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ARTICLE

Hygromycin B, carboxin and nourseothricin susceptibility of polyunsaturated fatty acid producing *Mortierella* and *Umbelopsis* strains

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ABSTRACT *Mortierella* and *Umbelopsis* species are particularly active in polyunsaturated fatty acid (PUFA) synthesis as they are able to produce many ω-3 and ω-6 PUFAs. Genetic manipulation of the lipid production to generate PUFA overproducing strains and strains with altered PUFA profile requires well-established transformation systems and reliable selectable markers. Therefore, we screened different antifungal agents, which can be used for selection in further transformation experiments. Hygromycin B, carboxin, pyrithiamine and nourseothricin susceptibility of several *Mortierella* and *Umbelopsis* isolates was investigated using a broth microdilution method. Pyrithiamine was totally ineffective against all isolates while the other three antifungal agents were active against *Mortierella* and *Umbelopsis* strains. Several *Mortierella* isolates represented high sensitivity to hygromycin B whilst nourseothricin was rather active against *Umbelopsis* species. Carboxin inhibited the hyphal growth and the spore germination of all isolates completely in low concentrations.
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KEY WORDS
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Introduction

Members of the genus *Mortierella* and *Umbelopsis* are filamentous fungi belonging to Mucoromycotina and are particularly active in polyunsaturated fatty acid (PUFA) synthesis. PUFAs are elemental structural and functional components of biological membranes and they are precursors of a wide variety of metabolites including prostaglandins, leukotrienes and hydroxy-fatty acids regulating critical biological functions (Innis 1991; Horrobin 1995). The ω-3 fatty acids reduce the risk of the development of cardiovascular diseases as they have several potentially cardioprotective effects, such as antiarrhythmic, antithrombotic, antiatherosclerotic and anti-inflammatory effects. Besides this, they can lower the triglyceride concentration and blood pressure and improves endothelial functions (Din et al. 2004). Docosahexaenoic acid plays an important role in the proper development of brain and retina in infants (Ward and Singh 2005), and recent studies demonstrated its anti-cancer effects and its possible role in the prevention of Alzheimer disease as well (Connor and Connor 2007; Serini et al. 2011; Siddiqui et al. 2011). Arachidonic acid (AA) presents in organs, muscle and blood tissue and has a major role as a structural lipid. It is also the main ω-6 fatty acid in the brain, so it is also important in infants' brain development. AA is also a direct precursor of eicosanoids, which play important roles in the lipoprotein metabolism and platelet activation (Ward and Singh 2005). Besides this, it protects pancreatic β cells against alloxan-induced diabetes and the harmful effects of oxidative stress (Suresh and Das 2006).

Oleaginous microorganisms, as alternatives to agricultural and animal oil products, have been intensively studied. *Mortierella alpina* is one of the most important industrial PUFA producers synthesizing mainly AA besides other ω-3 and ω-6 PUFAs, which can be used as pharmaceuticals or food additives (Higashiyama et al. 2002; Dyal and Narine 2005; Sakuradani et al. 2009). A number of other *Mortierella* species also seemed to be promising producers (Eroshin et al. 1996; Higashiyama et al. 2002).

The long-term aim of our work is to modify the lipid production of different *Mortierella* and *Umbelopsis* strains to generate PUFA overproducing strains and strains with altered PUFA profile. This genetic manipulation requires well-established and reliable selectable markers and transformation systems. In previous transformation experiments only *M. alpina* and *Umbelopsis isabellina* strains were investigated. These experiments mainly aimed at testing of different selection and transformation methods (MacKenzie et al. 2000; Takeno et al. 2005b; Zhang et al. 2007b; Ando

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et al. 2009a; Wei et al. 2010). However, some publications reported on the practical use of transformation methods to investigate or modify the PUFA production (Takeno et al. 2005a; Zhang et al. 2007a; Ando et al. 2009b). At the same time, no publication has been found about the transformation or the development of appropriate selection methods for other *Mortierella* and *Umbelopsis* species. As antibiotics are the most commonly used selective agents, we would like to elaborate an efficient selection method based on dominant antibiotic resistance markers for these fungi. Accordingly, the first step of our work was the screening for susceptibility against several antifungal agents.

In previous transformations of *M. alpina* and *U. isabellina*, hygromycin B (HygB) was an often used selective agent (MacKenzie et al. 2000; Zhang et al. 2007b; Wei et al. 2010). HygB is an aminoglycoside-type antibiotic produced by *Streptomyces hygroscopicus* inhibiting the protein synthesis of bacteria, fungi and higher eukaryotic cells as well (Gonzalez et al. 1978). Its growth inhibitory activity has been generally investigated in the range from 10 to 1000 μg/ml, and the suggested concentration range as a selective agent is 150-400 μg/ml for higher eukaryotes. Resistance to HygB is conferred by hygromycin B phosphotransferase gene (*hph*) isolated from *Escherichia coli* (Gritz and Davies 1983; Kuhstoss and Rao 1983).

Some *M. alpina* strains proved to be resistant to HygB, therefore, other antifungals or pesticides, such as zeocin and carboxin (CBX) were screened as a possible selective agent (Takeno et al. 2005b; Ando et al. 2009a). CBX is a systemic anilide fungicide inhibiting the respiratory chain of complex II (Ulrich and Mathre 1972). It is often used as a seed treatment to protect plants from smut, rot and blight. CBX is generally effective against basidiomycetes, ascomycetes and zygomycetes already at low concentrations; however, several basidiomycetes are CBX-resistant (Moore 2009). An amino acid substitution in the iron-sulphur protein subunit (SdhB) of succinate dehydrogenase confers CBX resistance (Skinner et al. 1998), so the mutant *sdhB* gene can be used as selectable marker in transformation experiments (Honda et al. 2000; Shima et al. 2008). The *sdhB* gene was originally isolated from *Ustilago maydis* (Keon et al. 1991); however, to transform *M. alpina*, the *CBXB* gene isolated from its genome was applied (Ando et al. 2009a).

Nourseothricin (NTC) is an aminoglycoside antibiotic produced by *Streptomyces noursei*, which is very effective against Gram-positive and Gram-negative bacteria, mycoplasma, protozoa, certain viruses and plants. It exerts weaker growth inhibitory effect against yeasts and filamentous fungi but it is exceptionally suitable for the selection of recombinant yeasts (Goldstein and McCusker 1999). NTC resistance is based on the nourseothricin N-acetyl-transferase (*nat1*) gene isolated from *S. noursei* (Krügel et al. 1993). NTC and the *nat1* gene were also successfully used for the selection of filamentous fungi as well (Kück and Hoff 2006; Alshahni et al. 2010), but it has never been applied at *Mortierella* species.

Pyrithiamine (PT) is a potent antagonist of thiamine, its administration inhibits the thiamine metabolism inducing serious neurological symptoms in human (Liu et al. 2006). PT has been found to inhibit the growth of yeasts and other ascomycetes. Its effect was investigated extensively in case of different *Aspergillus* species (Kubodera et al. 2002). The *ptrA* gene responsible for PT resistance was isolated from *Aspergillus oryzae*, its increased expression results in thiamine overproduction compensating the antagonistic effect of PT (Kubodera et al. 2000). The *ptrA* gene was successfully applied as selectable marker in transformation of various *Aspergillus* species, *Trichoderma reesei* and *Penicillium chrysogenum* (Kubodera et al. 2002; Janus et al. 2009).

As no data about NTC and PT susceptibility of *Mortierella* and *Umbelopsis* species was found in the literature, the aim of the present work was to investigate the *in vitro* antifungal activities of HygB, CBX, NTC and PT against various isolates of these species. Strains belonging to certain closely related oleaginous genera, such as *Dissophora*, *Gamsiella* and *Lobosporangium*, were also involved in the study.

Materials and Methods

Fungal strains

The investigated strains (31 *Mortierella*, 5 *Umbelopsis*, 2 *Dissophora*, 1 *Gamsiella* and 1 *Lobosporangium*) are listed in Table 1. All strains were maintained on malt extract agar (MEA: 1% malt extract, 0.5% yeast extract, 1% glucose, 2% agar) slants at 4 °C. Glucose-yeast medium (GY: 1% yeast extract, 2% glucose) was used for antifungal susceptibility testing. *Mortierella* strains were cultivated at 20 °C, except *M. histoplasmatoides* CBS 321.78, which together with *Lobosporangium transversale* CBS 357.67 and *Umbelopsis* isolates were cultivated at 25 °C.

Antifungal drugs

HygB (InvivoGen) and NTC (Jena Bioscience) were purchased as stock solutions (100 mg/ml in deionized water). CBX (Sigma-Aldrich) and PT (Sigma-Aldrich) were provided by the manufacturer as standard powders. CBX was dissolved in acetone at a concentration of 80 mg/ml, and PT was dissolved in distilled water at a concentration of 1 mg/ml. Stock solutions were stored at -20 °C until needed.

In vitro antifungal susceptibility testing

The *in vitro* antifungal activities of HygB, CBX, NTC and PT were determined using a broth microdilution method, which

Table 1. MICs of the investigated antifungal agents.

Name	Strain code	MIC values (µg/ml)			
		HygB	CBX	NTC	PT
Dissophora ornata	CBS 348.77	1600-3200	n.d.	200-400	>32
Dissophora decumbens	CBS 592.88	25	3.125	50-100	>32
Gamsiella multidivaricata	CBS 227.78	1600-3200	3.125	>400	>32
Lobosporangium transversale	CBS 357.67	6.25	n.d.	25	4-8
Mortierella acrotona	CBS 386.71	1600	3.125	400	n.d.
Mortierella alpina	CBS 210.32	3200	100-200	>400	>32
Mortierella alpina	FSU 2698	>3200	25-50	>400	>32
Mortierella amoeboidea	CBS 889.72	>3200	n.d.	>400	>32
Mortierella antarctica	CBS 609.70	400-800	25-50	100	>32
Mortierella beljakovae	CBS 123.73	n.d.	6.25-12.5	n.d.	n.d.
Mortierella capitata	CBS 648.68	25	>1600	>400	>32
Mortierella chlamydospora	CBS 120.34	100	n.d.	>400	>32
Mortierella cystojenkinii	CBS 456.71	>3200	25-50	>400	>32
Mortierella echinosphaera	CBS 575.75	50-100	12.5	200	n.d.
Mortierella exigua	CBS 655.68	1600-3200	50-100	>400	n.d.
Mortierella gamsii	CBS 749.68	1600-3200	100	>400	n.d.
Mortierella gamsii	CBS 253.36	200	3.125	100	n.d.
Mortierella gemmifera	CBS 134.45	100-200	25-50	1.56	n.d.
Mortierella globulifera	CBS 417.64	6.25	25-50	50-100	>32
Mortierella histoplasmatoides	CBS 321.78	6.25	3.125	6.25	n.d.
Mortierella indohii	CBS 720.71	25-50	n.d.	12.5	>32
Mortierella lignicola	CBS 313.52	200	12.5	400	>32
Mortierella minutissima var. *dubia*	CBS 307.52	200-400	n.d.	>400	n.d.
Mortierella parazychae	CBS 868.71	800	6.25	>400	n.d.
Mortierella parvispora	CBS 311.52	3200	25	>400	>32
Mortierella polycephala	CBS 456.66	25	50-100	25	>32
Mortierella rishikesha	CBS 652.68	3200	50-100	>400	n.d.
Mortierella sarnyensis	CBS 122.72	1600	25	>400	n.d.
Mortierella schmuckeri	CBS 295.95	>3200	100	>400	n.d.
Mortierella selenospora	CBS 452.88	>3200	100-200	>400	>32
Mortierella strangulata	CBS 455.67	1600	3.125-6.25	400	n.d.
Mortierella stylospora	CBS 211.32	100	200	>400	>32
Mortierella tuberosa	CBS 210.72	6.25	3.125	0.78	n.d.
Mortierella verticillata	CBS 374.95	3200	100-200	>400	>32
Mortierella zychae	CBS 102879	>3200	25	>400	>32
Umbelopsis angularis	CBS 603.68	400	>1600	50	>32
Umbelopsis autotrophica	CBS 310.93	400-800	>1600	>400	>32
Umbelopsis isabellina	NRRL 1757	100	100-200	100-200	>32
Umbelopsis ramanniana	NRRL 1296	400	>1600	200	>32
Umbelopsis vinacea	CBS 222.29	100	>1600	100	>32

Abbreviations: MIC – minimal inhibitory concentration, HygB – hygromycin B, CBX – carboxin, NTC – nourseothricin, PT – pyrithiamine, CBS – Centraalbureau voor Schimmelcultures, The Netherlands, FSU – Friedrich Schiller University, Jena, NRRL – Agricultural Research Service Culture Collection, USA, n.d. not determined.

was performed in accordance with the guideline of the Clinical and Laboratory Standards Institute (CLSI M38-A2 2008) with some modifications. Minimal inhibitory concentration (MIC) values were determined in 96-well flat-bottomed microtiter plate bioassays by measuring the optical density of the fungal cultures at 620 nm. The antifungal susceptibility of the fungal strains was investigated in GY medium instead of the suggested RPMI 1640 medium. For the investigation of the antifungal activity of CBX GY medium adjusted to pH 7.5 was used. The cultivation of the fungal strains was carried out at their optimal growth temperature.

Fungal spore suspensions were prepared from 7-day-old cultures grown on malt extract agar slants, and suspensions were diluted in GY medium to give a final inoculum of 1×10^5 spores/ml. Series of twofold dilutions were prepared in GY medium from HygB, NTC and PT stock solutions. Series of twofold dilutions of CBX were performed from the stock solution in acetone to yield one-hundredfold the final strength required for the tests and the intermediate solutions were further diluted in GY medium to twice the final strength. The drug dilutions were mixed with equal amounts of sporangiospore suspensions in the microtiter plates. In the wells,

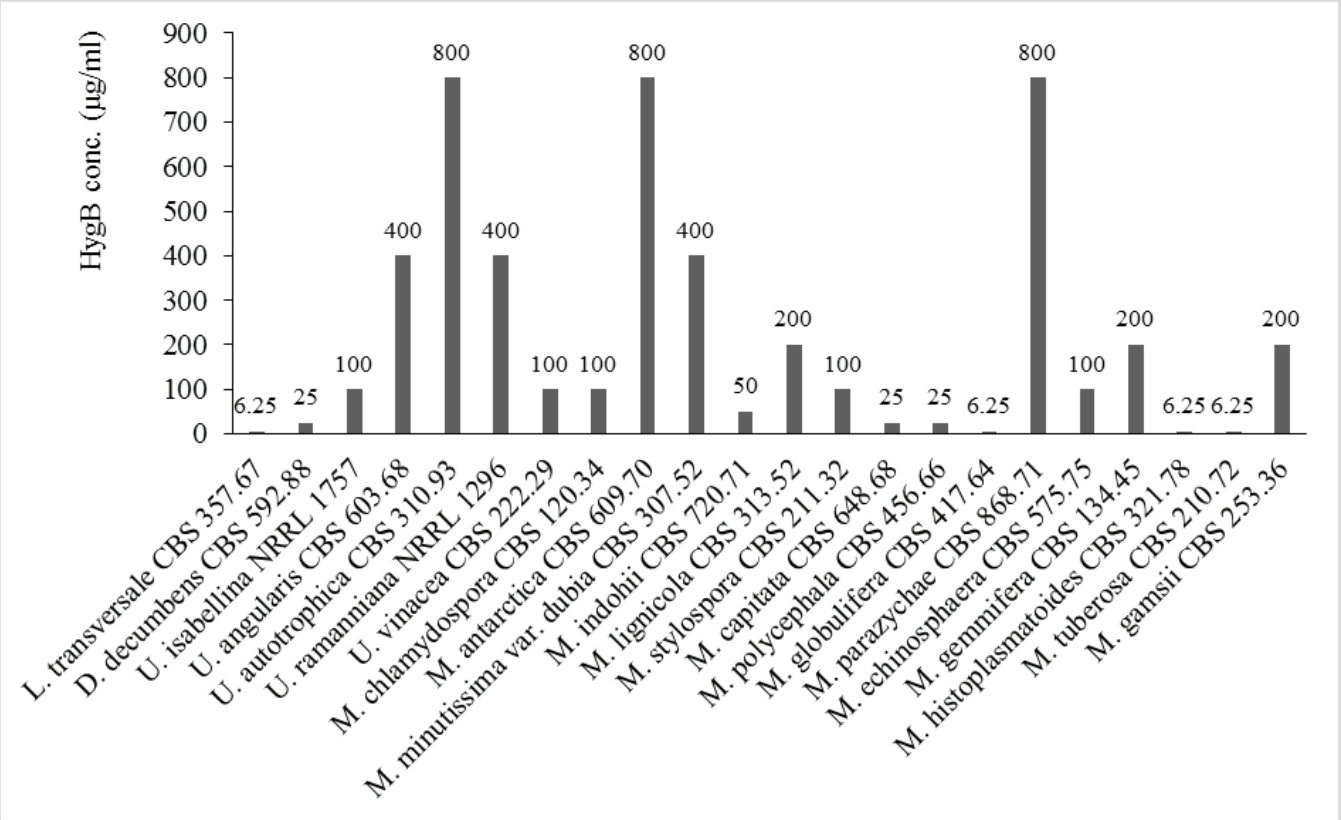

Figure 1. MIC values of HygB against the sensitive isolates.

the final concentrations for HygB ranged from 6.25 to 3200 µg/ml, for NTC ranged from 0.78125 to 400 µg/ml, for CBX ranged from 3.125 to 1600 µg/ml and for PT ranged from 0.0625 to 32 µg/ml, respectively.

The microtiter plates were incubated for 7 days at 20 or 25 °C, and the OD was measured at 620 nm with a microtiter plate reader (SPECTROstar Nano; BMG Labtech) after 42, 96 and 168 hour of incubation. Uninoculated medium was used as the background for the spectrophotometric calibration; the growth control wells contained inoculum suspension in the drug-free medium. The solvent control wells contained inoculum suspension in the drug-free acetone-containing (1%) medium to prove that acetone had no inhibitory effect on the investigated fungi at the applied concentration. For calculation of the extent of inhibition, the OD_{620} readings of the drug-free control cultures were referred to 100% growth. MICs for the antifungal agents were the lowest concentration of drugs that produced an optically clear well (over 90% growth inhibition). All experiments were repeated 3 times.

For the determination of the minimal fungicide concentrations (MFC) 10-10 µl samples were streamed over drug-free GY medium from the cultures incubated in the microtiter plates and plates were incubated for 7 days at 20 or 25 °C before evaluation.

Results and Discussion

Antifungal susceptibility of 40 isolates of *Umbelopsis*, *Mortierella* and related species was tested against four antifungal agents. The *in vitro* antifungal activities of HygB, CBX, NTC and PT were determined using a broth microdilution method, where the MIC values were determined by measuring the OD of the fungal cultures. The MICs of HygB, CBX, NTC and PT against the investigated fungal isolates are presented in Table 1. Minimal fungicide concentration (MFC) values were also investigated in order to determine the antifungal effect (fungistatic or fungicide) of the drugs (data not shown).

In our study, the inhibitory potential of HygB was studied in the range from 6.25 to 3200 µg/ml. Several isolates represented high sensitivity to HygB (Table 1) but about half of the isolates proved to be resistant to that in the tested concentration range. In Figure 1, the MIC values of HygB against the susceptible strains are presented. The most sensitive isolates were *L. transversale* CBS 357.67, *M. globulifera* CBS 417.64, *M. tuberosa* CBS 210.72 and *M. histoplasmatoides* CBS 321.78. Their growth could be inhibited by 6.25 µg/ml HygB. Other *Mortierella* strains were also sensitive to HygB in the range of 25-200 µg/ml. HygB was also effective against

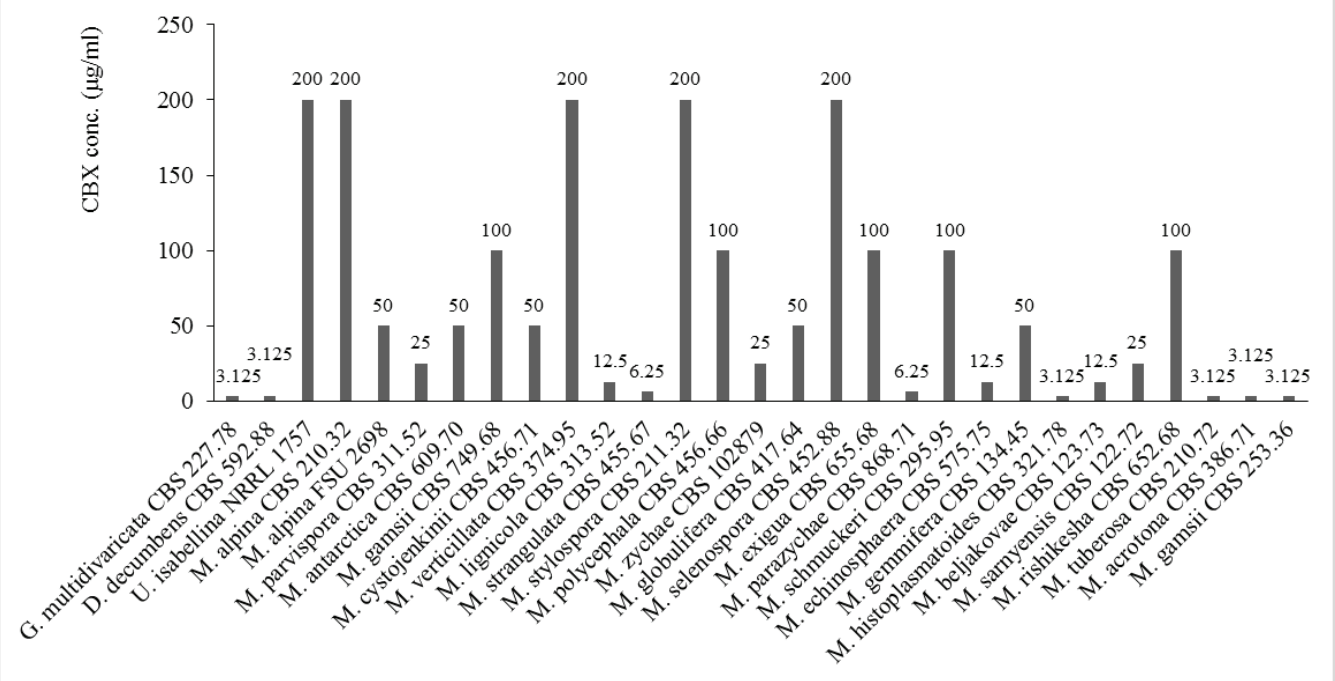

Figure 2. MIC values of CBX against the sensitive isolates.

Umbelopsis species in the range of 100-400 µg/ml, although, the growth of *U. autotrophica* CBS 310.93 could be inhibited just by 800 µg/ml HygB. Some *Mortierella* isolates also could be inhibited by higher HygB concentrations; for example, *M. parazychae* CBS 868.71 was sensitive to 800 µg/ml HygB and its spores could be totally killed only by 3200 µg/ml HygB. Anyway, the MIC and MFC values were found to be identical in all other cases, so HygB was proved to be fungicide for most of the sensitive isolates. HygB did not inhibit the growth of the investigated *M. alpina* isolates either at the highest administered concentration. Previous studies also reported that most of the *M. alpina* strains were resistant to HygB, only *M. alpina* CBS 224.37 was susceptible (MIC: 100-200 µg/ml) (MacKenzie et al. 2000; Takeno et al. 2005b). In the study of Zhang et al. (2007b), *U. isabellina* M6-22 isolate (named as *Mortierella isabellina* in that paper) was also resistant to HygB but a sensitive mutant (M6-22-4) was generated by N-methyl-N'-nitro-N-nitrosoguanidine treatment and this strain was used in the further transformation experiments. So, HygB seemed to inappropriate as a selective agent for *M. alpina* isolates, however it can be used for selection in case of certain *Mortierella* and *Umbelopsis* species.

CBX was very effective in the study of Ando et al. (2009a), as 100 µg/ml CBX inhibited the growth of the *M. alpina* 1S-4 *ura5*$^-$ strain. In our study, the inhibitory potential of CBX was studied in the range from 3.125 to 1600 µg/ml. CBX exerted its antifungal effect already at low concentrations (3.125-50 µg/ml) against most of the *Mortierella* isolates (Fig. 2). At the same time, *Umbelopsis* isolates showed high resistance against CBX. It was only effective against *U. isabellina* NRRL 1757 with a MIC of 200 µg/ml (Fig. 2). *M. alpina* CBS 210.32 also could be inhibited by 200 µg/ml CBX while *M. alpina* FSU 2698 was more sensitive (MIC: 50 µg/ml). Interestingly, CBX inhibited not only the hyphal growth and the spore germination but it completely killed the spores of the *M. alpina* isolates. For other isolates, CBX proved to be fungistatic, since it inhibited the hyphal growth already in low concentration, but the spores were able to germinate after the administration of 1600 or 3200 µg/ml CBX.

The inhibitory potential of NTC was studied in the concentration range from 0.78125 to 400 µg/ml. The antifungal effect of NTC was investigated previously against several yeasts and filamentous fungi and most of them showed susceptibility to NTC. The growth of *Saccharomyces cerevisiae* isolates was inhibited in the presence of 25 µg/ml NTC (Goldstein and McCusker 1999), and it was similarly effective against different filamentous ascomycetes fungi, like *Trichophyton mentagrophytes* (50 µg/ml) or *Acremonium chrysogenum* (25 µg/ml) (Kück and Hoff 2006; Alshahani et al. 2010). Although *Candida albicans* proved to be moderately susceptible to NTC (250 to 450 µg/ml) (Shen et al. 2005), it has successfully been used for the transformation of *C. albicans* and other *Candida* species. In our experiments, more than half of the investigated strains were resistant to

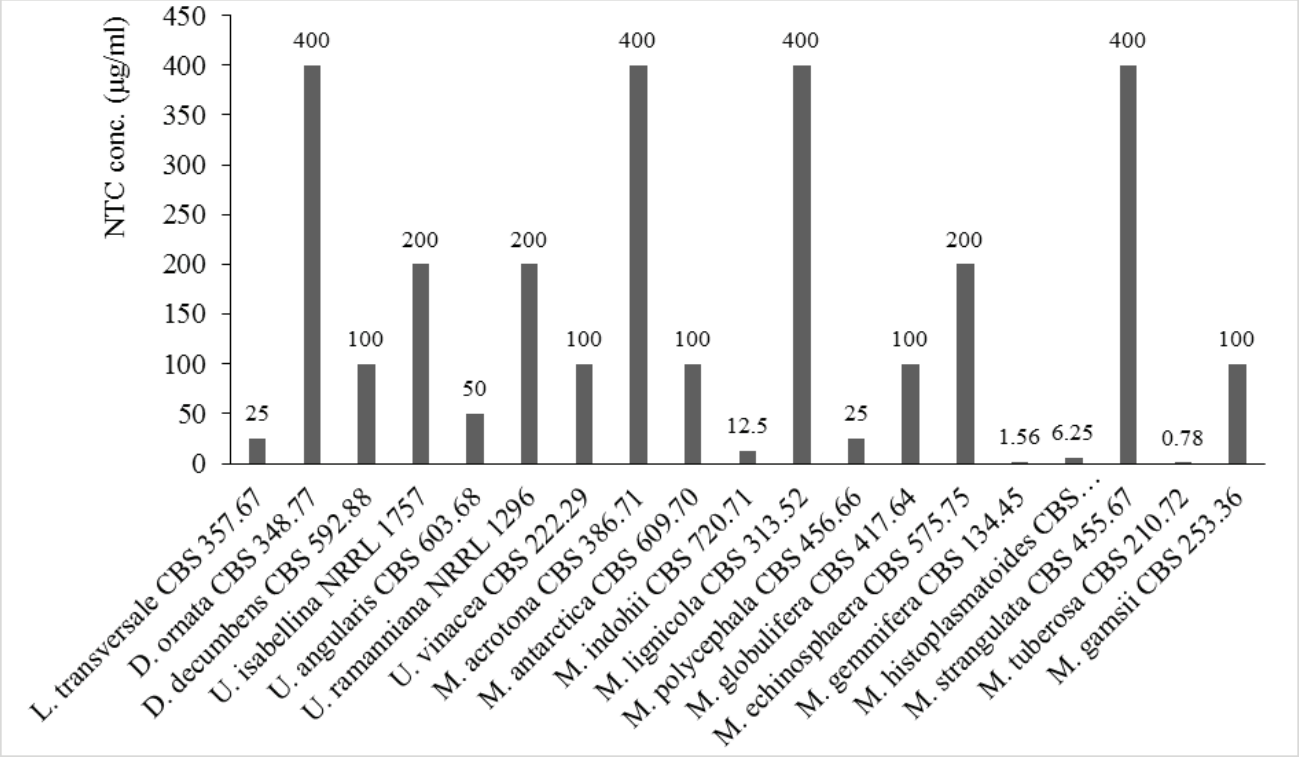

Figure 3. MIC values of NTC against the sensitive isolates.

NTC albeit some strains were extremely susceptible. In Figure 3 the MIC values of NTC in case of the susceptible strains are presented. The most susceptible isolates were *M. tuberosa* CBS 210.72 and *M. gemmifera* CBS 134.45; their growth was already inhibited in the presence of 0.78125 and 1.5625 µg/ml NTC. Some other *Mortierella* isolates, like *M. histoplasmatoides* CBS 321.78 (MIC: 6.25 µg/ml), *M. indohii* CBS 720.71 (MIC: 12.5 µg/ml) and *M. polycephala* CBS 456.66 (MIC: 25 µg/ml) showed similar susceptibility. The NTC-sensitive *Mortierella* isolates were also sensitive to HygB and CBX. NTC will be especially useful for the selection of *M. antarctica* CBS 609.70, which was only moderately susceptible to HygB (MIC: 800 µg/ml). *Umbelopsis* isolates (except *U. autotrophica* CBS 310.93) were also sensitive to NTC; however, the inhibitory effect could be achieved with the administration of higher NTC concentrations (50-200 µg/ml). NTC was fungicide as MIC and MFC values were found to be identical.

PT has been found previously to inhibit the growth of yeasts and filamentous fungi as well. It was tested against several members of the *Aspergillus* genus in the concentration range from 0.05 to 10 mg/l, and the fungal growth was inhibited even by 0.1 mg/l PT (Kubodera et al. 2002). In our work, the inhibitory potential of PT was studied in the range from 0.0625 to 32 µg/ml, but it did not affect the growth of *Mortierella* and *Umbelopsis* strains at all in the administered concentration range as most of the isolates could grow at 32 µg/ml PT as well as in the drug-free medium. Only two isolates, *L. transversale* CBS 357.67 and *M. antarctica* CBS 609.70 proved to be susceptible to PT. The growth of *L. transversale* CBS 357.67 could be totally inhibited in the presence of 8 µg/ml PT. This PT concentration also killed the spores of *L. transversale* CBS 357.67, so PT had a fungicide effect. *M. antarctica* CBS 609.70 proved to be moderately sensitive to this compound. Treatment with 0.5 µg/ml PT caused 50% growth inhibition, however, complete inhibition was not achieved in the tested concentration range. PT proved to be effective antifungal agent against *Aspergillus* species but other fungi, such as *Fusarium solani* and *Penicillium citrinum* also showed high PT resistance (Kubodera et al. 2002).

Summarizing our results, we can say that CBX seems to be an appropriate and widely usable selective agent in further transformation of different *Mortierella* species. In certain cases, HygB and NTC also can be applicable. However, HygB is unsuitable for the selection of transformants from the industrially used PUFA producing *M. alpina* isolates. This drug can be applied as selective agent for the transformation of other *Mortierella*, *Dissophora* and *Umbelopsis* species. NTC also

can be used in case of some *Mortierella* species, for example *M. antarctica*. *Umbelopsis* isolates proved to be resistant to CBX, however, HygB and NTC seems to be suitable for the selection of their transformants.

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