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Isolation of fungicide-resistant mutants from cold-tolerant *Trichoderma* strains and their *in vitro* antagonistic properties

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Abstract: Among 128 isolates of *Trichoderma*, two cold-tolerant strains possessing excellent *in vitro* antagonistic properties against plant pathogenic *Microdochium nivale*, *Fusarium culmorum*, *F. oxysporum* and *Pythium debaryanum* strains were selected for this study. Seven of the 16 pesticides tested – $CuSO_4$, Carbendazim, Mancozeb, Tebuconazol, Imazalil, Captan and Thiram – showed significant inhibition on the *Trichoderma* strains, the minimal inhibitory concentrations were approximately 300, 0.4, 50, 100, 100, 100 and 50 µg/ml, respectively. Mutants resistant to $CuSO_4$, Carbendazim, Mancozeb and Tebuconazol were isolated from a *T. harzianum* and a *T. aureoviride* strain by ultraviolet light mutagenesis. The cross-resistance capabilities and *in vitro* antagonistic properties of the mutants were determined in the presence of sublethal concentrations of different fungicides. Carbendazim-resistant mutants showed total cross-resistance to benomyl and thiabendazole at a concentration of 20 µg/ml, while tebuconazole-resistant strains tolerated epoxiconazole at the same level as tebuconazole. A great number of fungicide-resistant strains were found to be potential candidates for application in integrated pest management.

Key words: *Trichoderma*, mutagenesis, fungicide resistance, plant pathogenic fungi, antagonism

Introduction

Plant pathogenic fungi, e.g. *Fusarium*, *Pythium* and *Microdochium* species are able to cause great losses in wheat and corn fields even below the temperature of 10°C (Nakajima & Abe, 1996). Various pesticides are used against them successfully, but these chemicals are harmful to other organisms as well.

Trichoderma species are imperfect filamentous fungi with teleomorphs belonging to the *Hypocreales* order of the Ascomycota division. Their effective antagonistic abilities against plant pathogenic filamentous fungi are based on different mechanisms including competition, antibiosis and mycoparasitism (Manczinger et al., 2002; Papavizas 1985). Several *Trichoderma* species are effective agents to control plant pathogenic fungi, e.g. *Fusarium* (Sivan & Chet, 1986), *Pythium* (Naseby et al., 2000) and *Rhizoctonia* (Lewis & Papavizas, 1987) species, which allows for the development of biocontrol strategies.

As *Trichoderma* strains are potential biofungicides, integrated pest management strategies can be worked out based on their combined application with reduced amounts of fungicides. Furthermore, the presence of *Trichoderma* strains may have a positive effect on the germination of cereal seeds and on the growth of crops (Altomare et al., 1999). As commercial fungicides have an inhibitory effect on mycelial growth of biocontrol *Trichoderma* strains, the aim of this study was to isolate fungicide-resistant mutants for the purposes of integrated pest management.

Materials and methods

Strains and culture conditions

A number of 128 *Trichoderma* strains have been isolated from Hungarian soil samples and screened for cold-tolerance (Antal et al., 2000). Two cold tolerant isolates, *Trichoderma harzianum* T66 and *T. aureoviride* T122 were involved in this study. Plant pathogenic *Fusarium culmorum, F. oxysporum, Microdochium nivale* and *Pythium debaryanum* strains were selected from the culture collection of the Department of Microbiology, Faculty of Sciences, University of Szeged. All fungi were maintained on solid YEG medium (2 g/l yeast extract, 5 g/l glucose, 5 g/l KH_2PO_4, 20 g/l agar in distilled water). *In vitro* antagonism experiments were carried out on media containing 1 g/l yeast extract, 2 g/l glucose, 1 g/l KH_2PO_4, 1 g/l $MgSO_4$ and 20 g/l agar in distilled water.

Pesticides tested and determination of their minimal inhibitory concentration values

Minimal inhibitory concentration (MIC) values of the following 16 pesticides were determined for the two *Trichoderma* strains: MBC (carbendazim), captan, mancozeb, thiram (TMTD), tebuconazol, imazalil, fludioxonil, metalaxil, triadimefon, imidacloprid, Atrazin 500 FV, Acenit A880 EC, Erunit Profi, Prometrex 50 SC, Carboxin (all dissolved in dimethyl sulphoxide, 3 mg/ml) and $CuSO_4$ (dissolved in distilled water, 10 mg/ml). MIC-values were determined on solid YEG medium supplemented with pesticides in descending concentrations. Presence or absence of growth was examined after 4 days of incubation. Further 10 fungicides – thiabendazole, benomyl, propiconazol mixture, epoxiconazol, penconazol, bromuconazol, diniconazol, cyproconazol, itraconazol (Orungal) and ketoconazol (Nizoral) – were involved in cross-resistance studies.

Isolation of fungicide-resistant* Trichoderma *strains by UV mutagenesis

From the conidial suspension of the wild-type strains, 10^7 conidia were inoculated as a stripe onto solid YEG medium containing 1 µg/ml MBC, 500 µg/ml $CuSO_4$, 50 µg/ml mancozeb or 100 µg/ml tebuconazol and exposed to UV radiation for 30 sec.

Cross resistance of MBC-resistant and Tebuconazol-resistant mutants

From suspensions of MBC-resistant mutants 10^6 conidia were put in a drop onto solid YEG media containing MBC, benomyl or thiabendazole at the concentrations of 20, 10, 5 and 2.5 µg/ml. Tebuconazol-resistant mutants were inoculated similarly onto solid YEG media containing tebuconazol, diniconazol, epoxiconazol, cyproconazol, bromuconazol, penconazol, ketoconazol, propiconazol mixture or itraconazol at the concentrations of 100, 50, 25, 12.5 and 6.25 µg/ml. Growth of colonies was examined after 5 days of incubation.

In vitro *antagonism experiments*

Mycelial disks derived from the colonies of *F. culmorum, F. oxysporum, M. nivale* and *P. debaryanum* were put onto solid YEG media containing MBC or $CuSO_4$ in sublethal concentrations given in Table 1. After incubation for 2-5 days (depending on the growth of the plant pathogens), mycelial disks from MBC- and $CuSO_4$-resistant *Trichoderma* strains were put onto the medium 30 mm apart from the plant pathogenic strains.

Table 1. Sublethal concentrations of fungicides applied in the media

	F. culmorum	*F. oxysporum*	*M. nivale*	*P. debaryanum*
MBC (µg/ml)	1	1	0.8	0.05
$CuSO_4$ (µg/ml)	250	250	125	80

Table 2. MIC-values of fungicides on *T. harzianum* T66, *T. aureoviride* T122 and on plant pathogenic fungi (μg ml^{-1})

	MBC	$CuSO_4$	Mancozeb	Tebuconazol	Captan	Thiram	Imazalil
T. harzianum	0.4	300	50	100	100	50	100
T. aureoviride	0.4	300	50	100	100	50	100
F. culmorum	2	500	2	0.5			
F. oxysporum	2	500	2	3			
M. nivale	1.5	300	0.7	2			
P. debaryanum	0.1	200	1	1			

Results and discussion

MIC-values of the distinct pesticides

Fludioxonil, Metalaxil, Triadimefon, Imidacloprid, Atrazin 500 FV, Acenit A880 EC, Erunit Profi, Prometrex 50 SC and Carboxin showed no significant inhibition on the *Trichoderma* strains. Table 2 shows the MIC-values of seven fungicides on two *Trichoderma* strains and four fungicides on the examined plant pathogenic fungi. The MIC-values of MBC and $CuSO_4$ proved to be higher for three and two plant pathogenic fungi, respectively, than for the wild-type *Trichoderma* strains. Mancozeb and tebuconazole had much higher MIC-values for the *Trichoderma* strains than for the examined plant pathogenic fungi, suggesting that these compounds could be appropriate for the combination with biocontrol *Trichoderma* strains within the frames of complex integrated pest management.

The purpose of the further work was to develop *Trichoderma* strains resistant to MBC and $CuSO_4$, which proved to be the fungicides with the greatest inhibitory effect on the examined *Trichoderma* strains.

Isolation of fungicide-resistant Trichoderma strains by UV mutagenesis

Resistant sectors showing intensive growth were isolated and maintained on solid YEG medium. The level of resistance was checked several times and it proved to be stable in the case of the mutant strains. The numbers of stable resistant mutants isolated from *T. harzianum* T66 and *T. aureoviride* T122 are indicated in Table 3. These mutant strains seemed to be appropriate for *in vitro* antagonism experiments against plant pathogenic fungi in the presence of the corresponding fungicides.

Cross resistance

MBC-resistant mutants showed total cross-resistance to benomyl and thiabendazole, while tebuconazole-resistant strains tolerated epoxiconazole at the same level as tebuconazole. We suppose that similar resistance mechanisms may reveal the background of cross resistance to compounds with similar structures and modes of action.

In vitro *antagonism in the presence of MBC and $CuSO_4$*

Growth of the examined plant pathogenic fungi in the presence of MBC-resistant and $CuSO_4$-resistant *Trichoderma* strains and the corresponding fungicides in sublethal concentrations is shown in Table 4. A significant reduction of mycelial growth of the pathogen was observed in all examined mutant - plant pathogen combinations.

As the fungicide tolerance and antagonistic properties of the examined fungicide-resistant *Trichoderma* mutants are promising, they will be used in further studies aiming the development of fungicide-poliresistant strains for the purposes of integrated pest management.

Table 3. Number of stable resistant mutants isolated from *Trichoderma* strains T66 and T122

	MBC	$CuSO_4$	Mancozeb	Tebuconazol
T66	3	3	1	5
T122	10	3	2	4

Table 4. Growth of plant pathogenic fungi in the presence of MBC-resistant (MBC^R) and $CuSO_4$-resistant ($CuSO_4^R$) *Trichoderma* strains and the corresponding fungicides in sublethal concentrations

	F. culmorum	*F. oxysporum*	*M. nivale*	*P. debaryanum*
MBC + MBC^R T66	22[a]	13	46	21
MBC + MBC^R T122	27	13	33	19
$CuSO_4$ + $CuSO_4^R$ T66	33	29	16	0
$CuSO_4$ + $CuSO_4^R$ T122	33	31	20	0

[a] Data are presented in percentage of growth on media without fungicides and *Trichoderma*.

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