH, Cervantes M, Springer DJ, Boekhout T, Ruiz-Vazquez RM, Torres-Martinez, SR, Lee SC (2011) Sporangiospore size dimorphism is linked to virulence of *Mucor circinelloides*. *PLoS pathogens* 7:e1002086.

Li D, Tang Y, Lin J, Cai W (2017) Methods for genetic transformation of filamentous fungi. *Microb Cell Fact* 16:168.

Li L, Chang SS, Liu Y (2010) RNA interference pathways in filamentous fungi. *Cell Mol Life Sci* 67:3849-3863.

Li Y, Wadsö L, Larsson L (2009) Impact of temperature on growth and metabolic efficiency of *Penicillium roqueforti*; correlations between produced heat, ergosterol content and biomass. *J App Microbiol* 106:1494–1502.

Liang PH, Ko TP, Wang AHJ (2002) Structure, mechanism and function of prenyltransferases. *Eur J Biochem* 269:3339-3354.

Liao P, Hemmerlin A, Bach TJ, Chye ML (2016) The potential of the mevalonate pathway for enhanced isoprenoid production. *Biotechnol Adv* 34:697-713.

Lima JO, dos Santos JK, Pereira JF, de Resende ML, de Araújo EF, de Queiroz MV (2003) Development of a transformation system for *Crinipellis perniciosa*, the causal agent of witches' broom in cocoa plants. *Curr Genet* 42:236-240.

Lin X, Momany M (2004) Identification and complementation of abnormal hyphal branch mutants *ahbA1* and *ahbB1* in *Aspergillus nidulans*. *Fungal Genet Biol* 41:998-1006.

Linden H, Ballario P, Macino G (1997) Blue light regulation in *Neurospora crassa*. *Fungal Genet Biol* 22:141-150.

Liu H, Cottrell TR, Pierini LM, Goldman WE, Doering TL (2002) RNA interference in the pathogenic fungus *Cryptococcus neoformans*. *Genetics* 160:463-470.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–408.

Lo Re V, III, Carbonari DM, Lewis JD, Forde KA, Goldberg DS, Reddy KR, Haynes K, Roy JA, Sha D, Marks AR, Schneider JL, Strom BL, Corley DA (2016) Oral azole antifungal medications and risk of acute liver injury, overall and by chronic liver disease status. *Am J Med* 129:283–291.

Lombraña M, Moralejo FJ, Pinto R, Martín JF (2004) Modulation of *Aspergillus awamori* thaumatin secretion by modification of bipA gene expression. *Appl Environ Microbiol* 70:5145-5152.

Long N, Xu X, Zeng Q, Sang H, Lu L (2017) *Erg4A* and *Erg4B* are required for conidiation and azole resistance via regulation of ergosterol biosynthesis in *Aspergillus fumigatus*. *Appl Environ Microbiol* 83:e02924-16.

Lopes FJF, Queiroz MV, Lima JO, Silva VAO, Araújo EF (2008) Restriction enzyme improves the efficiency of genetic transformations in *Crinipellis perniciosa*, the

causal agent of witches' broom disease in *Theobroma cacao*. *Braz Arch Biol Technol* 51:27–34.

Lorenz RT, Parks LW (1990) Effects of lovastatin (mevinolin) on sterol levels and on activity of azoles in *Saccharomyces cerevisiae*. *Antimicrob Agents Chemother* 34:1660–1665.

Loyse A, Dromer F, Day J, Lortholary O, Harrison TS (2013) Flucytosine and cryptococcosis: time to urgently address the worldwide accessibility of a 50-year-old antifungal. *J Antimicrob Chemother* 68:2435–2444.

Lukács Gy, Papp T, Nyilasi I, Nagy E, Vágvölgyi C (2004) Differentiation of *Rhizomucor* species on the basis of their different sensitivities to lovastatin. *J clin microbiol* 42:5400-5402.

MacCallum DM, Coste A, Ischer F, Jacobsen MD, Odds FC, Sanglard D (2010) "Genetic dissection of azole resistance mechanisms in *Candida albicans* and their validation in a mouse model of disseminated infection,"*Antimicrob Agents Chemother* 54:1476–1483.

Maciejak A, Leszczynska A, Warchol I, Gora M, Kaminska J et al (2013) The effects of statins on mevalonic acid pathway in recombinant yeast strains expressing human HMG-CoA reductase. *BMC Biotechnol* 30:13:68.

Majumdar R, Rajasekaran K, Cary JW (2017) RNA Interference (RNAi) as a potential tool for control of mycotoxin contamination in crop plants: concepts and considerations. *Front Plant Sci* 14:200.

Malonek S, Meinhardt F (2001) *Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*. *Curr Genet* 40:152–155.

Makushok T, Alves P, Huisman SM, Kijowski AR, Brunner D (2016) Sterol-rich membrane domains define fission yeast cell polarity. *Cell* 165:1182-1196.

Mans R, van Rossum HM, Wijsman M, Backx A, Kuijpers NG, van den Broek M, Daran-Lapujade P, Pronk JT, van Maris AJ, Daran JM (2015) CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of a multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 15:fov004.

Mantzouridou F, Roukas T, Kotzekidou P (2002) Effect of the aeration rate and agitation speed on beta-carotene production and morphology of *Blakeslea trispora* in a stirred tank reactor: mathematical modeling. *Biochem Eng J* 10:123–135.

Marcoleta A, Niklitschek M, Wozniak A, Lozano C, Alcaíno J, Baeza M, Cifuentes V (2011) Glucose and ethanol-dependent transcriptional regulation of the astaxanthin biosynthesis pathway in *Xanthophyllomyces dendrorhous*. *BMC Microbiol* 11:190.

Marr KA, Lyons CN, Rustad T, Bowden RA, White TC (1998) "Rapid, transient fluconazole resistance in *Candida albicans* is associated with increased mRNA levels of CDR," *Antimicrob Agents Chemother* 42:2584–2589.

Marty FM, Ostrosky-Zeichner L, Cornely OA, Mullane KM, Perfect JR et al (2016) Isavuconazole treatment for mucormycosis: a single-arm open-label trial and case–control analysis. *Lancet Infect Dis* 16:828–837.

Masadeh M, Mhaidat N, Alzoubik K, Alazzams S, Alnasser Z (2012) Antibacterial activity of statins: a comparative study of atorvastatin, simvastatin, and rosuvastatin. *Ann Clin Micro biol Antimicrob* 2012:11-13.

Mehta BJ, Obraztsova IN, Cerdá-Olmedo E (2003) Mutants and intersexual heterokaryons of *Blakeslea trispora* for production of β-carotene and lycopene. *Appl Envir Microbiol* 69:4043-4048.

Mélida H, Sain D, Stajich JE, Bulone V (2015) Deciphering the uniqueness of Mucoromycotina cell walls by combining biochemical and phylogenomic approaches. *Environ Microbiol* 17:1649-1662.

Mertens JA, Skory CD, Ibrahim AS (2006) Plasmids for expression of heterologous proteins in *Rhizopus oryzae*. *Arch Microbiol* 186:41–50.

Mesa-Arango AC, Rueda C, Román E, Quintin J, Terrón MC, Luque D, Netea M, Pla J, Zaragoza O (2016) Cell wall changes in amphotericin B-resistant stain from *Candida tropcalis* and relationship with immune response elicted by the host. *Antimicrob Agents Chemother* 60:2326–2335.

Meyer V (2008) Genetic engineering of filamentous fungi Progress, obstacles and future trends. *Biotechnol Advances* 26:177-185.

Miao L, Chi S, Tang Y, Su Z, Yin T, Guan G, Li Y (2011) Astaxanthin biosynthesis is enhanced by high carotenogenic gene expression and decrease of fatty acids and ergosterol in a *Phaffia rhodozyma* mutant strain. *FEMS Yeast Res* 11:192-201.

Michielse CB, Salim K, Ragas P, Ram AF, Kudla B, Jarry B, Punt PJ, van den Hondel CA (2004) Development of a system for integrative and stable transformation of the zygomycete *Rhizopus oryzae* by *Agrobacterium*-mediated DNA transfer. *Mol Genet Genomics* 271:499–510.

Middleton B (1972) The kinetic mechanism of 3-hydroxy-3-methylglutaryl-coenzyme A synthase from baker's yeast. *Biochem J* 126:35–47.

Mikosch TS, Lavrijssen B, Sonnenberg AS, van Griensven LJ (2001) Transformation of the cultivated mushroom *Agaricus bisporus* (LANGE) using T-DNA from *Agrobacterium tumefaciens*. *Curr Genet* 39:35–39.

Millati R, Edebo L, Taherzadeh MJ (2005) Performance of *Rhizopus*, *Rhizomucor*, and *Mucor* in ethanol production from glucose, xylose, and wood hydrolyzates. *Enzyme Microb Technol* 36:294–300.

Misawa N, Shimada H (1997) Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. *J Biotechnol* 59:169-181.

Miziorko HM, (2011) Enzymes of the mevalonate pathway of isoprenoid biosynthesis. *Arch Biochem Biophys* 505:131–143.

Morace G, Borghi E (2012) Invasive mold infections: virulence and pathogenesis of *Mucorales*. *Int J Microbiol* ID:349278 .

Moralejo FJ, Cardoza RE, Gutierrez S, Lombraña M, Fierro F, Martín JF (2002) Silencing of the aspergillopepsin B (pepB) gene of *Aspergillus awamori* by antisense RNA expression or protease removal by gene disruption results in a large increase in thaumatin production. *Applied Environment Microbiol* 68:3550-3559.

Morelli G, Nelson MA, Ballario P, Macino G (1993) Photoregulated carotenoid biosynthetic genes of *Neurospora crassa*. *Methods Enzymol* 214:412-424.

Mosqueda-Cano G, Gutierrez-Cororna JF (1995) Environmental and developmental regulation of carotenogenesis in the dimorphic fungus *Mucor rouxii*. *Curr Microbiol* 31:141-145.

Mouyna I, Henry C, Doering TL, Latgé JP (2004) Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. *FEMS Microbiol Lett* 237:317-324.

Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathol* 91:173–180.

Muszewska A, Pilsyk S, Perlińska-Lenart U, Kruszewska JS (2017) Diversity of cell wall related proteins in human pathogenic fungi. *J Fungi* 4:6.

Nagy A, Vágvölgyi C, Balla E, Ferenczy L (1994) Electrophoretic karyotype of *Mucor circinelloides*. *Curr Genet* 26:45.

Nagy G, Csernetics Á, Bencsik O, Szekeres A, Vágvölgyi CS, Papp T (2012) Carotenoid composition of Mucorales fungi. *Afr J Microbiol Res* 6:7265–7270.

Nagy G, Farkas A, Csernetics Á, Bencsik O, Szekeres A, Nyilasi I, Vágvölgyi C, Papp T (2014) Transcription of the three HMG-CoA reductase genes of *Mucor circinelloides. BMC Microbiol* 14:93.

Nagy G, Szebenyi C, Csernetics Á, Vaz AG, Tóth EJ, Vágvölgyi C, Papp T (2017) Development of a plasmid free CRISPR-Cas9 system for the genetic modification of *Mucor circinelloides*. *Sci Rep* 7:16800.

Nakayashiki H (2005) RNA silencing in fungi: mechanisms and applications. *FEBS Lett* 579:5950-5957.

Navarro E, Lorca-Pascual JM, Quiles-Rosillo MD, Nicolás FE, Garre V, Torres-Martínez S, Ruiz-Vázquez RM (2001) A negative regulator of light inducible carotenogenesis in *Mucor circinelloides*. *Mol Genet Genomics* 266:463-470.

Nicolás FE, Torres-Martínez S, Ruiz-Vázquez RM, (2003) Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes. *EMBO J* 22:3983–3991.

Nicolas FE, Vila A, Moxon S, Cascales MD, Torres-Martínez S, Ruiz-Vázquez RM, Garre V (2015) The RNAi machinery controls distinct responses to environmental signals in the basal fungus *Mucor circinelloides*. *BMC Genomics* 16:237.

Nielsen JC, Nielsen J (2017) Development of fungal cell factories for the production of secondary metabolites: linking genomics and metabolism. *Synth Syst Biotechnol* 2:5–12.

Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS One* 10:e0133085.

Nyilasi I, Acs K, Papp T, Nagy E, Vagvolgyi C (2005) *Agrobacterium tumefaciens*-mediated transformation of *Mucor circinelloides*. *Folia Microbiologica* 50:415–420.

Nyilasi I, Kocsubé S, Krizsán K, Galgóczy L, Papp T, Pesti M, Nagy K, Vágvölgyi C (2014) Susceptibility of clinically important dermatophytes against statins and different statin-antifungal combinations. *Med Mycol* 52:140-148.

Oh J, Kim JH, Park JG, Yi YS, Park KW, Rho HS, Lee MS, Yoo JW, Kang SH, Hong YD, Shin SS, Cho JY (2013) Radical scavenging activity-based and AP-1-targeted anti-inflammatory effects of lutein in macrophage-like and skin keratinocytic cells. *Mediat Inflamm* 787042.

Oide S, Moeder W, Krasnoff S, Gibson D, Haas H, Yoshioka K, Turgeon BG (2006) NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. *Plant Cell* 18:2836–2853.

Orset S, Young AJ (1999) Low-temperature-induced synthesis of α-carotene in the microalga *Dunaliella salina* (chlorophyta). *J Phycology* 35:520-527.

Pagano L, Cornely OA, Busca A, Caira M, Cesaro S, Gasbarrino C et al. (2013) Combined antifungal approach for the treatment of invasive mucormycosis in patients with hematologic diseases: a report from the SEIFEM and FUNGISCOPE registries. *Haematologica* 98:e127-30.

Palmer CNA, Hsu MH, Griffin KJ, Johnson EF (1995) Novel sequence determinants in peroxisome proliferator signaling. *J Biol Chem* 270:16114-16121.

Papaioannou EH, Liakopoulou-Kyriakides (2012) Agro food waste utilization by *Blackeslea trispora* for carotenoids production. *Acta Biochim Pol* 59:151-153.

Papp T, Csernetics A, Nagy G, Bencsik O, Iturriaga EA, Eslava AP, Vágvölgyi C (2013) Canthaxanthin production with modified *Mucor circinelloides* strains. *Appl Microbiol Biotechnol* 97:4937–4950.

Papp T, Csernetics Á, Nyilasi I, Ábrók M, Vágvölgyi C (2010) Genetic transformation of Zygomycetes fungi. In *Progress in Mycology* (pp. 75-94), Springer, Dordrecht.

Papp T, Velayos A, Bartok T, Eslava AP, Vagvolgyi C, Iturriaga EA (2006) Heterologous expression of astaxanthin biosynthesis genes in *Mucor circinelloides*. *Applied Microbiol Biotechnol* 69:526–531.

Perea S, López-Ribot JL, Kirkpatrick WR, McAtee RK, Santillán RA, Martínez M, Calabrese D, Sanglard D, Patterson TF (2001) "Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients," *Antimicrob Agents Chemother* 45:2676–2684.

Pfaller MA, Diekema DJ (2005) Unusual fungal and pseudofungal infection of humans. *J Clin Mirobiol* 43:1495-504.

Platauf, AP (1885) Mycosis mucorina. *Virchows Arch* 102:543–564.

Prabhu RM, Patel R (2004) Mucormycosis and entomophthoramycosis: a review of the clinical manifestations, diagnosis and treatment. *Clin Micobiol Infect* 1:31-47.

Qi XF, Zheng L, Lee KJ, Kim DH, Kim CS, Cai DQ, Wu Z, Qin JW, Yu YH, Kim SK (2013) HMG-CoA reductase inhibitors induce apoptosis of lymphoma cells by promoting ROS generation and regulating Akt, Erk and p38 signals via suppression of mevalonate pathway. *Cell Death Dis* 28:4:e518.

Redberry GW (2006) Gene silencing: new research, Grace W. Redberry (editor). New York: Nova Science Publishers.

Rep M, Krantz M, Thevelein JM, Hohmann S (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway dependent genes. *J Biol Chem* 275:8290–8300.

Ribes JA, Vanover-Sams CL, Baker DJ (2000) Zygomycetes in human disease *Clin Microbiol Rev* 13:236-301.

Richardson M (2009) The ecology of the Zygomycetes and its impact on environmental exposure. *Clin Microbiol Infect* 5:2-9.

Riley TT, Muzny CA, Swiatlo E, Legendre DP (2016) Breaking the mold: A review of mucormycosis and current pharmacological treatment options. *Ann Pharmacother* 50:747-757.

Ringel AE, Ryznar R, Picariello H, Huang K, Lazarus AG, Holmes SG (2013) Yeast Tdh3 (Glyceraldehyde 3-Phosphate Dehydrogenase) Is a Sir2 Interacting factor that regulates transcriptional silencing and rDNA recombination. *PLoS Genet* 9:e1003871.

Rodriguez-Amaya DB, Kimura M (2004) Harvestplus Handbook for Carotenoid Analysis, HarvestPlus Technical Monograph Series 2; IFPRI: Washington, DC, USA, and CIAT: Cali, Colombia.

Roncero MIG, Jepsen LP, Strøman P, van Heeswijck R (1989) Characterization of a *leu*A gene and an ARS element from *Mucor circinelloides*. *Gene* 84:335-343.

Rosenfeld E, Beauvoit B (2003) Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast* 20:1115–1144.

Roze LV, Linz JE (1998) Lovastatin triggers an apoptosis-like cell death process in the fungus *Mucor racemosus*. *Fungal Genet Biol* 25:119–133.

Ruiz-Diez B, (2002) Strategies for the transformation of filamentous fungi. *J Appl Microbiol* 92:189-195.

Ruiz-Vázquez RM, Nicolás FE, Torres-Martínez S, Garre V (2015) Chapter two-distinct RNAi pathways in the regulation of physiology and development in the fungus *Mucor circinelloides*. *Adv Genet* 91:55–102.

Salas V, Pastor FJ, Calvo E, Alvarez E, Sutton DA, Mayayo E, Fothergill AW, Rinaldi MG, Guarro J (2012) *In vitro* and *in vivo* activities of posaconazole and amphotericin B in a murine invasive infection by *Mucor circinelloides*: poor efficacy of posaconazole. *Antimicrob Agents Chemother* 56:2246–2250.

Sambrook J, Fitsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor.

Sandmann G (1994) Carotenoid biosynthesis in microorganisms and plants. *Eur J Biochem* 223:7-24.

Sanglard D (2002) "Clinical relevance of mechanisms of antifungal drug resistance in yeasts," *Enfermedades Infecciosasy Microbiol Clinica* 20:462–479.

Sant DG, Tupe SG, Ramana CV, Deshpande MV (2016) Fungal cell membrane promising drug target for antifungal therapy. *J Appl Microbiol* 121:1498-1510.

Sautour M, Soares Mansur C, Divies C, Benssousan M, Dantigny P (2002) Comparison of the effects of temperature and water activity on growth rate of food spoilage moulds. *J Ind Microbiol Biotechnol* 28:311–315.

Schachtschabel D, David A, Menzel K-D, Schimek C, Wöstemeyer J, Boland W (2008) Cooperative biosynthesis of trisporoids by the (+) and (−) mating types of the zygomycete *Blakeslea trispora*. *Chembiochem* 9:3004–3012.

Schmidt-Dannert C (2015) Biosynthesis of terpenoid natural products in fungi. *Adv Biochem Eng Biotechnol* 148:19-61.

Schrettl M, Kim HS, Eisendle M, Kragl C, Nierman WC, Heinekamp T, WernerER, Jacobsen I, Illmer P, Yi H, Brakkhage AA, Haas H (2008) SreA-mediated iron regulation in *Aspergillus fumigatus*. *Mol Microbiol* 70:27–43.

Schumacher J (2017) How light affects the life of *Botrytis*. *Fung Genet Biol* 106:26-41.

Schumann U, Ayliffe M, Kazan K, Wang MB (2010) RNA silencing in fungi. *Frontiers Biol* 5:478-494.

Selvakumar D, Miyamoto M, Furuichi Y, Komiyama T (2006) Inhibition of fungal β-1,3-glucan synthase and cell growth by HM-1 killer toxin single-chain anti-idiotypic antibodies. *Antimicrob Agents Chemother* 50:3090–3097.

Seo K, Akiyoshi H, Ohnishi Y (1999) Alteration of cell wall composition leads to amphotericin B resistance in *Aspergillus flavus*. *Microbiol Immunol* 43:1017–1025.

Sheng C, Zhenyuan M, Ji H, Yao J, Wang W, Che X, Dong G, Lu J, Gio W, Zhang W (2009) Three-dimentional model of lanosterol-α-demethylase from *Cryptococcus neoformans*: active-site characterization and insights into azole binding. Antimicrob *Agents Chemother* 53:3487- 3495.

Shetty PK, Sohn HY, Shin KS, Kim E, Seo BI (2000) Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermohyalospora* MTCC 1384. *Curr Sci* 79:1381-1383.

Shields RK, Nguyen MH, Du C, Press E, Cheng S, Clancy CJ (2011) Paradoxical effect of caspofungin against *Candida* bloodstream isolates is mediated by multiple pathways but eliminated in human serum. *Antimicrob Agents Chemother* 55:2641-2647.

Shrivastava S, Chattopadhyay A (2007) Influence of cholesterol and ergosterol on membrane dynamics using different fluorescent reporter probes. *Biochem Biophys Res Commun* 356:705-710.

Silva F, Torres-Martínez S, Garre V (2006) Distinct white collar-1 genes control specific light responses in *Mucor circinelloides*. *Mol Microbiol* 61:1023–1037.

Simister RL, Poutasse CM, Thurston AM, Reeve JL, Baker MC, White HK (2015) Degradation of oil by fungi isolated from Gulf of Mexico beaches. *Mar Pollut Bull* 100:327-333.

Simova ED, Frengova GI, Beshkova DM (2004) Synthesis of carotenoids by *Rhodotorula rubra* GED8 co-cultured with yogurt starter cultures in whey ultrafiltrate. *J Ind Microbiol Biotechnol* 31:115–121.

Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML, Bonito G, Corradi N, Grigoriev I, Gryganskyi A, James TY, O'Donnell K, Roberson RW, Taylor TN, Uehling J, Vilgalys R, White MM, Stajich JE (2016) A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* 108:1028–1046.

Stancu C, Sima A (2001) Statins: mechanism of action and effects. *J Cell Mol Med* 5:78–387.

Stover KR, Cleary JD (2015) The Eagle-like effect of the echinocandins: is it relevant for clinical decisions? *Curr Fungal Infect Rep* 9:88-93.

Sudoh M, Yamazaki T, Masubuchi K, Taniguchi M, Shimma N, Arisawa M, Yamada-Okabe H (2000) Identification of a novel inhibitor specific to the fungal

chitin synthase; inhibition of chitin synthase 1 arrests the cell growth, but that of chitin synthase 1 and 2 is lethal in the pathogenic fungus *Candida albicans*. *J Biol Chem* 275:32901-32905.

Sugar AM (2000) Agents of mucormycosis and related species. In: Mandell, GI, BennettJE, DolinR, eds. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 5th edn. New York: *Churchill Livingstone*; 2685–2695.

Sugar AM, Liu XP (2000) Combination antifungal therapy in treatment of murine pulmonary mucormycosis: roles of quinolones and azoles. *Antimicrob Agent Chemotherap* 44:2004-2016.

Sutter RP (1970) Effect of light on beta-carotene accumulation in *Blakeslea trispora*. *J Gen Microbiol* 64:215-221.

Szkopińska A, Płochocka D (2005) Farnesyl diphosphate synthase: regulation of product specificity. *Acta Biochim Pol* 52:45–55.

Tabata K, Hashimoto SI (2004) Production of mevalonate by a metabolically-engineered *Escherichia coli*. *Biotechnol Lett* 26:1487–1491.

Tanabe Y, Saikawa M, Watanabe MM, Sugiyama J (2004) Molecular phylogeny of Zygomycota based on EF-1 and RPB1 sequences: limitations and utility of alternative markers to rDNA. *Mol Phylogenet Evol* 30:438–449.

Tanabe Y, Watanabe MM, Sugiyama J (2005) Evolutionary relationships among basal fungi (Chytridiomycota and Zygomycota): Insights from molecular phylogenetics. *J General and Applied Microbiol* 51:267–276.

Terry KL, Casey PJ, Beese LS (2006) Conversion of protein farnesyltransferase to a geranylgeranyl transferase. *Biochemistry* 1545:9746-9755.

Torres-Martínez S, Ruiz-Vázquez RM, Garre V, López-García S, Navarro E, Vila A (2012) Molecular tools for carotenogenesis analysis in the zygomycete *Mucor circinelloides. Methods Mol Biol* 898:85–107.

Trieu TA, Navarro-Mendoza MI, Pe´rez-Arques C, Sanchis M, Capilla J, et al. (2017) RNAi-based functional genomics identifies new virulence determinants in mucormycosis. *PLOS Pathog* 13:e1006150.

Tudzynski B, K Hölter (1998) Gibberellin biosynthetic pathway in *Gibberella fujikuroi*: evidence for a gene cluster. *Fungal Genet Biol* 25:157-170.

Ukibe K, Hashida K, Yoshida N, Takagi H (2009) Metabolic engineering of *Saccharomyces cerevisiae* for astaxanthin production and oxidative stress tolerance. *Appl Environ Microbiol* 75:7205–7211.

Valcarce G, Munoz L, Nusblat A, Nudel C, Florin-Christensen J (2001) The improvement of milk by cultivation with ciliates. *J Dairy Sci* 2001 84:2136-2143.

Van de Veerdonk FL, Kullberg BJ, Netea MG (2010) Pathogenesis of invasive candidiasis. *Curr Opin Crit Care* 16:453-464.

van der Linden, JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJ, Verweij PE (2011) Clinical implications of azole resistance in *Aspergillus fumigatus*. *Emerg Infect Dis* 17:1846–1854.

van-Heeswijck R, Roncero MIG (1984) High frequency transformation of *Mucor* with recombinant plasmid DNA. *Carlsberg Res Commun* 49:691-702.

Vandeputte P, Ferrari S, Costa AT (2012) Antifungal resistance and new strategies to control fungal infections. *Int J Microbiol* 2012:713687.

Vastag M, Kasza Z, Ács K, Papp T, Schwab H, Vágvölgyi C (2004) Cloning and sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase gene from the zygomycetes fungus *Rhizomucor miehei. Antonie van Leeuwenhoek* 86:111–119.

Vaupotic T, Plemenitas A (2007) Osmoadaptation-dependent activity of microsomal HMG-CoA reductase in the extremely halotolerant black yeast *Hortaea werneckii* is regulated by ubiquitination. *FEBS Lett* 581:3391–3395.

Vaupotič T, Veranic P, Petrovič U, Gunde–Cimerman N, Plemenitaš A (2008) HMG-CoA reductase is regulated by environmental salinity and its activity is essential for halotolerance in halophilic fungi. *Stud Mycol* 61:61–66.

Velayos A (2000) Carotenogenesis en *Mucor circinelloides*. PhD Thesis. Universidad de Salamanca, Salamanca, Spain.

Velayos A, Blasco JL, Alvarez MI, Iturriaga EA, Eslava AP (2000a) Blue-light regulation of the phytoene dehydrogenase (*carB*) gene expression in *Mucor circinelloides*. *Planta* 210:938-946.

Velayos A, Eslava AP, Iturriaga EA (2000b) A bifunctional enzyme with lycopene cyclase and phytoene synthase activities is encoded by the *carRP* gene of *Mucor circinelloides. Eur J Biochem* 267:1-12.

Velayos A, Fuentes-Vicente M, Aguilar-Elena R. Eslava AP. Iturriaga EA (2004) A novel fungal prenyl diphosphate synthase in the dimorphic zygomycete *Mucor circinelloides. Curr Genet* 45:371-377.

Velayos A, Papp T, Aguilar-Elena R, Fuentes-Vicente M, Eslava AP, Iturriga EA, Alvarez MI (2003) Expression of the *carG* gene, encoding geranylgeranyl

pyrophosphate synthase, is upregulated by blue light in *Mucor circinelloides. Curr Genet* 43:112-120.

Vinokur JM, Korman TP, Cao Z, Bowie JU (2014) Evidence of a novel mevalonate pathway in archaea. *Biochemistry* 53:4161–4168.

Walker LA, Gow NA, Munro CA (2010) Fungal echinocandin resistance. *Fungal Genet Biol* 47:117–126.

Wang GY, Keasling JD (2002) Amplification of HMG-CoA reductase production enhances carotenoid accumulation in *Neurospora crassa*. *Metab Eng* 4:193–201.

Wang L, Lin X (2012) Morphogenesis in fungal pathogenicity: shape, size, and surface. *PLoS Pathog* 8:e1003027.

Warrilow AG, Melo N, Martel CM, Parker JE, Nes WD, Kelly SL, Kelly DE (2010) Azole Binding Properties of *Candida albicans* Sterol 14- œ Demethylase (CaCYP51). *Antimicrob Agents Chemother* 54:4225.

Wawrzyn GT, Bloch SE, Schmidt-Dannert C (2012) Discovery and characterization of terpenoid biosynthetic pathways of fungi. *Methods Enzymol* 515:83-105.

Weete JD, Abril M, Blackwell M (2010) Phylogenetic distribution of fungal sterols. *PLoS ONE* 5:e10899.

Weld RJ, Plummer KM, Carpenter MA, Ridgway HJ (2006) Approaches to functional genomics in filamentous fungi. *Cell Research* 16:31-44.

Werner N, Gómez M, Baeza M, Cifuentes V, Alcaíno J (2016) Functional characterization of thiolase-encoding genes from *Xanthophyllomyces dendrorhous* and their effects on carotenoid synthesis. *BMC Microbiol* 16:278.

Whittaker RH (1969) New concepts of kingdoms of organisms. *Science* 163:150–160.

Wolff AM, Arnau J (2002) Cloning of glyceraldehyde-3-phosphate dehydrogenase-encoding genes in *Mucor circinelloides* (Syn. racemosus) and use of the gpd1 promoter for recombinant protein production. *Fungal Genet Biol* 35:21-29.

Yang J, Xian M, Su S, Zhao G, Nie Q, Jiang X, Zheng Y (2012) Enhancing production of bio-isoprene using hybrid MVA pathway and isoprene synthase in *E. coli. PLoS ONE* 7 e33509.

Yasmin S, Alcazar-Fuoli L, Grundlinger M, Puempel T, Cairns T, Blatzer M, Lopez JF, Grimalt JO, Bignell E, Haas H (2011) Mevalonate governs inter dependency of ergosterol and siderophore biosynthesis in the fungal pathogen *Aspergillus fumigatus*. *Proc Natl Acad Sci USA* 109:E497-E504.

Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol* 208:2819-2830.

Yelton MM, Hamer JE, Timberlake WE (1984) Transformation of *Aspergillus nidulans* by using a trpC plasmid. *Proc Natl Acad Sci USA* 81:1470–1474.

Zhang DH, Jiang LX, Li N, Yu X, Zhao P, Li T, Xu JW (2017) Overexpression of the squalene epoxidase gene alone and in combination with the 3-hydroxy-3-methylglutaryl coenzyme A gene increases ganoderic acid production in *Ganoderma lingzhi*. *J Agr Food Chem* 65:4683-4690.

Zhang Y, Eusebio Navarro, José T, Cánovas-Márquez, Almagro L, Chen H, Chen YQ, Zhang H, Torres-Martínez S, Chen W, Garre V (2016) A new regulatory mechanism controlling carotenogenesis in the fungus *Mucor circinelloides* as a target to generate β-carotene over-producing strains by genetic engineering. *Microb Cell Fact* 15:99.

Zhang Y, Luan X, Zhang H, Garre V, Song Y, Ratledge C (2017) Improved γ linolenic acid production in *Mucor circinelloides* by homologous overexpressing of delta-12 and delta-6 desaturases. *Microb cell Fact* 16:113.

9. ACKNOWLEDGEMENT

To complete a project of such magnitude requires a network of support and I am obliged to many people and would like to thank all of them, but to that Almighty first, without whose blessings nothing is possible.

Word thankful is a piece of my deep gratitude towards my supervisor Prof. Dr. Csaba Vágvölgyi, Head of the Department of Microbiology, Faculty of Science and Informatics, University of Szeged (Hungary Europe), for his constant support, excellent guidance and positive outlook in science as well as in life. His broad knowledge, dynamic and friendly nature inspired me to develop scientific temper. He has provided with independence, encouragement and motivation throughout the course of this work. His time, patience and scientific discussion were assets to my research and his opinion goes beyond any measurable value.

I am extremely thankful to Dr. Tamás Papp and Dr. Árpád Csernetics, for his guidance to plan experiment and make them successfully. I always try to be like him constant inspiration, scientific interest, great insight and guidance throughout my research. His patience and willingness to always help me will be remembered with sincere gratitude. I am also thankful to Dr. Ildikó Nyilasi and Dr. Gábor Nagy (Department of Microbiology, Faculty of Science and Informatics, University of Szeged) for their support, advice and suggestions. I thank all my lab members Ms. Eszter J. Tóth, Ms. Tünde Kartali, Ms. Amanda Grace Vaz, Ms. Csilla Szebenyi, and Mr. Olivér Jáger for their help, support and providing a delightful environment during the long hours in the lab.

During my studies on functional characterization of the mevalonate-isoprenoid biosynthesis pathway genes in filamentous fungi, Dr. Árpád Csernetics and Dr. Gábor Nagy deserve my special thank for thier inspiration, moral support, and friendly nature. They helped me in many ways to complete my dissertation. I would also like to thank to all of the departmental colleagues and staffs for their support.

I would like to thank my lovely wife Mrs. Sariks Singh and my dearest colleagues and friends Mr. Tanmoy Chakravarty, Mr. Dhirendra Singh, Mr. Kabi Chandran Singh, and Payal Chakravarty, for their help, support and friendship throughout my studies.

I would like to thank jointly The University Grant Commission New Delhi India, and Stipendium Hungaricum Tempus Public Foundation Hungary, for awarding me the fellowship in form of Doctoral Research Fellowship.

10. SUMMARY

Members of the subdivision Mucoromycotina, order Mucorales (such as *Lichtheimia, Mucor, Rhizomucor* and *Rhizopus* species) are saprotrophic fungi, which also have medical, industrial, biotechnological and agricultural importance. Some species may cause post-harvest damages in agriculture; some members are used as producers of extracellular enzymes, organic acids and carotenoids. Several species belonging to this fungal group are also considered to opportunistic pathogens, which can cause fatal systemic infections (so-called zygomycosis or mucormycosis) in immunocompromised patients. Today ergosterol and its biosynthesis is the major target of the antimycotic agents used in clinics to treat infections caused by Mucoromycotina fungi. The therapy of mucormycosis is still limited because of the intrinsic resistance of these fungi to the majority of the currently used antimycotics (such as azoles).

Metabolites synthesized via the mevalonate-isoprenoid pathway (such as sterols, functional groups of proteins and carotenoids) play an important role in signal transduction, morphogenesis, adaptation to environmental changes and protection against free radicals. In the pathway three molecules of acetyl-CoA are condensed by 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase to form HMG-CoA, which is then reduced to mevalonate by HMG-CoA reductase. Next, conversion of mevalonate to isopentenyl pyrophosphate (IPP, which is the binding block of all isoprenoids) is catalyzed by three enzymes, mevalonate kinase, mevalonate-5-phosphate kinase and diphosphomevalonate decarboxylase. In the isoprenoid pathway, formation of dimethylallyl pyrophosphate (DMAPP) is catalyzed by IPP isomerase, followed by condensation of IPP blocks, result in elongation of the prenyl chain. These steps are managed by prenyltransferases, such as farnesyl- (FPP) and geranylgeranyl pyrophosphate (GGPP) synthases, and form the intermediate geranyl pyrophosphate (GPP), FPP and GGPP. FPP and GGPP are the precursors of ergosterol, carotenoids and functional groups of farnesylated and geranylgeranylated proteins.

To date limited information is available about the function and regulation of the mevalonate-isoprenoid biosynthesis pathway genes in Mucoromycotina fungi. Thus, our aim was to characterize six genes of the *M. circinelloides* mevalonate-isoprenoid pathway, encoding the HMG-CoA synthase (*hmgS*), mevalonate kinase (*mvk*), diphosphomevalonate decarboxylase (*dmd*), IPP isomerase (*ipi*), FPP synthase (*isoA*) and GGPP synthase (*carG*). One of our goals was to improve the carotenoid production of the fungus with modification

of the mevalonate-isoprenoid pathway. Our next aim was to examine the effect of the overexpression and silencing of the genes on the ergosterol content of *M. circinelloides* and it's effect on the susceptibility to different antifungals, which may lead to identify targets for new antifungal therapy. Little is known about the efficiency of gene silencing can be achieved with different plasmid constructs in *M. circinelloides*, thus among our aims was to compare the characteristics of the mutants harbouring different plasmids for gene knockdown as well.

The following specific objectives have been addressed:

1. Investigation of the transcription of selected six genes under different cultivation conditions involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides.*

Effect of cultivation conditions, such as temperature, oxygen tension, light sources, medium composition and incubation time on the transcription of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes.

2. Overexpression and silencing of six genes involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides* and characterization of the transformants

Development of different plasmid constructs for overexpression and silencing of the *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes in *M. circinelloides.* Transformation experiments with the *M. circinelloides* MS12 strain. Characterization of the transformants in detail, such as analyzes of micromorphology, carotenoid and ergosterol content, antifungal susceptibility and interaction with macrophages. Comparison of the overexpression and gene silencing efficiency achieved with the different plasmid constructs.

Results

1. Transcription of six genes under different cultivation conditions involved in the mevalonate-isoprenoid biosynthesis in *M. circinelloides.*

Before the present thesis, our research group designed primers to study the transcription of *hmgS*, *mvk*, *dmd*, *ipi*, *isoA* and *carG* genes. The *M. circinelloides* MS12 strain was cultivated under different cultivation conditions, such as on different media composition, different temperature, light conditions, oxygen tension, which was followed by RNA extraction and cDNA synthesis; the relative transcription levels was analyzed with qPCR. The transcription of six mevalonate-isoprenoid pathway genes was compared

to each other. The fungus was cultivated in liquid or on solid YNB medium for four days. In both cases, the isoprenoid pathway genes (*ipi*, *isoA* and *carG*) showed higher transcription level than mevalonate pathway genes (*hmgS*, *mvk* and *dmd*), moreover we found that *mvk* showes the lowest, while *ipi* the highest transcription level. Light was also played key role and reduced the transcription level when the fungus was cultivated in continuous dark, in comparison with cultures cultivated in continuous light. The short light exposure increased significantly the transcription of *carG*. The *isoA* showed higher transcipt level, when *M. circinelloides* MS12 was cultivated under warm white (contains more components in the yellow-red wavelength range), than under daylight (contains more components in the blue wavelength range) source; while the *carG* showed higher transcription level under daylight condition. Previous study verified that blue light induce the transcription of *carG*, moreover our findings presume that red color has similar effect to the transcription of *isoA. M. circinelloides* is a dimorphic fungus, which can grow in yeast-like morphology under anaerobic conditions. All selected six genes showed an increased transcription level upon cultivation under anaerobic conditions in comparison with cultivation under aerobic conditions. Interestingly, anaerobic followed by one hour aerobic growth increased the transcription of *dmd* significantly and decreased the transcription of *carG* compared to cultures under continuous anaerobic condition, which presume the importance of these genes in the biosynthesis of isoprenoids and metabolites playing role in morphological switch. *M. circinelloides* grows intensively on 25 °C. Growth temperature at 35 °C increased the transcription of all six genes, moreover similar effect was observed at suboptimal temprerature at most of the genes in comparison with the control conditions (25 °C). Genes showed the highest transcription level at four hours and eight hours postinoculation, except *carG*, which showed the highest transcription in 96 hours old cultures. The increased glucose concentration generally resulted decrease in most of the gene transcription, while DHA (in previous studies modified and increased the carotenoid production of mutant *M. circinelloides* strains) significantly reduced the transcription of *hmgS* and *mvk* and increased that of *ipi* in comparison with cultivation on glucose. Addition of NaCl to the media generally did not changed the transcription of the genes significantly, at the same time fluvastatin significantly increased the transcription of all six genes in comparison with the control.

2. Construction of plasmids for overexpression and silencing of *M. circinelloides* six genes involved in the mevalonate-isoprenoid biosynthesis and transformation experiments.

For overexpression and silencing of the *hmgS, mvk, dmd, ipi, isoA* and *carG* genes five plasmid were constructed for all six genes. For overexpression the genes were amplified with their own promoter and terminator regions or were placed under the control of the *M. circinelloides* glyceraldehyde-3-phosphate dehydrogenase (*gpd1*) promoter and terminator (*own* and *gpd* plasmid constructs, respectively). For gene silencing three different plasmid were constructed for all six genes: (1) the genes were inserted between the *Mucor gpd1* and *zrt1* promoter regions (*pMAT* constructs); (2) a fragment of a gene was inserted between the *Mucor gpd1* promoter and terminator in inverted orientation (*as* constructs); (3) a fragment of a gene and its reverse complement together with an intron were placed under the regulation of the *gpd1* promoter and terminator (*hpRNA* constructs). The *Mucor pyrG* and *leuA* genes were used as selection markers, which complement the uracil and the leucine auxotrophy, respectively. Successful transformation of *M. circinelloides* MS12 strain was carried out with the plasmids; the mutants were selected based on the auxotrophy complementation.

The presence of the plasmids in the mutants was verified with PCR. The plasmid copy number was investigated in the mutants and found as to be 0.3–10 copy/genom; at the same time fluctuation in the copy number was observed. The relative transcription levels of the overexpressed and silenced genes were also analyzed. We observed an increase in the relative transcription level of the overexpressed genes, which was more prominent in those transformants, harbouring the genes in extra copies under the control of the *gpd1* promoter and terminator, while gene silencing was most effective when the *hpRNA* and *as* constructs were used. Significant decrease was found in the colony forming unit in all transformants, including those which harbour the plasmids with the *pyrG* and *leuA* genes, in comparison with *M. circinelloides* MS12, which can be due to mitotic instability of the plasmids. This was more prominent in transformant harbouring the *hpRNA* plasmids. In case of several transformants (mainly in four days or older colonies) increased number of hyphal branching and cytoplasmic effusion was observed.

Overexpression of all six genes (primary the *mvk, ipi* and *carG*) increased the carotenoid content, while silencing of mevalonate pathway genes decreased that in comparison with the wild type. Similarly, significant decrease was found in the ergosterol content with silencing of the genes with *hpRNA* plasmids. Antifungal susceptibility of the

mutants was also investigated. Primarily modification of the *dmd* and *ipi*, responsible for the formation and isomerization of IPP, respectively, resulted significant difference in the susceptibility to azoles and statins in comparison with *M. circinelloides* MS12. Overexpression of the *hmgS*, *ipi* and *carG* and silencing of the *mvk* and *isoA* resulted decrease in phagocytic indexes, while overexpression of the *mvk* and *dmd* increased phagocytic indexes.

11. ÖSSZEFOGALÁS

A Mucormycotina altörzs Mucorales rendjébe tartozó gombák (pl. *Lichtheimia, Mucor, Rhizomucor* és *Rhizopus* fajok) elsősorban szaprotrófok, amik ipari, mezőgazdasági és klinikai szempontból is nagy jelentőséggel bírnak. A rend egyes tagjai raktári kártevők, mezőgazdasági termények károsítói lehetnek; másokat extracelluláris enzimek, szerves savak és karotinoidok előállítására használnak az iparban. A csoportba tartozó számos faj ismert, mint opportunista humánpatogén gomba, amik halálos kimenetelű gombafertőzések (ún. zigomikózisok vagy mukormikózisok) kórokozói lehetnek immunszuppresszált betegekben. A klinikumban a mukormikózisok kezelésére alkalmazott antifungális szerek leggyakoribb támadáspontja az ergoszterin és annak bioszintézise, ugyanakkor a mukormikózist okozó gombák többsége rezisztens a legtöbb ma alkalmazott antifungális szerre (így pl. azolokra).

A mevalonsav-izoprén bioszintézis útvonalon képződő metabolitok (így pl. szterinek, karotinoidok, egyes fehérjék funkciós csoportjai) fontos szerepet játszanak a jelátviteli folyamatokban, a morfogenezisben, a megváltozott környezeti feltételekhez történő alkalmazkodásban és a szabadgyökök elleni védelemben. Az útvonal kezdeti szakaszában a HMG-KoA szintáz általt katalizált reakció során három acetil-koenzim A kondenzációjával jön létre a 3-hidroxi-3-metilglutaril-koenzim A (HMG-KoA), ami a HMG-KoA reduktáz hatására mevalonsavvá alakul. Ezt követően a mevalonsav a mevalonsav kináz, foszfomevalonsav kináz és a difoszfoszfomevalonsav dekarboxiláz által katalizált reakcióban izopentenil pirofászfáttá (IPP) alakul, azon öt szénatomos egységgé, ami minden izoprénvázas vegyület alap építőmolekulája. A bioszintézis izoprén szakaszában egy izomerizációs lépés során jön létre a dimetilallil pirofoszfát (ezt a lépést az IPP izomeráz katalizálja), majd a poliprenil transzferázok katalizálta reakcióban IPP egységek beépítésével hosszabbodik a lánc. Ennek során a farnezil pirofoszfát (FPP) és geranilgeranil pirofoszfát (GGPP) szintáz enzimek katalízise révén jön létre a geranil pirofoszfát, farnezil pirofoszfát (FPP) és geranilgeranil pirofoszfát; utóbbiak gombákban a prekurzorai az ergoszterin, karotinoidok, farnezilált és geranilgeranilált fehérjék funkciós csoportjai képződésének.

Kevés ismerettel rendelkezünk a Mucoromycotina gombák mevalonsav-izoprén bioszintézisében szerepet játszó gének szabályozásáról és az egyes metabolitok bioszintézisében betöltött szerepéről. Ezért célul tűztük ki, hogy jellemezzük a *M. circinelloides* ezen bioszintézis útvonalában szerepet játszó géneket, köztük a HMG-KoA

szintázt (*hmgS*), mevalonsav kinázt (*mvk*), difoszfomevalonsav dekarboxilázt (*dmd*), IPP izomerázt (*ipi*), FPP szintázt (*isoA*) és a GGPP szintázt (*carG*) kódoló géneket. Céljaink között szerepelt továbbá, hogy a bioszintézis útvonal módosításával karotinoid túltermelő mutáns törzseket hozzunk létre, valamint megvizsgáljuk a gének túlműködtetésének és csendesítésének hatását a gomba ergoszterin tartalmára és antifungális szerekkel szemben mutatott érzékenységére, ami a későbbiekben hozzájárulhat új antifungális terápiák kifejlesztéséhez. Célul tűztük ki továbbá azt is, hogy megvizsgáljuk a különböző plazmidkonstrukciókkal megvalósítható géncsendesítés hatékonyságát *M. circinelloides*-ben.

A munkánk során a következő konkrét célokat fogalmaztuk meg:

1. A *M. circinelloides* mevalonsav-izoprén bioszintézisében szerepet játszó gének transzkripciójának vizsgálata különböző tenyésztési körülmények mellet.

Különböző környezeti tényezők, így pl. az inkubációs idő, hőmérséklet, oxigéntenzió, fényforrás, táptalaj összetétel hatása a *hmgS, mvk, dmd, ipi, isoA* és *carG* gének transzkripciójára.

2. A *M. circinelloides* mevalonsav-izoprén bioszintézisében résztvevő hat gén túlműködtetése és csendesítése, valamint a transzformánsok jellemzése.

Különböző plazmidkonstrukciók létrehozása a *hmgS, mvk, dmd, ipi, isoA* és *carG* gének túlműködtetéséhez és csendesítéséhez. Transzformációs kísérletek a *M. circinelloides* MS12 törzzsel. A mutáns törzsek részletes jellemzése, így pl. a mikromorfológia, karotin- és ergoszterin tartalom, antifungális szerekkel szembeni érzékenység, valamint makrofágokkal történő interakció vizsgálata. A különböző plazmidkonstrukciókkal elérhető túlműködtetés és géncsendesítés hatékonyságának összehasonlítása.

Eredmények

1. A *M. circinelloides* mevalonsav-izoprén bioszintézisében szerepet játszó gének transzkripciójának vizsgálata különböző tenyésztési körülmények mellet.

Jelen doktori munkát már megelőzően a tanszéki kutatócsoportunk primereket tervezett a *M. circinelloides* MS12 törzs *hmgS, mvk, dmd, ipi, isoA* és *carG* gének transzkripciójának vizsgálatához. Az MS12 törzset különböző körülmények között (így pl. különböző összetételű táptalajon, különböző hőmérsékleten, eltérő fényforrás és oxigéntenzió mellett) tenyésztettük, majd RNS tisztítást és cDNS szintézist követően

kvantitatív valós-idejű PCR segítségével megvizsgáltuk a gének transzkripciós szintjét. Megállapítottuk, hogy négy nap tenyésztést követően, mind folyadéktenyészetben, mind szilárd táptalajon, a mevalonsav útvonalban résztvevő *hmgS*, *mvk* és *dmd* gének alacsonyabb transzkripciós szintet mutatnak, mint az *ipi*, *isoA* és *carG*. Az *mvk* gén esetében határoztuk meg a legalacsonyabb, míg az *ipi* esetében a legmagasabb relatív transzkripciós szintet. Megállapítottuk, hogy az állandó megvilágítás pozitívan hat a gének transzkripciójára a sötétben történő neveléshez képest, továbbá, hogy a sötétben történő nevelést követő rövid idejű megvilágítás különösen a *carG* gén transzkripciójára van hatással. Az alacsonyabb színhőmérsékletű, a sárga-vörös hullámhossz tartományban intenzívebb warm white fényforrás az *isoA*, míg a magasabb színhőmérsékletű, a kék tartományban intenzívebb daylight fényforrás a *carG* gén transzkripciójára volt pozitív hatással. Korábban már igazolták, hogy a *carG* gén transzkripcióját a kék fény indukálja, ugyanakkor eredményeink szerint a vörös fény az *isoA* kifejeződésére van hasonló hatással. A *M. circinellodies* egy dimorf gomba, vagyis bizonyos körülmények között (így pl. anaerob tenyésztés esetén) az amúgy fonalas gomba élesztőszerű növekedésre képes. Anaerob körülmények között mind a hat gén esetében magasabb transzkripciót mértünk, mint aerob körülmények között, továbbá az anaerob körülmények között történő tenyésztést követő, egy órán át tartó aerob nevelés fokozta a *dmd*, valamint csökkentette a *carG* transzkripcióját, ami feltételezi ezen gének fontos szerepét a morfológiai váltásban szerepet játszó metabolitok bioszintézisében. A gomba legintenzívebben 25 °C-on nő. 35 °C-on mindegyik, illetve az optimálistól alacsonyabb hőmérsékleten a legtöbb gén esetében magasabb transzkripciót mértünk, mint 25 °C-on. A tenyésztési idő tekintetében, a *carG* gén kivételével, a leoltást követő négy és nyolc óra után mértük a legmagasabb relatív transzkripciós szinteket, míg a *carG* esetében ez négy nap tenyésztést követően volt megfigyelhető. A glükózkoncentráció fokozása általában csökkentette a gének kifejeződését, míg a különböző szénforrások közül a dihidroxi-aceton (amely korábbi vizsgálatokban fokozta, illetve módosította egyes mutáns *M. circinelloides* törzsek karotinoid termelését) az *ipi* és *isoA* gének kifejeződését fokozta, míg a *hmgS* és *mvk* génekét csökkentette a glükózhoz képest. Míg a különböző koncentrációban alkalmazott NaCl általában nem, ugyanakkor a fluvasztatin szignifikánsan növelte a gének transzkripcióját a kontrollhoz viszonyítva.

2. Plazmidok létrehozása a *M. circinelloides* mevalonsav-izoprén bioszintézisében résztvevő hat gén túlműködtetéséhez és csendesítéséhez, valamint a gomba transzformációja a plazmidokkal

A *hmgS, mvk, dmd, ipi, isoA* és *carG* gének túlműködtetéséhez és csendesítéséhez öt-öt plazmidot hoztunk létre. A gének túlműködtetéséhez azokat saját szabályozó régióikkal szaporítottuk fel vagy a *M. circinelloides* glicerinaldehid-3-foszfát dehidrogenáz 1 (*gpd1*) promóter és terminális régiók szabályozása alá helyeztük (*own* és *gpd* konstrukciók). A gének csendesítéséhez három konstrukciót hoztunk létre: (1) a géneket az egymással szemben elhelyezkedő *M. circinelloides gpd1* és *zrt1* promóter régiói közé építettük (*pMAT* konstrukciók); (2) a gének egy szakaszát a *gpd1* promóter és terminális régiók közé építettük fordított orientációban (*as* konstrukciók); (3) a gének egy szakaszát, valamint annak reverz komplementerét egy intronnal együtt építettük a *gpd1* szabályozó régiók közé (*hpRNA* konstrukciók). A plazmidok az uracil vagy a leucin auxotrófia komplementálásáért felelős *M. circinelloides pyrG* vagy *leuA* szelekciós markergéneket hordozták. A plazmidokkal sikeresen transzformáltuk a *M. circinelloides* MS12 törzsét, a transzformánsokra az auxotrófia komplementáció alapján szelektáltunk.

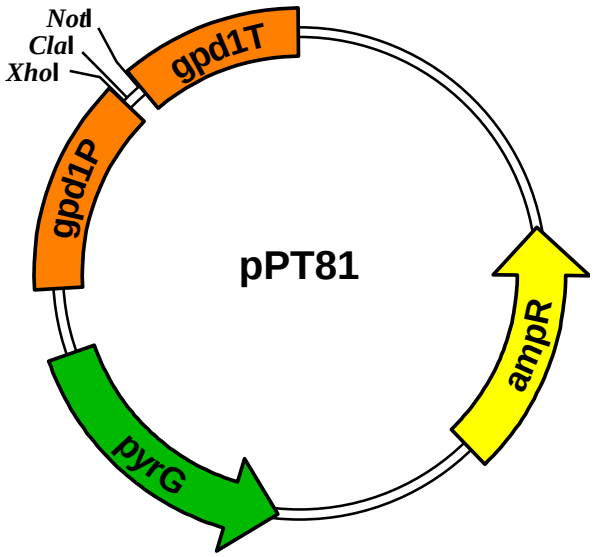
A létrehozott mutánsokat részletesen jellemeztük. Igazoltuk, hogy a bejuttatott plazmidok jelen vannak a gombában. Megállapítottuk, hogy azok relatív kópiaszáma 0,3 és 10 kópia/genom között mozog, ugyanakkor az átoltások során ingadozást találtunk a plazmidok kópiaszámában. Megvizsgáltuk a gének transzkripciójában bekövetkezett változásokat. Megállapítottuk, hogy a gének túlműködtetése általában akkor hatékonyabb, amikor a gének a *gpd1* promóter szabályozása alatt állnak, a gének csendesítése pedig a *hpRNA* és *as* konstrukciók alkalmazása esetén bizonyult a leghatékonyabbnak. Megállapítottuk, hogy az MS12 törzshöz képest minden transzformáns esetében, így a leucin és uracil auxotrófia komplementálásáért felelős géneket tartalmazó plazmidokat hordozó transzformánsok esetében is, szignifikáns csökkenés tapasztalható a spórák telepképző képességében, ami feltehetőleg a plazmidok mitótikus instabilitásából eredhet. A gének *hpRNA* konstrukciókkal történő csendesítése esetében ez a csökkenés még nagyobb mértékű volt. Számos transzformáns esetében, főként idősebb tenyészeteknél, a hifa elágazások és citoplazma kiáramlások számának növekedését tapasztaltuk.

A gének túlműködtetése (különösen az *mvk, ipi* és *carG* gének) a karotintermelés fokozódását, továbbá a mevalonsav gének csendesítése annak csökkenését eredményezte, valamint csökkenést tapasztaltunk az ergoszterin tartalomban a gének *hpRNA* konstrukciókkal történő csendesítése során. Megvizsgáltuk a mutánsok antifungális

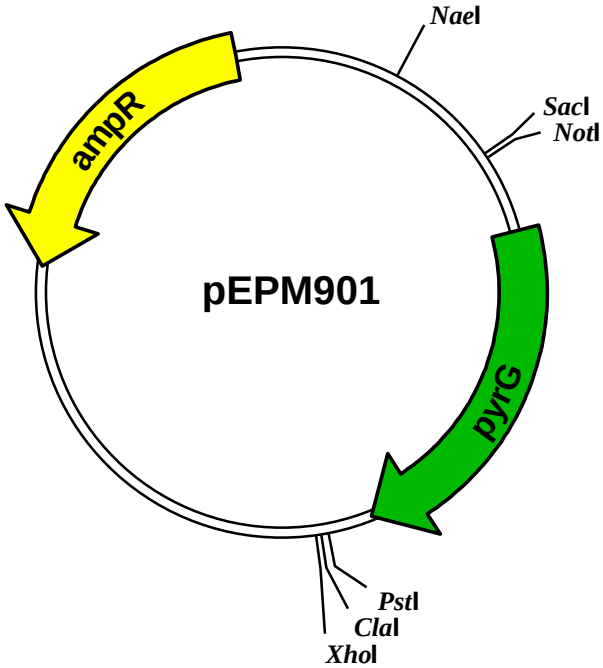
szerekkel szembeni érzékenységét is. Megállapítottuk, hogy különösen az izoprén út kezdeti szakaszában résztvevő, az IPP szintéziséért és izomerizációjáért felelős *dmd* és *ipi* gének módosítása eredményezett jelentős változást a gomba azolokkal és sztatinokkal mutatott érzékenységében. Megvizsgáltuk továbbá a mutánsok MH-S makrofágokkal történő interakcióját, és megállapítottuk, hogy a *hmgS, ipi* és *carG* gének túlműködtetése, valamint az *mvk* és *isoA* csendesítése a fagocitózis index csökkenését, míg az *mvk* és *dmd* túlműködtetése annak növekedését eredményezte.

12. SUPPLEMENTARY MATERIALS

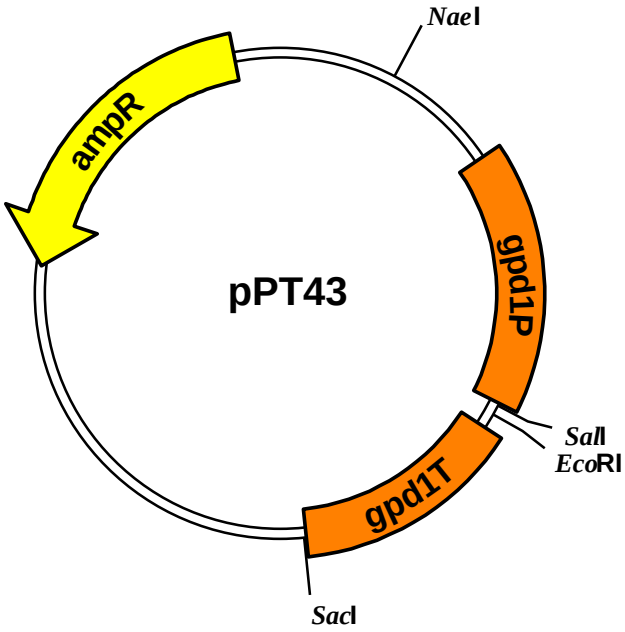
Suppl 1. Plasmids used in this study for construction of vectors.



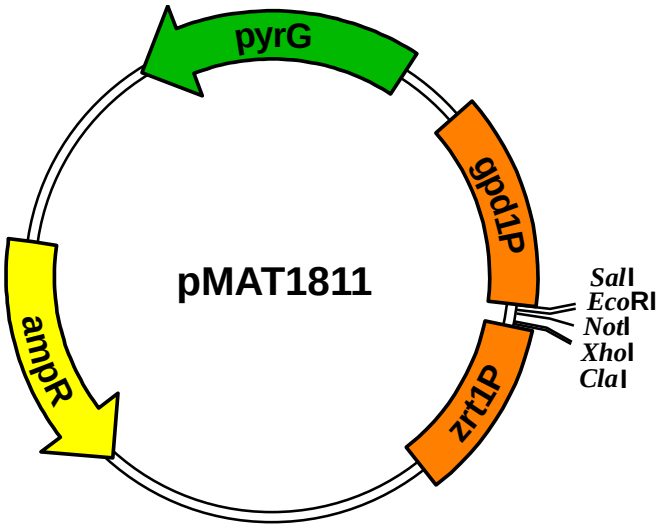
Plasmid pPT81 harbours the *Mucor gpd1* promoter and terminator (*gpd1*P and *gpd1*T), the ampicillin resistance gene (*ampR*) and *pyrG*, responsible for the complementation of the uracil auxotrophy.



Plasmid pEPM901 harbours the *Mucor pyrG* gene, responsible for the complementation of the uracil auxotrophy and ampicillin resistance gene (*ampR*).



Plasmid pPT43 harbours the *Mucor gpd1* promoter and terminator (*gpd1*P and *gpd1*T) and the ampicillin resistance gene (*ampR*).



Plasmid pMAT1811 harbours the *Mucor gpd1* promoter (*gpd1*P) and *zrt1* promoter (*zrt1*P) facing to each other, the *Mucor pyrG* gene, responsible for the complementation of the uracil auxotrophy and ampicillin resistance gene (*ampR*).

Suppl 2. Nucleotide sequences of *M. circinelloides* genes used in this study and these sequences were collected from the *M. circinelloides* genome database (https://genome.jgi.doe.gov/Mucci2/Mucci2.home.html).

Grey – upstream and downstream from the coding region, red – coding region, blue – untranslated region, black – intron.

hmgS

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ipi (NCBI Acc. No.: AM903092)

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isoA (NCBI Acc. No.: AJ496299)

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2141127 CTTTCTCGATTTCGTACCTGAAATTTGACATACAGTTTCCATGACAACCAGCTTTTTGACACATTTGCCG 2141196
2141197 TTTGCAATGAACCCCATACTCATATTGCGTCCAATCAAACATGAAGCAAGCGTATTTGAACATCAGTAGC 2141266
2141267 CTTTGATTTAGGACACTATTCAGTGTACG 2141295
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carG (NCBI Acc. No.: AJ276129)

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4761907 ATTTTTTGCATCAAGATGTTTGTGTTTTCAAGCAAGATAGATACCTACTGTAATTACAAGCTGAGCATGC 4761976
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4762117 CAAATACCATGCTCAACTCACACAACAGAACCGAAGAAAGATCGACCGAAGACGTAAGAGTGTATTTCTC 4762186
4762187 GGAGGGCTAATCTCTGATGACATGCTTGCTAACTGGTAGAAACAGATCATTTTGGAGCCTTACACCTACT 4762256
4762257 TGATATCACAGCCTGGCAAAGATATCCGGGCAAAGTTAATTTCGGCATTCGACCTGTGGCTGCATGTGCC 4762326
4762327 CAAGGACGTGCTGTGCGTAATCAACAAGATTATCGGCATGTTGCATAATGCTAGTTTAATGTAAGTAGTG 4762396
4762397 CTTGTTATCTCGCCATGAGGGGGAGGAAAATGGATACAATTGCTGATATCGATCCCAATACAGGATCGAC 4762466
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4762537 CTATCAACACTGCAAATTATGTCATCTTCTTGGCATTGCAAGAAGTGATGAAGCTGAACATCCCCAGCAT 4762606
4762607 GATGCAAGTGTGCACGGAAGAGCTGATCAATCTGCATCGAGGCCAGGGCATCGAGCTGTACTGGAGAGAC 4762676
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4762747 CGTGCATTGACTCATGAGTAACACGATCGGGCAGAAACCAGCGGTTTATTACGATTGGCGGTGCGATTAA 4762816
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4763587 TCCTCTTGTCAAG 4763599
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