Species Composition of Indigenous *Trichoderma* Fungi Affected by Cd, Ni and Zn Heavy Metals in Calcareous Chernozem Soil

[1]Z. NAÁR and [2]B. BIRÓ

[1]Eszterházy Károly College, Department of Plant Sciences, Eger (Hungary) and
[2]Research Institute for Soil Science and Agricultural Chemistry (RISSAC) of the Hungarian Academy of Sciences, Budapest

The interest has been growing recently for studying the role and functioning of various microbial communities under environmental stress conditions. Among the abiotic stress factors, the heavy metals and/or toxic elements are the most frequently investigated (BIRÓ et al., 1996, 2005; KANDELER et al., 2000; ELLIS et al., 2001; MÜLLER et al., 2001; VÁRADY et al., 2002; VIVAS et al., 2003). The processes contributing to soil conditions, however, have mainly remained unexplored, due to the complex relationships among the compartments of soil and the different microbial, soil biological parameters. The *Trichoderma* population of soil seems to be a good candidate for such studies, as their assessment is simple both quantitatively and qualitatively. *Trichoderma* fungi are among those few saprophytic soil microbes that are extensively investigated nowadays regarding their inevitable influence on the various microbial populations, more particularly the plant symbiotic and pathogenic fungi (NAÁR et al., 2002; KUBICEK et al., 2001). Their coexistence in various soils has proven to be a common phenomenon (DOMSCH et al., 1980; WIDDEN, 1980; NAÁR & DOBOS, 2006). Considering the actively growing hyphae of *Trichoderma* species, the habitat competence among the various environmental (stress) factors can be more successful for these fungi, in comparison with other microbial components of the soil–plant environment (PIANKA, 1978). The effect of soil temperature (WIDDEN & ABITBOL, 1980; WIDDEN, 1984) and some other abiotic parameters (NAÁR & DOBOS, 2006) were investigated as potentials for niche (habitat) separation. Among the heavy metals and/or toxic elements copper (DUFFY et al., 1997), manganese (BULLUCK et al., 2002) and zinc (NAÁR & DOBOS, 2006) were reported to have influence on the frequency of the introduced *Trichoderma* fungi in the soil. NAÁR et al. (2002) found a significant species-loss at some low metal loads, where the loss of rare species, such as the *T. viride* could be recorded after 8 years of metal application.

In the present study the effect of long-term Cd, Ni and Zn loads on the most frequent six *Trichoderma* species were investigated at different metal ratios to clarify the role of metals in the interactions with other soil biotic, abiotic factors.

Correspondence to: ZOLTÁN NAÁR, Eszterházy Károly College, Department of Plant Sciences, H-3300 Eger, Leányka u. 4. Hungary. *E-mail:* naarzoltan@t-online.hu

Materials and Methods

Soil samples originated from a long-term field experiment of RISSAC set up in Nagyhörcsök (Hungary) (KÁDÁR, 1995). The calcareous chernozem soil (pH_{KCl}: 7.2; $CaCO_3$: 5.5%; humus: 3%; sand:silt:clay ratio: 2:2:1) was artificially contaminated with salts of trace elements in 1991 at rates of 90, 270 and 810 $kg \cdot ha^{-1}$ (30, 90 and 270 mg $metals \cdot kg^{-1}$ dry soil). Samples collected in 2003 were air-dried and remoistened to 60% of the maximum water holding capacity, and were kept on this level throughout the experiment. The samples were incubated at room temperature for two weeks, to reach equilibrium beyond the fluctuating environmental conditions.

To assess the density and species composition of the *Trichoderma* population, soil particles (about 1–2 mm) were placed on a *Trichoderma*-selective agar (ASKEW & LAING, 1993). Twenty particles were placed on each of the three agar plates in a 120 mm diameter. Cultures were incubated for 14 days at room temperature in daylight. Preceeding the isolation of all developed colonies, the rate of *Trichoderma*-colonized soil particles was also recorded. Isolates were maintained on potato-dextrose agar (PDA) slants until identification. Colonies growing on 2% malt extract agar and oatmeal agar were examined macro- and microscopically and identified according to RIFAI (1969) and BISSETT (1991a,b,c, 1992). Frequency of species was calculated as the percent of colonized soil particles, originating from the metal polluted soils.

Interrelations between the particular heavy metal loads and the indigenous *Trichoderma* species and frequency were calculated with correlation regression analysis (CA). The differences between the initial two-factorial correlation data and the partial correlation coefficients were estimated after eliminating the effects of heavy metals. Such techniques are also commonly used in casual modelling studies. The partial correlation among the independent (i) and the dependent (j) factors is estimated, when controlling or equalising a third (k) or other additional factors. The partial correlation, r(ij,k) may be equal, lower, or higher than the original correlation, r(ij). There is no effect when the original (CA) and partial correlations (PCA) are equivalent in their value. Suppression occurs when PCA data are higher than the two-factorial (CA) correlation, r(ij) (MCPHERSON, 1990). In the present trial the differences between the two correlation types were treated as follows: If $[r(ij)-r(ij,k)] < 0.25$, then no heavy metal effect on the particular intraspecific relationship was suspected. The effect of metals was partly considered when $0.25 < [r(ij)-r(ij,k)] < 1.0$ was found. A strong suppression of metals was accepted, if $[r(ij)-r(ij,k)]>1.0$. Statgraphics 5.1 software was used for all analyses.

Results

Trichoderma fungi were present in all soil samples, with the exception of the highest cadmium load (270 mg $Cd \cdot kg^{-1}$ soil), from which only two strains were identified as *Fusarium* sp. on the applied medium. Among 278 isolates 93.5%

proved to be *Trichoderma*, while others belonged to the genera of *Gliocladium* and *Fusarium*. Six species of *Trichoderma* fungi were identifiable from the control soil (Fig. 1). The abundance of *Trichoderma* fungi was quite low (25%) in the uncontaminated control soil. Some heavy metal treatments, however, significantly affected both the density and the species composition of the *Trichoderma* population. The frequency ranged between 0–3.3%, 0–31.7%, 0–3.3%, 0–1.7%, 0–16.7% and 0–26.7% for *T. atroviride* KARSTEN, *T. harzianum* RIFAI; *T. pubescens* BISSETT; *T.*

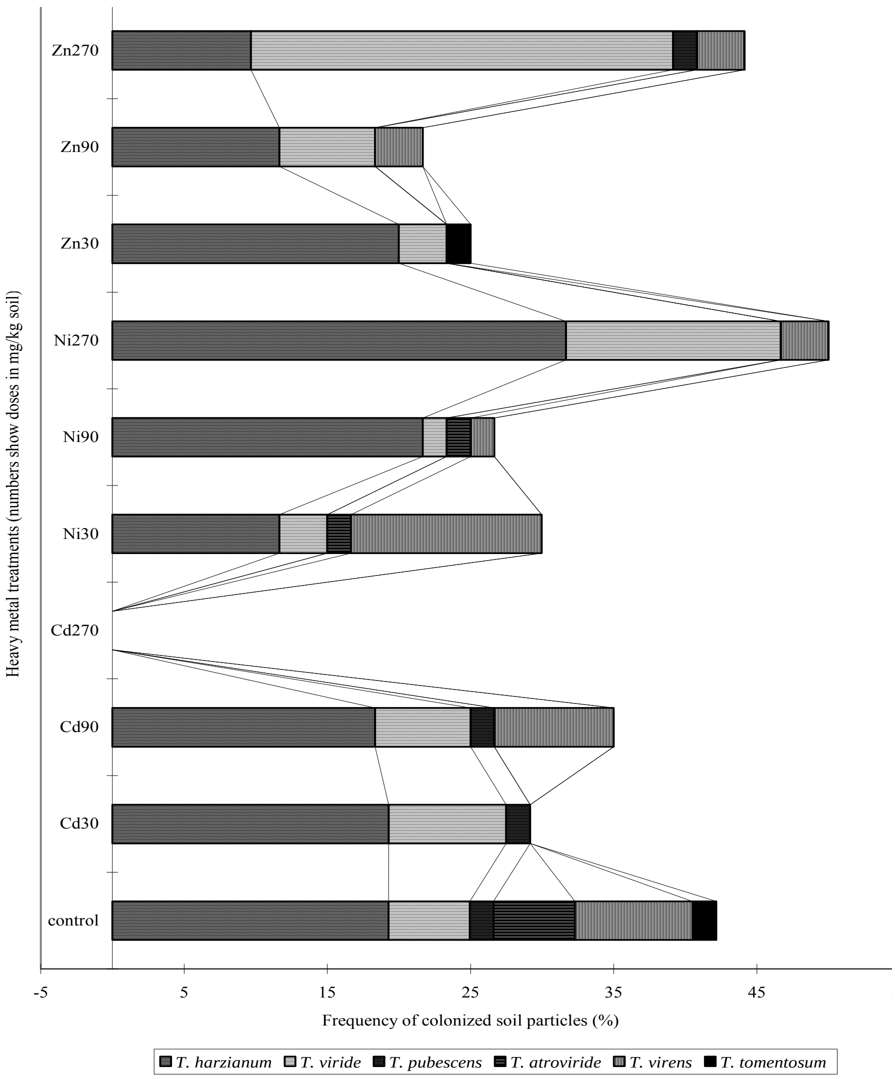

Fig. 1

Species composition of the *Trichoderma* population in the calcareous chernozem soil (Nagyhörcsök) 12 years after applying Zn, Ni and Cd loads (30, 90 and 270 $mg \cdot kg^{-1}$ dry soil) *tomentosum* BISSETT; *T. virens* MILLER von ARX and *T. viride* PERS ex GRAY, respectively. The highest density occurred in the case of the highest nickel and zinc loads (270 mg·kg^{-1} soil), indicating the relatively high general environmental tolerance of *Trichoderma* fungi to heavy metals.

A significant correlation was established between the available heavy metal content of soil and the *species composition* of the *Trichoderma* fungi (Table 1). The

Table 1
Correlation between the available heavy metal content of soil and the frequency of *Trichoderma* spp.

Heavy metal	*Trichoderma* species					
	T. harzianum	*T. virens*	*T. pubescens*	*T. atroviride*	*T. viride*	*T. tomentosum*
Cd	-0.931*	-0.953*	-0.952*	-0.975*	n.s.	n.s.
Ni	0.955*	0.964*	n.s.	n.s.	n.s.	n.s.
Zn	n.s.	0.955*	n.s.	n.s.	n.s.	-0.965*

density of *T. harzianum*, *T. viride*, *T. pubescens* and *T. atroviride* was strongly reduced concomittant with the increasing concentration of cadmium. The latter species seemed to be the most sensitive to metal contamination. No significant correlation was found, however in the case of *T. virens* and *T. tomentosum*. The relationship between nickel contamination and *Trichoderma* spp. showed an opposite tendency. The abundance of *T. harzianum*, *T. viride* significantly increased as a function of Ni concentration. The rate of Zn pollution, on the other hand, positively correlated with *T. viride* and negatively with *T. tomentosum*.

The heavy metals had various effects on the interspecific relationships of the indigenous *Trichoderma* spp. Cadmium suppressed the correlation between *T. harzianum–T. pubescens* and *T. harzianum–T. atroviride* pairs. The absolute value between the two-factorial correlation and partial correlation coefficients [r(ij)-r(ij,k)] was higher than 1.00 in a negative direction. Although the [r(ij)-r(ij,k)]value for the *T. harzianum–T. viride* pair was below the upper threshold of no effect, the very high partial correlation [r(ij,k) = 0.999] suggested that there might be a close positive relationship between these species under the unpolluted conditions. No marked influence of Cd is probable for the *T. pubescens–T. atroviride* pair and either pairs of *T. virens*. In other cases, various levels of the inhibitory effect of cadmium, as a partial explanation of the calculated correlation could be assumed (Table 2).

The effect of nickel on the interspecific relationships differed from that of cadmium. The *T. harzianum–T. pubescens* and *T. harzianum–T. atroviride* pairs were among those showing suppressive effect, but the positive partial correlation coefficients suggested an opposite influence of nickel as compared to cadmium. The relationship of *T. tomentosum* with *T. harzianum* and *T. viride* was also suppressively affected by nickel. Another difference from Cd was that all pairs of *T. virens* were influenced by Ni pollution. Pairs of *T. pubescens* with *T. atroviride* and *T. tomento-*

Table 2

Influence of the available Cd content of soil on the relationships among *Trichoderma* spp.

		Trichoderma species				
		T. viride	*T. pubescens*	*T. atroviride*	*T. virens*	*T. tomentosum*
T. harzianum	r(ij)	0.758	0.642	0.087	0.354	0.554
	r(ij,k)	0.999	-0.821	-0.98	0.46	-0.412
	r(ij)-r(ij,k)	0.241	1.463	1.067	0.106	0.966
T. viride	r(ij)		0.003	-0.577	0.485	0.010
	r(ij,k)		-0.814	-0.978	0.47	-0.412
	r(ij)-r(ij,k)		0.817	0.401	0.015	0.422
T. pubescens	r(ij)			0.815	0.172	0.707
	r(ij,k)			0.918	0.13	-0.182
	r(ij)-r(ij,k)			0.103	0.042	0.889
T. atroviride	r(ij)				-0.14	0.577
	r(ij,k)				-0.273	0.222
	r(ij)-r(ij,k)				0.133	0.355
T. virens	r(ij)					-0.485
	r(ij,k)					0.999
	r(ij)-r(ij,k)					0.514

Table 3

Influence of the available Ni-content of soil on the relationship among *Trichoderma* spp.

		Trichoderma species				
		T. viride	*T. pubescens*	*T. atroviride*	*T. virens*	*T. tomentosum*
T. harzianum	r(ij)	0.925	-0.218	-0.593	-0.692	-0.378
	r(ij,k)	0.057	0.947	0.820	-0.997	0.689
	r(ij)-r(ij,k)	0.868	1.165	1.413	0.305	1.067
T. viride	r(ij)		-0.530	-0.839	-0.465	-0.382
	r(ij,k)		-0.268	-0.525	-0.134	0.763
	r(ij)-r(ij,k)		0.262	0.314	0.331	1.145
T. pubescens	r(ij)			0.904	-0.501	0.577
	r(ij,k)			0.960	-0.919	0.419
	r(ij)-r(ij,k)			0.056	0.418	0.158
T. atroviride	r(ij)				-0.082	0.522
	r(ij,k)				-0.773	0.149
	r(ij)-r(ij,k)				0.691	0.373
T. virens	r(ij)					-0.289
	r(ij,k)					-0.743
	r(ij)-r(ij,k)					0.454

Remarks: Significance level: * p < 5%; n.s.: not significant correlations; r(ij): values of the correlation coefficients; r(ij,k): values of the partial correlation coefficients of given pairs of parameters when the effect of heavy metal was fixed; {r(ij)-r(ij,k)}: the absolute values of difference between correlation and partial correlation coefficients *sum* were the only cases in which nickel did not affect the interspecific relationships (Table 3).

The effect of zinc differed both from that of cadmium and nickel. Only this heavy metal modified markedly the relationship between the two most frequent species, *T. harzianum* and *T. viride*. The correlation between the frequency of *T. viride* and *T. tomentosum* was also suppressed. Eight of thirteen further species pairs were not affected by zinc, the others were partly influenced by zinc pollution (Table 4).

Table 4
Influence of the available Zn content of soil on the relationship among *Trichoderma* spp.

		Trichoderma species				
		T. viride	*T. pubes-cens*	*T. atro-viride*	*T. virens*	*T. tomentosum*
T. harzia-num	r(ij) r(ij,k) r(ij)-r(ij,k)	-0.477 0.739 1.216	-0.185 -0.383 0.198	-0.367 -0.221 0.146	-0.500 -0.797 0.297	0.855 0.637 0.218
T. viride	r(ij) r(ij,k) r(ij)-r(ij,k)		0.049 0.338 0.289	0.469 0.494 0.025	-0.447 -0.996 0.549	-0.625 0.990 1.615
T. pubes-cens	r(ij) r(ij,k) r(ij)-r(ij,k)			0.905 0.985 0.080	-0.246 -0.252 0.006	0.301 0.468 0.167
T. atro-viride	r(ij) r(ij,k) r(ij)-r(ij,k)				-0.408 -0.413 0.005	0.011 0.620 0.609
T. virens	r(ij) r(ij,k) r(ij)-r(ij,k)					-0.408 -0.973 0.565

Remarks: Significance level: * p < 5%; n.s.: not significant correlations; r(ij): values of the correlation coefficients; r(ij,k): values of the partial correlation coefficients of given pairs of parameters when the effect of heavy metal was fixed; {r(ij)-r(ij,k)}: the absolute values of difference between correlation and partial correlation coefficients

Discussion

The composition of *Trichoderma* population was quite variable, according to the type and severity of heavy metal contamination. It is hard to make a comparison of the results, due to the fact that no similar work has been published in the literature, as yet. The consideration of indigenous, native *Trichoderma* species is not frequent in the literature and not many field experiments exist with metal contaminated soils in the World (KÁDÁR, 1995). Several evidences are found for the structural and functional changes of some microbial communities, other than the *Trichoderma* in metal polluted soils (KANDELER et al., 2000; ELLIS et al., 2001; MÜLLER et al., 2001; KÁDÁR et al., 2001).

The rate of cadmium pollution significantly correlated with the frequency of four of six *Trichoderma* spp. Presumably, the Cd tolerance of isolates from differently contaminated plots is similar. This fact was supported by an earlier study, in which the metal (Cd) tolerance of *T. harzianum*, *T. virens*, and *T. viride* isolates, originating from soils with different Cd pollution level were studied. No considerable difference was found in the minimum inhibitory concentration (MIC) for *in vitro* growth rate on Cd containing medium. However, their antagonistic activity against *Pythium irregulare* under Cd stress correlated in some cases with the pollution level of their original habitat (NAÁR, 2006). In addition to the direct inhibitory effect of the metals and the physiological status of the fungi there are other elements that influence the density and species composition of the *Trichoderma* fungi.

The partial correlation analysis revealed that the heavy metals significantly alter the relationship among the indigenous *Trichoderma* species. The combination of affecting, stable abiotic soil factors could be explained by the variance of *Trichoderma* species composition in 24 arable soils only to a 66.2% extent in the investigation of NAÁR and DOBOS (2006). It seems obvious that a further part of the variances could be attributed to the differently structured soil and rhizosphere microbiota. The microbial tolerance to the varying inhibitory effect of the soils was found to be mainly responsible for the soil colonization of *Trichoderma* fungi (NAÁR & KECSKÉS, 1998). It is also expected that the shifts in the composition of microbiota at the heavy metal pollution may alter the inhibitory effect towards the *Trichoderma* fungi, as well. *In vitro* experiments have shown that the metals also used in our study could affect the mycelial growth (KREDICS et al., 2003), the differentiation (FRANK et al., 1993) and biomass distribution within the colony (GADD et al., 2001). Although they did not modify the activity of extracellular enzymes playing role in the mycoparasitism (KREDICS et al., 2001, 2004), the *in vitro* antibiosis of *Trichoderma* spp. against *Pythium irregulare* growing on heavy metal containing agar proved to be more sensitive than mycelial growth (NAÁR, 2006). Unfortunately, no such data are available from *in vivo* soil conditions.

Besides the soil microbiota, the coexisting *Trichoderma* fungi may also have an influence on each other, as their ecological niche may overlap due to similar environmental requirements. It was established that the competitive success of sympatric species depended on the season; they shared the available ecological space along the fluctuating temperature (WIDDEN, 1984). Competition, however, is not only the single way for the interaction between *Trichoderma* spp. VAJNA (1987) observed that some strains of *Trichoderma* could parasitize on other members of the genus under *in vitro* conditions. REAVES and CRAWFORD (1994) showed different growth intensities as influencing factors in the competition among 15 *Trichoderma* isolates. Although there is a lack of data on those phenomena in real soils, the heavy metal effect on the species composition of soil biota is obvious.

The present study is among the first to report the significant influence of long-term heavy metal applications on the *Trichoderma* fungal interactions under field conditions. Further studies are necessary to understand the structure and function of other soil and rhizosphere microbiota in those dynamic interactions, especially on the targeted long-term level.

Summary

Cadmium, nickel or zinc contaminated soils originating from a long-term heavy metal field experiment were used to assess the influence of those particular treatments on the coexistence of various *Trichoderma* species. The abundance of six indigenous *Trichoderma* spp. – *T. atroviride*, *T. harzianum*, *T. pubescens*, *T. tomentosum*, *T. virens* and *T. viride* – were studied 12 years after the application of Cd, Zn and Ni salts on four levels (0, 30, 90 and 270 $mg \cdot kg^{-1}$) in a calcareous chernozem soil. *Trichoderma* fungal colonies from the soil particles were estimated on selective media. The isolated strains were taxonomically characterized by microscopic visualization.

A reduced *Trichoderma* fungal colonization was found at the lower ratio of the studied metals. No colonization could be recorded in the case of Cd, and a slightly increased abundance at Ni and Zn metal salts at the highest 270 $mg \cdot kg^{-1}$ doses. The species composition of the fungi varied considerably in the contaminated samples as a function of the metals and the applied doses. Correlation analysis revealed that the population density of *T. atroviride*, *T. harzianum*, *T. pubescens*, *T. viride* was negatively affected by the available Cd concentration. The nickel content of the soil, however, correlated positively with the abundance of *T. harzianum* ($r = 0.955$) and *T. virens* ($r = 0.964$). In addition to this finding, the frequency of *T. viride* and *T. tomentosum* showed significant positive and negative correlation with the Zn treatment ($r = 0.955$; $r = -0.965$, respectively). Great differences between the correlation and partial correlation coefficients suggested that the heavy metals may alter not only the abundance of the fungi, but the interspecific relationships among the indigenous *Trichoderma* population, as well. This fact is considered to have further influence on some other biotic parameters and the soil functioning in heavy-metal-affected soils.

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Key words: *Trichoderma* species, heavy metal pollution, biotic interactions, partial correlations

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