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Increased activity of the sterol branch of the mevalonate pathway elevates glycosylation of secretory proteins and improves antifungal properties of *Trichoderma atroviride*

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ABSTRACT

Some *Trichoderma* spp. have an ability to inhibit proliferation of fungal plant pathogens in the soil. Numerous compounds with a proven antifungal activity are synthesized via the terpene pathway.

Here, we stimulated the activity of the mevalonate pathway in *T. atroviride* P1 by expressing the *Saccharomyces cerevisiae ERG20* gene coding for farnesyl pyrophosphate (FPP) synthase, a key enzyme of this pathway. *ERG20*-expressing *Trichoderma* strains showed higher activities of FPP synthase and squalene synthase, the principal recipient of FPP in the mevalonate pathway.

We also observed activation of dolichyl phosphate mannose (DPM) synthase, an enzyme in protein glycosylation, and significantly increased *O*- and *N*-glycosylation of secreted proteins. The hyper-glycosylation of secretory hydrolases could explain their increased activity observed in the *ERG20* transformants.

Analysis of the antifungal properties of the new strains revealed that the hydrolases secreted by the transformants inhibited growth of a plant pathogen, *Pythium ultimum* more efficiently compared to the control strain. Consequently, the biocontrol activity of the transgenic strains, determined as their ability to protect bean seeds and seedlings against harmful action of *P. ultimum*, was also improved substantially.

1. Introduction

Trichoderma spp. are used as biocontrol agents against fungal plant pathogens (Hjeljord and Tronsmo, 1998). Their antimicrobial activity is connected with the secretion of cell-wall-degrading enzymes but also with the production of antifungal substances including antibiotics, volatile metabolites, terpenes, peptaboils and piperazine derivatives (Sivasithamparam and Ghisalberti, 1998; Keszler et al., 2000; Benitez et al., 2004; Cardoza et al., 2007; Reino et al., 2008; Druzhinina et al., 2011; Crutcher et al., 2013). The mevalonate pathway (Fig. 1) product farnesyl diphosphate (FPP) is a substrate for the synthesis of the terpene compounds involved directly in the biocontrol (ergokonins and viridins), ergosterol essential for proper functioning of the cell membranes, and dolichol mainly acting as a carrier of carbohydrate residues for protein glycosylation. In this study we engineered the mevalonate pathway in *Trichoderma atroviride* towards an elevated activity of farnesyl diphosphate synthase.

Most of the information concerning the activity and regulation of the mevalonate pathway enzymes comes from studies on the yeast *Saccharomyces cerevisiae*. The pathway is mainly regulated at the level of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), the key regulated enzyme. HMGR is regulated at the transcriptional, translational and protein stability levels (Goldstein and Brown, 1990; Hampton et al., 1996; Donald and Hampton, 1997). The amount of the HMGR protein is directly dependent on the level of farnesyl diphosphate (FPP), a major product of the mevalonate pathway and a source of a positive signal for degradation of HMGR (Gardner and Hampton, 1999). Although HMGR is commonly accepted to play the key role in

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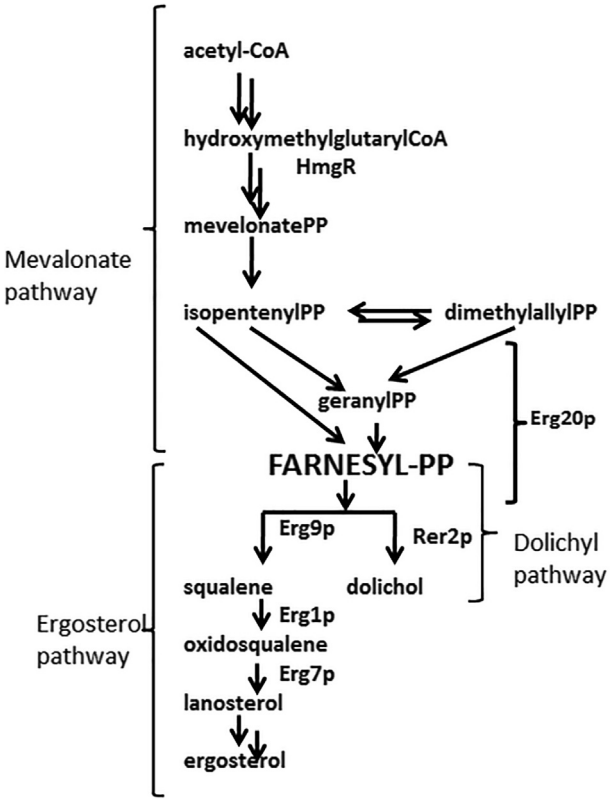


Fig. 1. Schematic representation of the mevalonate pathway. Abbreviations of enzymes presented in the scheme: HmgR − 3-hydroxy-3-methylglutaryl-CoA reductase; Erg20p (FPPS) - farnesyl pyrophosphate synthase; Rer2p - *cis*-prenyltransferase; Erg9p (SqS) - squalene synthase; Erg1p - squalene epoxidase; Erg7p - lanosterol synthase.

the mevalonate pathway, some authors tend to see FPP synthase as a pivotal enzyme (Dhar et al., 2012). FPP synthase catalyzes the formation of geranyl pyrophosphate (GPP) from two C5 monoterpene units, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), and its subsequent elongation to C15 FPP. In the yeast cell the end products of FPP synthase are FPP and GPP in a 75:25 ratio (Grabińska and Palamarczyk, 2002).

Since FPP serves as a substrate for the production of almost all isoprenoids larger than monoterpenes its unlimited availability is crucial for the overall productivity of the mevalonate pathway. Moreover, FPP is the last common substrate of two branches of the mevalonate pathway leading to linear polyisoprenoids (dolichyl branch) or pentacyclic sterols (ergosterol branch). Consequently, the distribution of FPP between these two branches needs to be regulated by the cell (Szkopińska et al., 1997; Grabińska and Palamarczyk, 2002; Ro et al., 2006; Paradise et al., 2008). In yeast the flux of FPP is directed primarily to the synthesis of ergosterol to keep its level constant (Karst et al., 2004). Also in *Trichoderma* limiting the activity of early steps of the mevalonate pathway induces overexpression of the *erg1* and *erg7* genes encoding squalene epoxidase and lanosterol:oxidosqualene cyclase, respectively (Cardoza et al., 2006; Cardoza et al., 2007), thereby channeling the mevalonate produced to the synthesis of triterpenoids which are essential components of the cell membrane and hence are required for cell survival. Thus, changes in the activity of the early steps of the mevalonate pathway affect differently the productivity of the dolichol and ergosterol branches of the pathway.

In this study, we stimulated the activity of the mevalonate pathway in *T. atroviride* to improve the supply of the substrate for the synthesis of all branches of the pathway. The *S. cerevisiae ERG20* gene coding for FPP synthase was expressed in the biocontrol strain of *T. atroviride* P1. Integration of the *ERG20* gene into the *Trichoderma* genome had a significant effect on FPP synthase activity. The *ERG20*- transformants also had a substantially enhanced DPM synthase activity and significantly higher *O*- and *N*-glycosylation of secretory proteins. The *ERG20* expression also improved the antifungal and biocontrol activities of *T. atroviride*.

2. Materials and methods

2.1. Strains and cultivation media

T. atroviride strain P1 ("*Trichoderma harzianum*" ATCC 74058) was used as the recipient strain for transformation. *Escherichia coli* strain JM 109 was used for plasmid propagation (Yanish-Perron et al., 1985). *T. atroviride* was cultivated at 28 °C on a rotary shaker (250 r.p.m.) in 2-l shake flasks containing 1 l of minimal medium (MM): 1 g $MgSO_4{\cdot}7H_2O$, 6 g $(NH_4)_2SO_4$, 10 g KH_2PO_4, 3 g sodium citrate·$2H_2O$, and trace elements (25 mg $FeSO_4{\cdot}7H_2O$, 2.7 mg $MnCl_2{\cdot}4H_2O$, 6.2 mg $ZnSO_4{\cdot}7H_2O$, 14 mg $CaCl_2{\cdot}2H_2O$) per liter and 1% (w/v) lactose or glucose as a carbon source. The flasks were inoculated with 42×10^6 conidia/l medium.

Pythium ultimum MUCL 16164, from the MUCL Culture Collection, Leuven, Belgium, was grown on potato-dextrose agar (PDA) (BioShop, Burlington, Canada).

2.2. Analysis of fungal growth

Fungal dry weight was determined by filtering culture samples through G1 sintered glass funnels, washing the biomass with water, and drying to a constant weight at 110 °C.

2.3. Expression of the S. cerevisiae ERG20 gene in T. atroviride

T. atroviride P1 was cotransformed with the yeast *ERG20* gene fused under the *A. nidulans gpdA* gene promoter (glyceraldehyde-3 phosphate dehydrogenase) and *trpC* (indole-3-glycerol phosphate synthase) terminator, using pAN52-1NotI plasmid (NCBI Acc. No. Z32697). The complete coding sequence of the *S. cerevisiae ERG20* gene was amplified by PCR using primers ErgBam-U and ErgBam-L (Table 1). The pAN521N plasmid was cut between the promoter and the terminator with BamHI, the PCR product was ligated with the plasmid and then the plasmid was cut with NcoI, the sticky ends were blunted with Mung Bean nuclease (Promega, Mannheim, Germany) and selfligated to remove the additional ATG from the NcoI restriction site (Fig. 1S). The pRMLex_30 plasmid with *E. coli* hygromycin B phosphotransferase gene (*hph*) fused between the promoter and terminator elements of the *Trichoderma pki1* (coding for pyruvate kinase) and *cbh2* (encoding cellobiohydrolase II) genes, respectively, was used as a partner in cotransformation (Mach et al., 1994). Transformants were selected for hygromycine B resistance on plates containing 1.2 M sorbitol and 200 μg/ml hygromycin B (Sigma-Aldrich, St. Louis, MO, USA). The transformants were then cultivated in liquid MM medium for preparation of DNA.

2.4. Molecular biology methods

Genomic DNA was isolated from *T. atroviride* using the Wizard Genomic DNA Purification kit (Promega, Mannheim, Germany). Total RNA was isolated using the single-step method described by Chomczynski and Sacchi (1987). Other molecular biology procedures were performed according to standard protocols (Sambrook et al., 1989).

2.4.1. ERG20 copies integrated into T. atroviride genome

Quantitative Real-Time PCR (qRT-PCR) analysis was performed to estimate the copy number of the transgenes in the *Trichoderma* genome

Table 1
Primers used in these studies.

Primer name	5′- sequence −3′	Amplification efficiency	Amplicon size bp	Purpose
ErgBam-U	CGGGATCCATGGCTTCAGAA		1075	Screening of *T. atroviride ERG20* transformants
ErgBam-L	CGGGATCCCTATTTGCTTCTC			
Primers used in the expression studies (qRT-PCR)				
Erg20F_a	TAATCACTGCACCTGAAGACAA	2.04	109	Expression of *ERG20* gene from *S. cerevisiae*
Erg20R_a	GCGACAGGCAAGTAGAAAGA			
Erg20F_b	TCAGTCGCAGAAGCCAAAT	2.02	98	
Erg20R_b	GCCTTCAAATCCTTGGCAATAG			
Erg20F_c	AGGAAGCATGTGACTGGTATG	2.03	81	
Erg20R_c	CCACAACGGACAAACCTCTAT			
LErgTa	TCGGCGAGTACTTCCAGATTC	2.05	203	Expression of *erg20* gene from *T. atroviride*
RErgTa	TCTTGACGACCTGCTCCTTG			
hmgrTaF1	CAAGGTTAAGCCCCAGGAGG	2.03	125	Expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase encoding gene from *T. atroviride*
hmgrTaR1	GAATCTTGCCACGCATCGAC			
dpmsTaF1	TTCAGCCACCACCCAAAGTT	1.98	135	Expression of dolichyl diphosphate mannose synthase (*dpm1*) encoding gene from *T. atroviride*
dpmsTaR1	GCTGACAAACTTGCGCTTCA			
erg9TaF1	ACCCCGTACTACTTTTCCGC	1.98	116	Expression of squalene synthase (erg9) encoding gene from *T. atroviride*
erg9TaR1	GTTTGAGGGGGAGGACGAAG			
erg7TaF1	ACCCAAAGTTGGTGGCAAGA	2.19	77	Expression of lanosterol synthase (*erg7*) encoding gene from *T. atroviride*
erg7TaR1	GATAGTGCCACGTATGGCGA			
erg1TaF1	GATCGCTACCACGAAGCAGA	2.12	113	Expression of squalene monooxygenase (*erg1*) encoding gene from *T. atroviride*
erg1TaR1	ATCCACCGCTCGAGGAGAA			
Primers homologous to the reference genes from *T. atroviride* used in qRT-PCR				
FActTa	AACCGTGAGAAGATGACCCAG	1.93	198	Expression of *act1* gene from *T. atroviride*
RActTa	CCATGTCCACACGAGCAATG			
gapdhTaF	CGTTCTCTCCAACGCCTCTT	1.95	108	Expression of glyceraldehyde 3-phosphate dehydrogenase encoding gene from *T. atroviride*
gapdhTaR	TAGGAGTGGACGGTGGTCAT			
ef1aTaF1	ACAGAAAGCTCTTGGGACGG	1.99	147	Expression of elongation factor 1 alpha encoding gene from *T. atroviride*
ef1aTaR1	GGTTTGGGAGCTGCTTTTGG			
uceTaF1	ACGGCGAAGACTTGAAGGAA	1.96	130	Expression of ubiquitin conjugating enzyme encoding gene from *T. atroviride*
uceTaR1	GTGACCTCTGGGGGCTCTAT			
e3ulTaF1	TTCGAACTCAATCACCCCCG	2.10	83	Expression of E3 ubiquitin ligase encoding gene from *T. atroviride*
e3ulTaR1	TGTAATCAGATCGGGCTGGC			
oxdcTaF1	GGCCTTGACACCGAGAATGA	2.25	100	Expression of oxalate decarboxylase encoding gene from *T. atroviride*
oxdcTaR1	GGGTATGAGCGATCCAGTCG			

using the relative standard curve method (Joshi et al., 2008; Ma and Chung, 2015; Salas-Marina et al., 2015). Serial 1:10 dilutions of standard samples containing from 300,000 to 300 copies of the *ERG20* gene were prepared by mixing the wild-type *Trichoderma* genomic DNA with pGEM-*ERG20* plasmid. Each reaction contained adequate amount of DNA calculated using the online tool (DNA Copy Number and Dilution Calculator, Thermo Fisher Scientific), 0.5 µM of each primer (Table 1), and 7,25 µl 3color 2xHS-qPCR Master Mix Sybr (A&A Biotechnology, Gdynia, Poland), in a total volume of 15 µl. All reactions were run on a LightCycler® 96 (Roche Diagnostics GmbH, Mannheim, Germany) using the following program: 360 s 95 °C, 40 times (15 s 95 °C; 60 s 60 °C), followed by melting curve generated from 65 °C to 90 °C. Four technical replicates were used for both standard curve and the test samples. A single product was amplified by all primer pairs tested. Raw data was processed using LightCycler® 96 software Version 1.1.0.1320 (Roche Diagnostics GmbH, Mannheim, Germany) and the transgene copy number was calculated automatically.

2.4.2. Expression quantitative PCR (RTq-PCR) and data analysis

One-step RT-qPCR assay was performed using a LightCycler® EvoScript RNA SYBR® Green I Master Kit (Roche Diagnostics GmbH, Mannheim, Germany) and was carried out in a 20 µl reaction which consisted of 5 µl mRNA (25 pg), 0.4 µM each of forward and reverse primers (Table 1), and 4 µl of Master Mix, 5x concentrated containing enzymes for reverse transcription and PCR, RT-qPCR Reaction Buffer, dATP, dCTP, dGTP, and dUTP, $Mg(OAc)_2$, SYBR® Green I dye, and proprietary additives. For the negative controls, mRNA was substituted with molecular grade water. The assay was performed using a LightCycler96® (Roche Diagnostics GmbH, Mannheim, Germany) with an initial incubation at 60 °C for 15 min for RT followed by pre-incubation at 95 °C for 10 min. Forty five cycles of amplification were performed using a thermal cycling profile of 95 °C for 10 s, 58 °C for 30 s. Subsequently, a melting curve was recorded by holding at 95 °C for 10 s, cooling to 65 °C for 60 s, and then heating at 0.1 °C/s up to 97 °C. The amplification and melting curve data were collected and analyzed using the LightCycler96® software 1.0.

2.5. Biochemical methods

2.5.1. FPP synthase (EC 2.5.1.10) activity

The FPP synthase activity was assayed in a cell free extract obtained from transformants and the control *T. atroviride* P1 strain. After 120 h of cultivation mycelia of *Trichoderma* were harvested by filtration, washed with water and suspended in 50 mM phosphate buffer, pH 7.5 containing 1 mM $MgCl_2$ and 5 mM iodoacetamide. Cells were homogenized in a beadbeater with glass beads (0.5 mm) and the homogenate was centrifuged at 5,000 × g for 10 min to remove cell debris and unbroken cells. The resulting supernatant was centrifuged again at 100 000 × g for 1 h to remove membrane pellet and the obtained supernatant was used as the source of FPP synthase. The whole procedure was performed at 4 °C.

The FPP synthase activity was measured in 100 µl of reaction mixture containing 50 mM phosphate buffer, pH 7.5, 1 mM $MgCl_2$, 5 mM iodoacetamide, 60 µM isopentenyl diphosphate (IPP), $1x10^5$cpm [^{14}C] IPP (specific activity 52 Ci/mol) (ARC, 101 ARC Drive, St. Louis, MO, USA), 120 µM dimethylallyldiphosphate (DMAPP), and 150 µg protein (Karst et al., 2004). After 5-min incubation at 37 °C samples were ice-chilled and 0.5 ml water was added, followed by 1 ml hexane and 0.2 ml of 1 M HCl to dephosphorylate the products. The samples were shaken for 30 min at 37 °C. The mixture was ice-chilled and vigorously mixed. The upper phase was washed three times with water and subjected to TLC on silica gel 60 plates in benzene-ethyl acetate 7:1. Radioactive spots were localized by autoradiography, identified by co-chromatography with unlabeled standards, then scraped off and the

radioactivity was measured in a scintillation counter (Karst et al., 2004).

2.5.2. Membrane preparation

After 120 h of cultivation mycelia were harvested by filtration, washed with water and suspended in 50 mM Tris-HCl, pH 7.4, containing 15 mM $MgCl_2$ and 9 mM β-mercaptoethanol. Cells were homogenized and processed as above. Following centrifugation at 100,000 × g for 1 h the membrane pellet was collected, homogenized in 50 mM Tris-HCl, pH 7.4, containing 3.5 mM $MgCl_2$ and 6 mM β-mercaptoethanol, and used as the source of enzyme. The whole procedure was performed at 4 °C (Perlińska-Lenart et al., 2006).

2.5.3. Cis-prenyltransferase (EC 2.5.1.20) activity

The enzyme activity was assayed in the membrane fraction by incubation (final volume 250 µl) of 500 µg of membrane proteins with 4 µg FPP, 50 mM sodium phosphate buffer pH 7.4, 0.5 mM $MgCl_2$, 20 mM β-mercaptoethanol, 10 mM KF and 3 × 10^5 cpm [^{14}C]IPP (specific activity 52 Ci/mol) (ARC, 101 ARC Drive, St. Louis, MO, USA). After 90-min incubation at 30 °C, the reaction was terminated by addition of 4 ml of chloroform - methanol (3: 2 v/v). The protein pellet was removed by centrifugation and the supernatant was washed three times with 1/5 vol of 10 mM EDTA in 0.9% NaCl. The organic phase was concentrated under a stream of nitrogen and subjected to thin-layer chromatography on HPTLC RP-18 plates (Merck Group, Darmstadt, Germany) developed in 50 mM H_3PO_4 in acetone. The zone containing the radiolabeled polyprenols was scraped off and the radioactivity was measured in a scintillation counter (Szkopińska et al., 1997; Perlińska-Lenart et al., 2006).

2.5.4. Squalene synthase (EC 2.5.1.21) activity

Squalene synthase activity was analyzed by incubation of 200 µg of membrane fraction with 100 mM potassium phosphate buffer pH 7.4, 5 mM $MgCl_2$, 5 mM CHAPS, 10 mM DTT, 2 mM NADPH and 10 µM [^{3}H] FPP(sp. act. 15–30 Ci/mmol) (ARC, 101 ARC Drive, St. Louis, MO, USA) in a total volume of 100 µl. Incubation was performed at 37 °C for 20 min, then reaction was stopped with 10 µl of 1 M EDTA pH 9.2 and 10 µl of unlabeled 0.5% squalene was added as a carrier. The reaction mixture was applied onto a silica gel 60 thin layer chromatography plate and developed with 5% toluene in hexane. The radioactive zone containing squalene (R_f = 0.5) was scraped off and measured in a scintillation counter (Shechter et al., 1992).

2.5.5. Dolichyl phosphate mannose (DPM) synthase (EC 2.4.1.83) activity

DPM synthase activity was measured in the pelleted membrane fraction by incubating it with GDP[^{14}C] Mannose (sp. act. 50–60 Ci/mol) (ARC, 101 ARC Drive, St. Louis, MO, USA) and 5 ng of dolichyl phosphate (Dol-P) (Danilov and Chojnacki, 1981) according to Kruszewska et al. (1989).

2.5.6. Ergosterol and squalene determination

Sterols were extracted according to the method proposed by Bernat et al. (2014) with some modifications. Lyophilized biomass (30 mg) was transferred into Eppendorf tube containing glass beads and 1 ml of methanol:chloroform (2:1, v/v). The homogenization process using a ball mill (FastPrep-24, MP-Biomedicals) was carried out for 2 min. Next, the sample was centrifuged (2 min, 6000 × g). The mixture was transferred to another Eppendorf tube. In order to facilitate the separation of two layers, 0.2 ml of H_2O was added. The lower layer was collected, evaporated and dissolved in 1 ml of methanol:chloroform (4:1, v/v). Sterols were measured using an Agilent 1200 HPLC system (Santa Clara, CA, USA) and a 3200 Q-TRAP mass spectrometer (Sciex, Framingham, MA, USA) equipped with an atmospheric-pressure chemical ionization (APCI) source operating in the positive ionization mode. Lipid extract (10 µl) was injected onto a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, Torrance, CA, USA) heated at 40 °C with the flow rate of 0.8 ml min^{-1}. Water (A) and methanol (B) were applied as a mobile phase, both containing 5 mM ammonium formate. The solvent gradient was initiated at 40% B, increased to 100% B during 1 min, and maintained at 100% B for 3 min before returning to the initial solvent composition over 2 min. The following instrumental settings were applied: curtain gas 25.0, ion spray voltage 5500, nebulizer gas 50, auxiliary gas 50, and ion source temperature of 550 °C, entrance potential 10.0. The monitored multiple reaction monitoring (MRM) pairs were *m/z* 379.3–69.1, 379.3–81.3 for ergosterol and 411.4–231.4, 411.4–163.3 for squalene. The data analysis was performed with the Analyst™ v1.5.2 software (Sciex, Framingham, MA, USA).

2.5.7. Protein concentration assay

Protein concentration was determined according to Lowry et al. (1951).

2.5.8. Identification and quantification of carbohydrates bound to secretory proteins

Secreted proteins were precipitated from post-culture medium with two volumes of 96% ethanol, washed twice with 70% ethanol (Ma et al., 1996). *O*-linked carbohydrates were removed from the protein pellet using mild alkaline hydrolysis according to Duk et al. (1997) and analyzed in the supernatant and then the *N*-linked carbohydrates were determined in the pellet.

O- and *N*- linked carbohydrates obtained as above were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 16 h. Monosaccharides were determined by high performance anion-exchange chromatography using Dionex ICS-3000 Ion Chromatography System (Thermo Fisher Scientific, Waltham, MA, USA) with a Carbo Pac PA10 analytical column. Neutral sugars were eluted with 18 mM NaOH at 0.25 ml/min (Zdebska and Kościelak, 1999).

2.5.9. Cellulase activity

The activity of cellulases was measured in the cultivation medium by incubation of 0.5 ml of carboxymethylcellulose (10 g/l) in 50 mM sodium citrate buffer, pH 5.0 at 50 °C for 10 min with 0.2 ml of culture filtrate. The reaction was stopped by boiling for 5 min. The amount of reducing sugars formed was determined by the method of Bernfeld (1955) and estimated using a standard curve prepared with glucose.

2.6. Growth inhibition of Pythium ultimum by hydrolases and metabolites secreted by Trichoderma strains to cultivation medium

To determine antagonistic activity, *Trichoderma* was grown on minimal medium with lactose covered with cellophane or dialyzing membrane (cut-off 10 kDa). Following the removal of the membrane with the mycelia, the effect of post-culture medium containing secreted hydrolytic enzymes and metabolites (cellophane) or only metabolites (dialyzing membranes) on the growth of *P.ultimum* was analyzed.

A 5-mm mycelial disk of *P. ultimum* was placed in the center of the *Trichoderma*-pretreated plate. Plates were incubated at 28 °C for eight days and the area of growth was measured. Three replicates were assayed for each *Trichoderma* strain. As a control, *P. ultimum* was cultivated on plates containing fresh minimal medium and *Trichoderma* strains were cultivated on *Trichoderma*-pretreated plates to exclude lack of nutrients in the medium (Fig. 3S).

2.7. Plate confrontation assay

Mycelial disks (5 mm) of *T. atroviride ERG20* transformants and *P. ultimum* were placed at opposite sides (7 cm apart) of agar plates with MM supplemented with 1% glucose. The plates were incubated at 28 °C and photographs were taken after 3 days of incubation. Three replicates were prepared for each experiment and for each transformant.

2.8. Biocontrol assay

Bean seeds (*Phaseolus vulgaris L.* (*var. nanus L.*)) were coated with 5×10^8 spores of *T. atroviride* per 10 g of seeds. Pathogen-infected soil was prepared by adding 2.2 g of fungal biomass of *P. ultimum* to 1 l of sterile soil. Seeds were allowed to germinate in the moist soil for ten days and the number and height of the seedlings was determined.

3. Results

3.1. Expression of S. cerevisiae ERG20 gene in T. atroviride

The biocontrol strain *T. atroviride* P1 was transformed with the *ERG20* gene from *S. cerevisiae* under the control of the *gpdA* gene promoter (encoding glyceraldehyde-3-phosphate dehydrogenase) from *A. nidulans*. Stable transformants were analyzed for the presence of the yeast gene by PCR using ErgBam-U and ErgBam-L primers and genomic DNA of the transformants as template. As positive and negative controls the PCR reactions were performed on the templates of DNA from *S. cerevisiae* and *T. atroviride* P1, respectively. Three transformants carrying the *ERG20* gene, SGUL59/11, SGUL125/11 and SGUL133/11 were selected for further analysis. qRT PCR analysis revealed that eight copies of *ERG20* gene were integrated into the genome of SGUL125/11, and three and one copy were found in the SGUL59/11 and SGUL133/11 transformants, respectively. Expression of the heterologous *ERG20* measured by RTqPCR showed very high expression of the gene in SGUL125/11 and much lower in the other two transformants (Fig. 2). These results are in agreement with the number of copies of *ERG20* integrated into the genome of the transformants. In addition, we found that expression of the heterologous gene from *S. cerevisiae* did not alter the expression of the native *erg20* gene.

3.2. Characterization of the T. atroviride ERG20 strains

The activity of FPP synthase encoded by the *ERG20* gene from *S. cerevisiae* was measured in cell-free extracts obtained from the transformants and compared with the endogenous FPP activity in the control non-transformed strain. All the transformed strains exhibited higher activity of FPP synthase compared to the control P1 strain (Fig. 3A). The increase in activity ranged from 55 to 104% of the control activity. The highest activity was detected for SGUL125/11 having the highest expression of the *ERG20* gene.

Since the higher activity of FPP synthase could affect the production of sterols and/or dolichols, we analyzed the activity of two enzymes

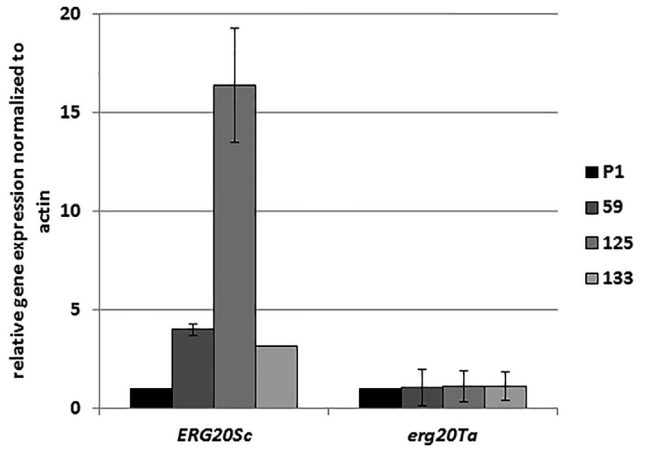


Fig. 2. Transcript levels of *ERG20* genes from *S. cerevisiae* (*ERG20Sc*) and *T. atroviride* (*erg20Ta*) determined by RT-qPCR in *ERG20*-transformed *T. atroviride*. *Trichoderma* strains (59-SGUL59/11; 125-SGUL125/11; 133-GSUL133/11) and control strain P1 were grown for 144 h in PDB medium.

representing the sterol and the dolichyl branch of the mevalonate pathway, squalene synthase (Erg9p) and *cis*-prenyltransferase (Rer2p), respectively, in membrane fractions obtained from the transformants and the control P1 strain. The activity of *cis*-prenyltransferase was unchanged in the SGUL59/11, SGUL125/11 and SGUL133/11 transformants compared to the control (Fig. 3B). A markedly different effect was found for squalene synthase. Its activity was increased substantially in all the transformed strains, by between 78 and 110% of the control activity for SGUL125/11 and SGUL133/11, respectively (Fig. 3C).

3.3. Ergosterol and squalene production

Since the higher activity of squalene synthase should alter sterol synthesis we analyzed ergosterol and squalene levels in the cells of transformants compared to the control. Although significant differences of the squalene level were found, the concentration of ergosterol in the mycelia remained unchanged in all the transformants. The level of squalene was 100% higher in the mycelium of SGUL133/11 compared to the control strain (Fig. 4). In SGUL59/11 and SGUL125/11 the squalene level increased by 51% and 46%, respectively, compared to the control. Although the activity of squalene synthase (Erg9p) (Fig. 3C) and the level of squalene (Fig. 4) were significantly elevated in the transformants, expression of the *erg9* gene was only slightly increased (Fig. 5). Since we did not observe an elevated level of ergosterol (Fig. 4), utilization of squalene had to be partially inhibited in the transformants. The decreased expression of *erg7* gene and only moderately increased expression of *erg1* (statistically significant only for the SGUL133/11 transformant) (Fig. 5) confirmed this hypothesis.

3.4. Activity of DPM synthase and glycosylation of secreted proteins

The higher activity of the sterol branch of the mevalonate pathway led to a higher production of squalene. Spanova et al. (1821) demonstrated that squalene in combination with ergosterol was an important parameter modulating the fluidity of the ER and plasma membrane. The fluidity of the cell membrane could in turn affect the activity of membrane-bound enzymes. One such membrane-bound enzyme is DPM synthase involved in the processing of hydrolases secreted by *Trichoderma*. DPM synthase is a key enzyme of the process of protein *O*-glycosylation and is also engaged in the assembly of the lipid-linked oligosaccharide substrate for protein *N*-glycosylation. The DPM synthase activity was measured in the membrane fraction and found to be higher by between 26 and 70% in SGUL133/11 and SGUL125/11, respectively, compared to the control strain (Fig. 6A).

We reasoned that the increased activity of DPM synthase found in the *ERG20* transformants could affect protein glycosylation and/or secretion (Kruszewska et al., 1999; Perlińska-Lenart et al., 2006; Zembek et al., 2011). To verify this assumption, protein secretion to the culture medium was examined during cultivation. Medium samples were collected every 24 h for 168 h and the concentration of secreted proteins was determined. The amount of proteins was elevated by 19% compared to the control (Fig. 2SA) in the culture medium of the SGUL133 (120 h of cultivation) and SGUL59/11 (168 h of cultivation) strains.

Proteins secreted by *Trichoderma*, mainly hydrolytic enzymes, are highly glycosylated (Kruszewska et al., 2008-rev). The significantly increased activity of DPM synthase in the transformants should result in higher production of dolichyl phosphate mannose conductive to hyperglycosylation of the proteins. To analyzed protein glycosylation *Trichoderma* strains were cultivated in minimal medium with lactose as a carbon source, proteins were precipitated from the medium after 72 and 96 h of cultivation, and the amount of protein-bound carbohydrates was determined. We found the highest amount of carbohydrates on proteins secreted after 96 h of cultivation (Fig. 6B,C). The increase, relative to the untransformed strain, in the amount of *N*-linked carbohydrates was more pronounced compared to the *O*-linked sugars. The highest level of protein glycosylation was found for strain SGUL125/11,

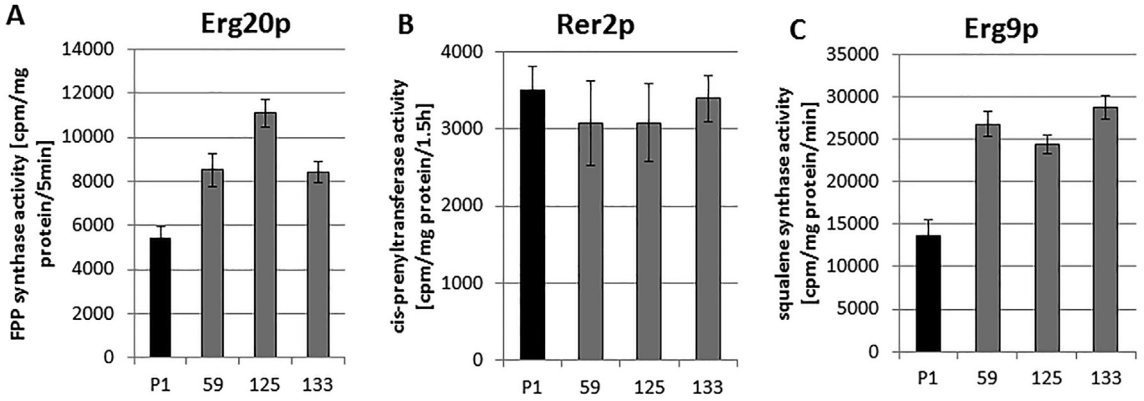


Fig. 3. Activities of FPP synthase (A), *cis*-prenyltransferase (B), squalene synthase (C) in cell free extract (A) or in membrane fraction (B, C) from *ERG20*-transformed *Trichoderma*. Transformant strains (59 - SGUL59/11; 125 -SGUL125/11; 133 - SGUL133/11) and control strain P1were processed and analyzed for enzymatic activities as described in Sections 2.5.1 and 2.5.4. Data are presented as mean ± standard deviation from six independent experiments each determined in triplicate. For FPP synthase (A) and squalene synthase (C) all differences between the control P1 strain and transformants are statistically significant ($P < 0.05$; *t* test).

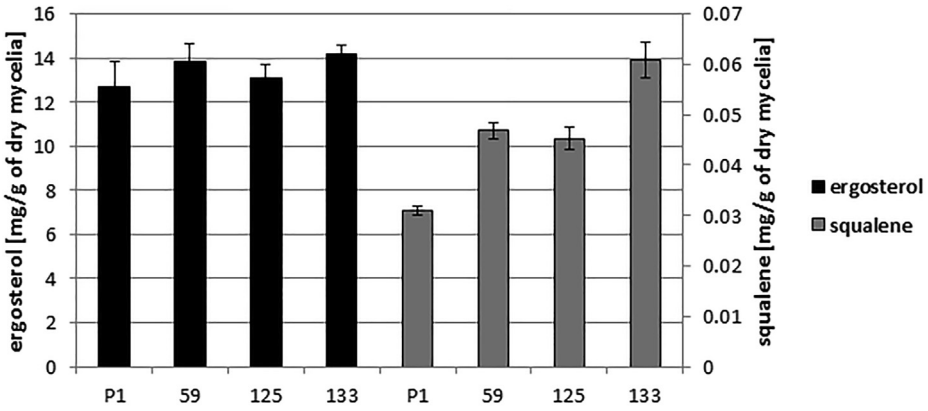


Fig. 4. Ergosterol and squalene content in membrane fraction from *ERG20* transformed strains and the control P1. Analysis was done using an Agilent 1200 HPLC system and a 3200 Q-TRAP mass spectrometer (see Section 2.5.6). Data are presented as mean ± standard deviation from three independent experiments. Differences in squalene concentration between control P1strain and the transformants are statistically significant ($P < 0.05$; *t* test) .

and it was 5.3-fold higher for *N*-glycosylation and 2.2-fold higher for *O*-glycosylation compared to the parental strain. The other two transformed strains also produced secretory proteins significantly more extensively *O*- and *N*-glycosylated than in the control P1 strain.

A qualitative analysis of the carbohydrates by high performance anion-exchange chromatography revealed that in both *O*- and *N*- linked carbohydrates mannose was the dominant sugar. The amount of mannose bound to proteins secreted by SGUL125/11 was 4.3- and 3.3-fold higher than for the P1 strain in *N*- and *O*-linked carbohydrates, respectively. This result correlates with the highest activity of DPM synthase in SGUL125/11. The both types of carbohydrates of the secretory proteins also contained glucose and galactose.

3.5. Hydrolytic activity of the ERG20-expressing strains

Since a major fraction of the proteins secreted to the cultivation medium are hydrolytic enzymes enabling the uptake of nutrients from complex carbon sources, and the secretory proteins were highly over-

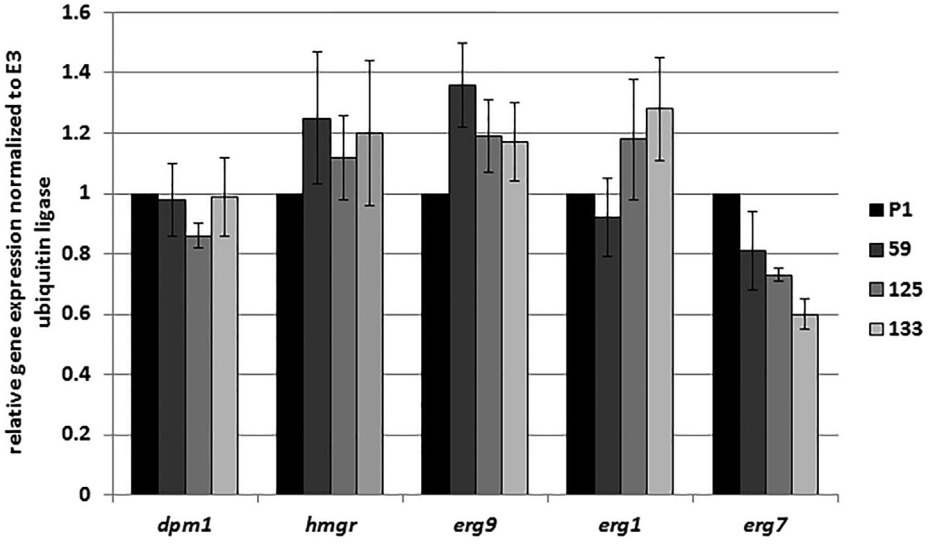


Fig. 5. Transcript levels of genes coding for DPM synthase (*dpm1*) and for enzymes from the mevalonate pathway: 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgr*), squalene synthase (*erg9*), squalene epoxidase (*erg1*), and lanosterol synthase (*erg7*) determined by RT-qPCR in the *ERG20*-transformed *T. atroviride*. *Trichoderma* strains (59-SGUL59/11; 125-SGUL125/11; 133-GSUL133/11) and control strain P1 were grown for 144 h in PDB medium, RNA was extracted and cDNA synthesized. qPCR reactions were performed using a LightCycler 96 instrument. Data obtained from three independent experiments each determined in triplicate.

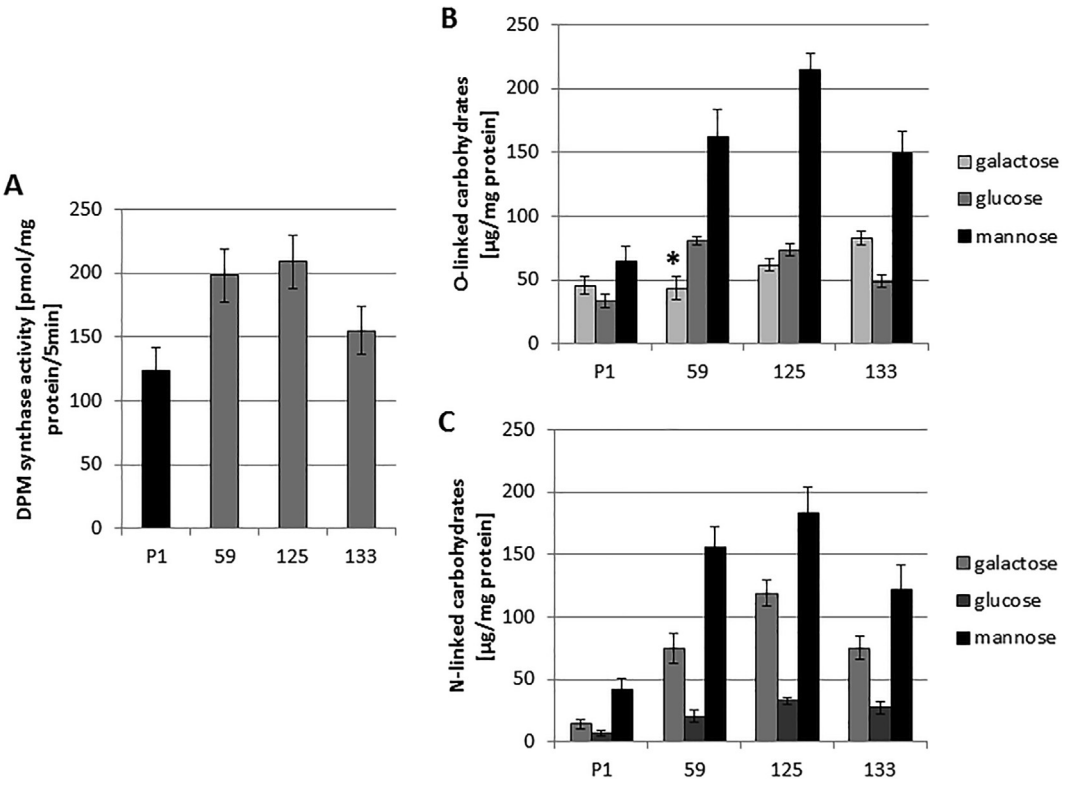


Fig. 6. Activity of DPM synthase (A) in membrane fraction and amount of carbohydrates *O*- (B) and *N*-linked (C) to the proteins secreted by *ERG20*-transformed *Trichoderma* strains. *O*- and *N*-linked carbohydrates were released selectively from proteins secreted by *Trichoderma* transformants (59 - SGUL59/11; 125 - SGUL125/11; 133 -SGUL133/11) and control strain P1 and determined by high performance anion-exchange chromatography as described in Section 2.5.8. Data are presented as mean ± standard deviation from three independent experiments. * Difference statistically insignificant. All other differences between the control P1 strain and transformants are statistically significant ($P < 0.05$; *t* test).

glycosylated, we decided to check the hydrolytic capacity of the transformants.

Analysis of the cellulolytic activity secreted into the cultivation medium showed that all the transformants exhibited maximal cellulolytic activity after 72 and 96 h of cultivation (Fig. 7). The highest activity was noticed for SGUL133/11 after 72 h of cultivation and it was higher by 45% than that of the control strain after 96 h. The lowest activity among the transformants was found for SGUL125/11 and it was only 9% higher than in the control.

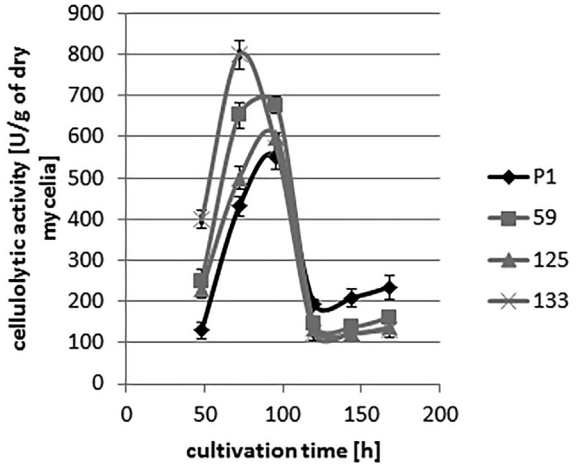


Fig. 7. Cellulolytic activity in cultivation medium from *ERG20*-transformed *T. atroviride* strains. Concentration of reducing sugars released from carboxymethylcellulose by cellulases secreted into cultivation medium from *Trichoderma* strains (59 -SGUL59/11; 125 - SGUL125/11; 133 - SGUL133/11) and the parental strain P1was measured as described in Section 2.5.9. Data are presented as mean ± standard deviation from six independent experiments.

3.6. Antifungal activity of ERG20 transformants

To determine the antifungal potential of the *ERG20* transformants we studied the effect of compounds liberated by the transformants on the growth of the plant pathogen *Pythium ultimum*. *Trichoderma* strains were cultivated on plates covered with cellophane or dialysis membranes (cut-off 10 kDa), then the membrane or cellophane was removed together with the *Trichoderma* and *P. ultimum* was inoculated on the pretreated plates containing both metabolites and hydrolases secreted by *Trichoderma* (cellophane) or only the metabolites (dialysis membrane). The growth of the pathogen on the *Trichoderma*-pretreated plates was compared with the growth on clean non-pretreated plates. The hydrolases and metabolites secreted through cellophane by all strains inhibited the growth of *P. ultimum* significantly, albeit the control strain inhibited the growth of *Pythium* slightly less effectively -by 70% while the transformants - by 88% (Fig. 8).

In contrast no differences in the inhibition of *Pythium* growth were found on plates containing only the secreted metabolites (covered with dialysis membrane) (data not shown). In fact the low-molecular-weight metabolites secreted though the dialysis membrane had little inhibitory effect (8%) on *Pythium* growth regardless of the *Trichoderma* strain used.

In addition, we made a confrontation assay between the *ERG20*-transformed *Trichoderma* strains and *P. ultimum* (Fig. 9). When *Trichoderma* and *Pythium* were cultivated on a single plate from separate inocula, the *Pythium* colony was overgrown more rapidly by all the transformants than by the control P1 strain, and the SGUL133/11 transformant was the most efficient. Since the cell wall of *Pythium*

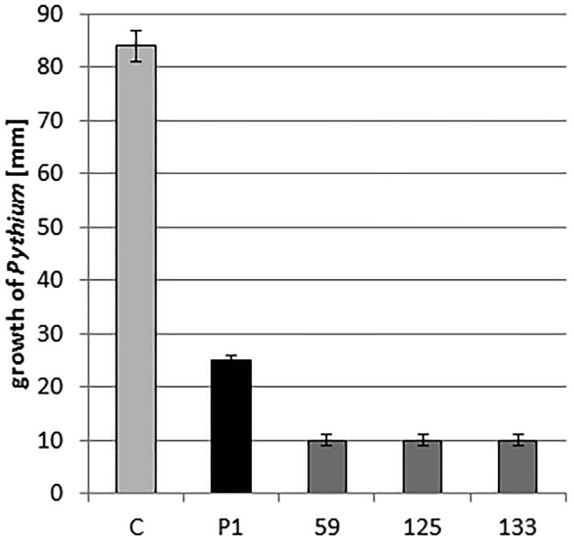


Fig. 8. Growth inhibition of *P. ultimum* cultivated on plates pretreated with *T. atroviride* strains. *Trichoderma* strains (59 - SGUL59/11; 125 - SGUL125/11; 133 -SGUL133/11) and control P1strain were cultivated on MM plates covered with cellophane for three days and then removed with the cellophane. *P. ultimum* was inoculated on the pretreated plates and its rate of growth was determined as colony diameter after two days. As a control (C), *P. ultimum* was cultivated on non-pretreated plates. Data are presented as mean ± standard deviation from six independent experiments. All differences between the control P1 strain and transformants are statistically significant ($P < 0.05$; *t* test).

contains β-(1,4) glucan (cellulose) (Latijnhouwers et al., 2003) and SGUL133/11 revealed the highest cellulolytic activity this result was expected. Inspection of the *Pythium*-overgrown area under a microscope showed that some hyphae of *Pythium* were devoid of organelles and contained mycelia of *Trichoderma* inside (Fig. 10).

3.7. Biocontrol properties of the ERG20- transformed strains

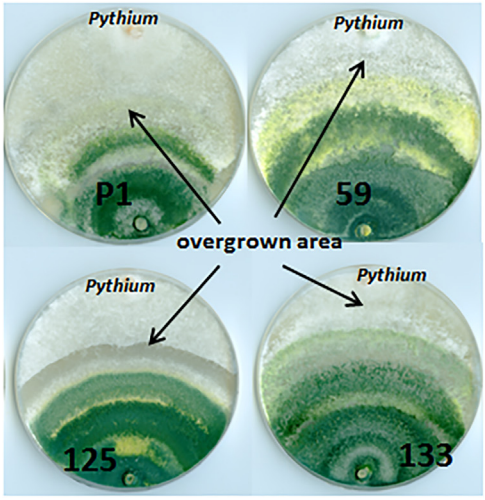
Since our transformants revealed a stronger antifungal activity and higher activity of the secretory hydrolases compared to the control strain it could be expected that also their biocontrol activity was more pronounced. To check this we tested seed germination and seedling growth of bean (*Phaseolus vulgaris L.* (*var. nanus L.*)) in the presence of *P. ultimum*. In the absence of *Trichoderma*, the pathogenic fungus decreased the bean germination rate to 28% of that observed in sterile soil (Fig. 11A). Pre-treatment of the seeds with the control *Trichoderma* strain P1increased the germination rate in the presence of *P. ultimum* to 44% while with the SGUL125/11 transformant the rate was 84%. Poorer protection was observed for SGUL133/11 and SGUL59/11, but still the rate was 72 and 63%, respectively. The presence of *P. ultimum* in the soil inhibited the plant growth by 64% (Fig. 11B). *Trichoderma* largely prevented that inhibition: with the untransformed P1 strain the growth was poorer by only 9% compared to fungi-free control, while the SGUL125/11 transformant actually increased the growth rate in the presence of *P. ultimum* by 13% above the control (P1) value.

4. Discussion

The aim of this study was to engineer *T. atroviride* toward improving its antifungal properties. The importance of the mevalonate pathway products in the antifungal activity had been shown before using *T. harzianum* with a partially silenced expression of the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Cardoza et al., 2007). The HMGR-silenced transformants showed a reduced antifungal activity against the plant-pathogen fungi *Rhizoctonia solani* and *Fusarium oxysporum*. Since we intended to obtain an opposite effect we up-regulated the mevalonate pathway in the biocontrol *T. atroviride* strain P1by expression of the yeast *ERG20* gene encoding a key enzyme of this pathway, FPP synthase.

We chose a heterologous system since the yeast enzyme exhibits a much higher specific activity than the homologous enzyme from *Trichoderma* (Szkopińska et al., 2000; Piłsyk et al., 2014).

The increased activity of FPP synthase caused further changes in the mevalonate pathway mainly by increasing the rate of sterol synthesis, while the activity of the dolichol branch represented by *cis*-prenyltransferase remained unchanged. Szkopińska et al. (2000) showed that overexpression of the *ERG20* gene in yeast elevated almost three-fold the rate of dolichol synthesis, while sterol synthesis was elevated only by 30%. The contrasting consequences of the *ERG20* over-expression in *S. cerevisiae* and *T. atroviride* could reflect its homologous and heterologous origins. Enzymes from the dolichyl branch of the mevalonate pathway have been postulated to form an enzymatic complex with FPP synthase (Szkopińska et al., 1997). The *cis*-prenyltransferase activity is found in the membrane fraction, while FPP synthase was initially claimed to be cytosolic, which would make formation of such a complex unlikely; however, later studies (Runquist et al., 1992) found the FPP synthase activity also in the microsomal fraction and thus removed that caveat. Since the same yeast FPP synthase expressed in *S. cerevisiae* and in *Trichoderma* had different effects on the respective endogenous *cis*-prenyltransferase activities one could infer that the Erg20 protein could only form an active complex for dolichol synthesis with the homologous (*S. cerevisiae*) but not the heterologous (*Trichoderma*) *cis*-prenyltransferase. Furthermore, the



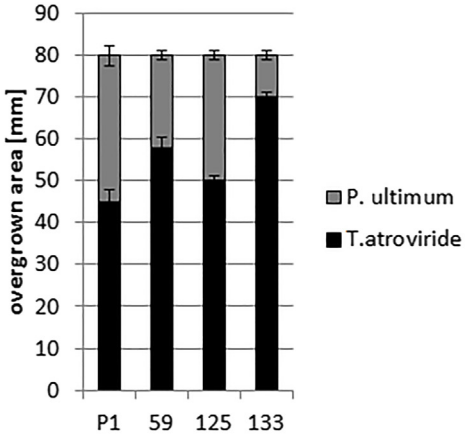


Fig. 9. Plate confrontation assay of *T. atroviride* against *P. ultimum*. Mycelial disks of SGUL59/11, SGUL125/11 and SGUL133/11 transformants and the control strain P1 and *P. ultimum* were placed at opposite sides of MM-agar plates and incubated at 28 °C. Pictures were taken three days after inoculation. Arrows mark the overgrown zone between the two fungi. Overgrown zones were measured and results are presented as mean ± standard deviation from three separate plates. Differences are statistically significant ($P < 0.05$; *t* test).

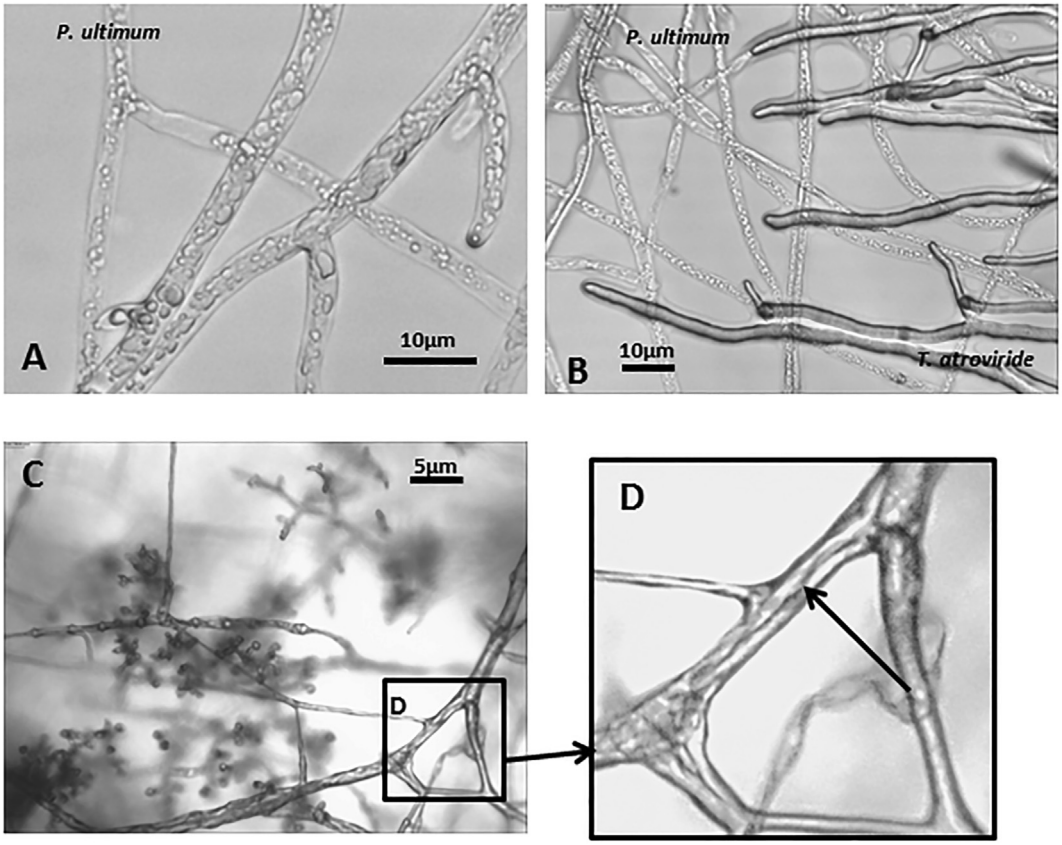


Fig. 10. Microscopic analysis of *Pythium*-overgrown area in the *P. ultimum* and *T. atroviride* confrontation assay. A- *Pythium* hyphae, B- *Pythium* and *Trichoderma* in the contact area, C-*Pythium* and *Trichoderma* in the *Pythium*-overgrown area, D-hyphae of *Pythium* attacked by *Trichoderma* Arrow marks *Trichoderma* hyphae inside of *Pythium* mycelium.

unchanged *cis*-prenyltransferase activity in the *Trichoderma* transformants could create a surplus of FPP for the sterol branch of the mevalonate pathway. This could explain the significant increase in the activity of squalene synthase in the transformants.

On the other hand, overexpression of the homologous *erg20* gene in *T.reesei* did not activate the two branches of the mevalonate pathway (Piłsyk et al., 2014). Although *cis*-prenyltransferase was activated in these transformants the activity of squalene synthase actually decreased 2-fold. Such an effect could be disadvantageous when attempting to improve the antifungal properties of *Trichoderma*. The level of sterols in the fungal cells could be crucial for activity of membrane-bound enzymes and secretion of proteins.

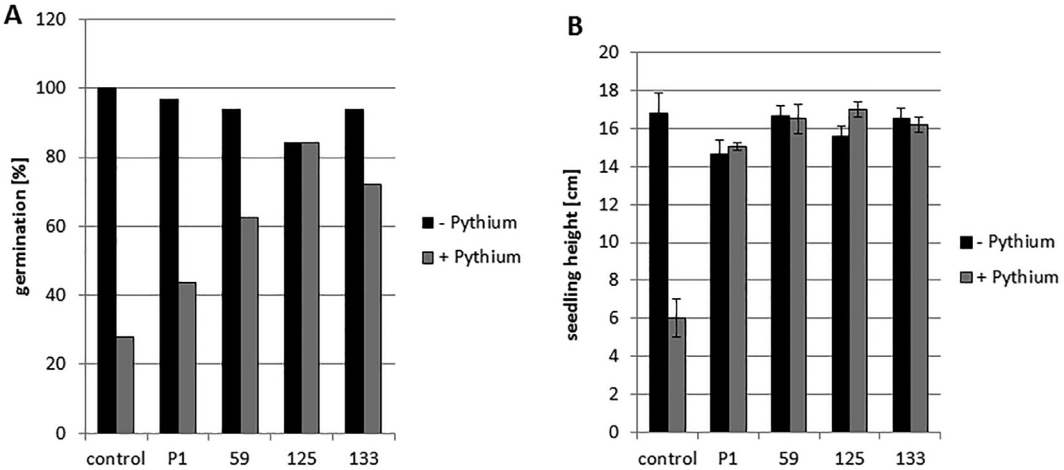


Fig. 11. Protective effect of *Trichoderma* on bean germination (A) and bean seedling growth (B) in soil infected with *P. ultimum*. A- Efficiency of germination presented as percentage of seeds sown was analyzed after ten days. Seeds were sown in sterile (black bars) or *P. ultimum*-infected (dark gray bars) soil in the absence or presence of *Trichoderma* strains as indicated. Each bar represents % of germinated seeds out of 32 seeds used in experiments and 100 seeds for the control with *Pythium* but no *Trichoderma*. B- Protective effect of *Trichoderma* on bean seedling growth. Seedlings height was measured after ten days. Seeds were sown in sterile (black bars) or *P. ultimum*- infected (dark grey bars) soil in the absence or presence of *Trichoderma* strains as indicated. Each bar represents mean ± standard deviation from different number of independent experiments, with 28 seedlings for control with *P. ultimum* but no *Trichoderma* and 30 seedlings for control without *P. ultimum*. Difference statistically significant ($P < 0.05$; *t* test).

It is known that sterols can affect the whole metabolism of the fungal cell since sterols, major lipids of the plasma membranes, interact with other lipids of the membrane bilayer influencing its rigidity, fluidity and permeability (Abe and Hiraki, 2009; Baumann et al., 2011; Dupont et al., 2011). It has been demonstrated that squalene in combination with ergosterol is an important modulator of the fluidity of ER and plasma membranes (Spanova et al., 1821). Our results revealed that the squalene content in membranes was nearly doubled in the *ERG20*-expressing *T. atroviride*. Squalene has a stabilizing effect making membranes more rigid, but the crucial parameter is the ratio of ergosterol to squalene (Spanova et al., 1821).

The changes in membrane properties could in turn modify the activity of membrane-bound enzymes such as DPM synthase, found to be stimulated in this study. The increased activity of DPM synthase could produce an excess of dolichyl phosphate mannose required for *O*- and *N*-glycosylation of secretory enzymes. Our previous studies showed that an elevated intracellular concentration of GDP-mannose, another form of mannose needed for glycosylation, caused hyper-glycosylation of secreted proteins (Zakrzewska et al., 2003). In addition, the activity of the hyper-glycosylated cellulases was elevated (Kruszewska and Palamarczyk, unpublished). A similar correlation could obey in this study since we observed a higher glycosylation of secretory proteins and higher activity of the secreted cellulases.

The mycoparasitic action of *T. atroviride* against fungal pathogens involves hydrolytic enzymes and low-molecular-mass metabolites (Seidl et al., 2009; Atanasova et al., 2013). The *ERG20* transformants showed an enhanced antifungal effect against *Pythium* which was due to the higher activity of hydrolases secreted by the transformants, as confirmed by a direct analysis of their activity. The metabolites secreted to the medium by the transformants did not differ in their antifungal action from those secreted by the non-transformed strain.

The most pronounced differences between the *ERG20* transformants and the non-transformed *Trichoderma* concerned their biocontrol activity against the plant pathogen *P. ultimum*. The supreme antifungal activity of the transformants compared to the control strain doubled the germination rate of bean seeds and slightly improved the growth of seedlings in *P. ultimum*-infected soil.

5. Conclusions

Present results indicate a crucial role of the mevalonate pathway for the biocidal properties of *Trichoderma*. By a simple manipulation of that pathway we have constructed *Trichoderma* strains with greatly enhanced antifungal and biocontrol properties. Our results showed that one copy of the yeast *ERG20* gene expressed in *Trichoderma* strain was optimal to raise the FPP synthase activity, which resulted in a moderately higher activity of DPM synthase and protein glycosylation. This *ERG20* transformant showed the highest secreted hydrolytic activity, was the most efficient in confrontation with *P. ultimum* and had the best antifungal and biocontrol properties.

CRediT authorship contribution statement

Sebastian Graczyk: Investigation. **Urszula Perlińska-Lenart:** Investigation. **Wioletta Górka-Nieć:** Investigation. **Renata Lichota:** Investigation. **Sebastian Piłsyk:** Investigation. **Patrycja Zembek:** Investigation. **Jacek Lenart:** Investigation. **Przemysław Bernat:** Investigation. **Elżbieta Gryz:** . **Justyna Augustyniak:** Investigation. **Grażyna Palamarczyk:** Conceptualization, Writing - review & editing. **Joanna S. Kruszewska:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2020.103334.

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