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Effects of abiotic and biotic factors on *Trichoderma* strains with biocontrol potential

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Abstract: The effects of low temperature, low water potential, pesticides, heavy metals and antagonistic bacteria on *Trichoderma* strains with biocontrol potential were examined *in vitro*.

Cold-tolerant strains growing well at 5°C were isolated from soil samples. In dual culture tests at 10°C, most strains antagonized plant pathogenic fungi. The activities of extracellular enzymes important for mycoparasitism were significant at 5°C in the cold-tolerant strains.

Nearly linear correlation was found between water potential and colony growth rate with higher growth rates at higher water potential. Secretion of the enzymes also depended on the water potential of the liquid media. However, significant *in vitro* activities were measured for most of the enzymes even at water potential values below the limit of mycelial growth.

The effects of seven pesticides on *Trichoderma* strains were also examined. The fungicide susceptibility of the strains may cause problems during their combined application with antifungal compounds. For such purposes fungicide resistant mutants should be applied.

The examined heavy metals showed inhibition of mycelial growth, but the extracellular enzyme systems of *Trichoderma* strains remained active even at heavy metal concentrations, where mycelial growth was already strongly inhibited. Heavy metal resistant mutants were isolated by UV-mutagenesis and tested for possible cross-resistances. Some of the mutants were effective antagonists of plant pathogenic fungi even on media containing the respective heavy metals.

Eighteen *Trichoderma* isolates were tested for their ability to degrade heat-inactivated Gram-positive and Gram-negative bacteria. In addition to trypsin-like protease, chymotrypsin-like protease and β-1,4-*N*-acetyl-glucosaminidase enzymes, muramidase-like activities were also present in the induced ferment broth of a *T. harzianum* strain.

Key words: *Trichoderma*, biocontrol, environmental factors, extracellular enzymes

Introduction

Trichoderma species are imperfect fungi, with teleomorphs belonging to the ascomycete order *Hypocreales*. Their mycoparasitic ability against plant pathogenic fungi allows for the development of biocontrol strategies based on *Trichoderma* strains (Manczinger, 1999; Manczinger *et al.*, 2002). Such strategies can be incorporated in a complex integrated plant protection. When planning the application of biocontrol strains, it is very important to consider the environmental stresses affecting microbial activities. Low temperature, low water potential, the presence of heavy metals or pesticides and antagonistic bacteria in the soil are among the most important stress factors. The study of the influence of such parameters on *Trichoderma* strains is of great importance: biocontrol strains should have better stress tolerance levels than the plant pathogens against which they are going to be used during biological control.

Materials and methods

Strains

The 360 *Trichoderma* strains were isolated from Hungarian soil samples. *Fusarium culmorum, Fusarium oxysporum* f. sp. *dianthi, Pythium debaryanum* and *Rhizoctonia solani* strains were obtained from the strain collection of the Cereal Research Non-Profit Company, Szeged. The strains of bacteria (*Bacillus cereus* var. *mycoides, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa* and *Serratia marcescens*) used in the experiments were derived from the Microbiological Collection of the University of Szeged (SZMC).

Experimental procedures

Culture media and methods applied for the investigation of the effects of low temperature (Antal *et al.*, 2000), water potential (Kredics *et al.*, 2000), heavy metals (Kredics *et al.*, 2001a, b) and antagonistic bacteria (Manczinger *et al.*, 2002) (colony growth measurement, test of *in vitro* antagonism, measurement of enzyme activities, isolation and characterization of mutants, testing of bacterium-degrading ability, Sephadex G-150 column chromatography, etc.) were described earlier.

Pesticides (benomyl, MBC, dicloran, fenuron, fluometuron, monuron and diuron) derived from Aldrich. Susceptibility testing was performed on solid medium (1% glucose, 0.5% KH_2PO_4, 0.1% $NaNO_3$, 0.2% yeast extract, 0.1% $MgSO_4 \times 7H_2O$) supplemented with pesticides in different concentrations in the following ranges: benomyl 0-2 μg/ml, MBC: 0-2 μg/ml, dicloran: 0-10 μg/ml, fenuron: 0-700 μg/ml, fluometuron: 0-1000 μg/ml, monuron: 0-1000 μg/ml, diuron: 0-50 μg/ml.

Results and discussion

Cold tolerance of biocontrol Trichoderma *strains*

Of 360 *Trichoderma* strains investigated, 14 - identified as *T. aureoviride*, *T. harzianum* and *T. viride* - grew well at 5°C on both minimal and yeast extract agar media. The incidence of cold-tolerant isolates was the highest in species group *T. viride*. In dual culture tests at 10°C, all cold tolerant strains produced appressoria and antagonized the plant pathogens *R. solani* and *F. oxysporum* f. sp. *dianthi*. *T. aureoviride* and *T. viride* strains were more effective against *P. debaryanum* than *T. harzianum* strains. The activities of extracellular β-1,4-*N*-acetyl-glucosaminidase (NAGase), β-glucosidase and trypsin- and chymotrypsin-like proteases - thought to be involved in the mycoparasitic process - were also examined and results showed that these enzymes were highly active at 5°C in the cold-tolerant strains (Antal *et al.*, 2000).

Influence of water potential on strain Trichoderma harzianum *T66*

Influence of water potential on linear mycelial growth, secretion and *in vitro* activities of *β*-glucosidase, cellobiohydrolase, β-xylosidase, NAGase and chymotrypsin-like protease enzymes of the cold-tolerant *T. harzianum* strain T66 was studied at different temperatures (Kredics *et al.,* 2000). Nearly linear correlation was found between water potential and colony growth rate at both 25°C and 10°C with higher growth rates at higher temperature and water potential. Optimal water potential values for the secretion of β-glucosidase, cellobiohydrolase, β-xylosidase, NAGase and chymotrypsin-like protease enzymes were different. Cellobiohydrolase and NAGase enzymes showed optimal secretion at the highest examined water potential, while the maximum activities of secreted β-glucosidase, β-xylosidase and chymotrypsin-like protease enzymes occurred at lower water potential values than those optimal for growth. The *in vitro* enzyme activities were affected by water potential, but significant enzyme activities were measured for most of the enzymes even at -14.8 MPa which is below the water potential, where mycelial growth ceased. These results suggest the possibility of using mutants with improved xerotolerance for biocontrol purposes in soils with lower water potential.

Effects of pesticides and heavy metals on biocontrol* Trichoderma *strains

The effects of three fungicides (benomyl, MBC, dicloran) and four herbicides (fenuron, fluometuron, monuron, diuron) were examined on the growth of *T. aureoviride* T122, *T. harzianum* T66 and T334, and *T. viride* T124 and T228 strains. The IC_{50} concentrations are presented in Table 1. In the case of diuron, 50% inhibition could not be reached, and the IC_{50} concentrations were found to be so high for the other herbicides, which values can not be present in the soil during their application. However, the fungicide susceptibility of the strains may cause problems during their combined application with antifungal compounds. For such purposes fungicide resistant mutants should be applied.

Table 1. IC_{50} concentrations of the examined pesticides in μg/ml. ND: not determinable

Strain	benomyl	MBC	dicloran	fenuron	fluometuron	monuron
T66	0.52	0.26	9.75	385.00	200.00	170.00
T114	0.38	0.21	11.10	618.00	580.00	430.00
T122	0.45	0.22	ND	580.00	430.00	275.00
T124	0.48	0.21	12.25	515.00	575.00	340.00
T228	0.45	0.24	7.80	515.00	535.00	215.00
T334	0.41	0.21	ND	420.00	380.00	325.00

The effect of ten metals (aluminium, copper, nickel, cobalt, cadmium, zinc, manganese, lead, mercury and iron) on mycelial growth and on the *in vitro* activities of trypsin-like protease, chymotrypsin-like protease, NAGase, β-1,3-glucanase, β-glucosidase, cellobiohydrolase, β-xylosidase and endoxylanase enzymes was also investigated in the case of these six strains (Kredics *et al.,* 2001a, b). Mycelial growth was significantly influenced by the heavy metals. The lowest IC_{50} values were found for copper, while the highest were for aluminium. In a concentration of 1 mmol only mercury inhibited the examined extracellular enzymes significantly, in the case of the other metals the enzymes of *Trichoderma* could remain active even at concentrations inhibiting mycelial growth, suggesting that breeding for heavy metal resistant *Trichoderma* strains could result in biocontrol agents effective against plant pathogenic fungi even under heavy metal stress.

A total number of 177 metal resistant mutants were isolated by UV-mutagenesis and tested for possible cross-resistances (Kredics *et al.*, 2001b). Significant cross-resistance was found in the case of the aluminium- and nickel-resistant mutants to copper and in the case of copper resistant ones to nickel. Some of the mutants were effective antagonists of plant pathogenic *F. culmorum*, *F. oxysporum* f. sp. *dianthi*, *P. debaryanum* and *R. solani* strains even on media containing the respective heavy metals. Such mutants might be the preferred choice for combined application with heavy metal-containing pesticides in the frame of a complex integrated plant protection.

Bacterium-degrading ability of biocontrol* Trichoderma *strains

Eighteen *Trichoderma* strains were screened for their ability to degrade bacterial cells (Manczinger *et al.*, 2002). The specificity spectrum and the intensity of degradation were highly variable. In the case of five strains showing outstanding degrading abilities towards *B. subtilis*, the NAGase, trypsin-like and chymotrypsin-like protease activities were determined under inductive and non-inductive circumstances. All strains were able to produce NAGase and proteases constitutively at a moderate level, which could be elevated by induction with *B. subtilis* cells: 3-6 times more NAGase and proteases were produced in inductive media.

The inductive ferment broth of an outstanding strain, *T. harzianum* T19, was fractionated on a Sephadex G-150 column. The strain produced at least 3 trypsin-like proteases, 6 chymotrypsin-like proteases, and 4 NAGases upon induction with *B. subtilis* cells. Muramidase-like activities were also present in the ferment broth of this *T. harzianum* strain.

These results indicate that bacterium-degrading ability is common, but highly variable among *Trichoderma* strains. Proteases, NAGases and muramidases seem to have great importance in the degradation of bacterial cells.

In addition to testing their ability to antagonize plant pathogenic fungi, the determination of their bacterium-degrading capabilities may also be useful in the evaluation of biofungicide *Trichoderma* strains, as perhaps this property can help the strains to be dominant microorganisms in the habitats where they are applied.

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