Toxicity of Some Field Applied Heavy Metal Salts to the Rhizobial and Fungal Microsymbionts of Alfalfa and Red Clover

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Introduction

Adverse effects of toxic metals

Repeated application of sewage sludge can result in elevated metal concentrations that persist on the plough layer (KÁDÁR, 1991). Due to the accumulation of heavy metals, however, the long-term application of sewage sludge may have adverse effects on some soil biological parameters, such as soil microbial biomass (BROOKES & MCGRATH, 1984), N_2 fixation by blue green algae (BROOKES et al., 1986) and N_2 fixation by legumes (MCGRATH et al., 1988). As a function of the applied doses other abiotic environmental factors (nitrate content, salinity, acidity, drought, etc.) were also found to have a harmful effect on the most important beneficial soil–plant microbes (*Rhizobium* bacteria, PGPR pseudomonads) (BIRÓ & KECSKÉS, 1991; BAYOUMI et al., 1995).

Decreases in plant and nodule size as well as in nitrogenase (acetylene reduction) activity have been observed in white clover grown in metal treated soils, too (GILLER et al., 1989; MARTENSSON & WITTER, 1990; ROTHER et al., 1983). The introduced effective clover rhizobia were unable to survive in free living state outside the protected root nodule in the metal contaminated soil. Alfalfa (*Medicago sativa* L.) on the other hand occasionally was less sensitive than white clover (*Trifolium repens* L) (GILLER et al., 1993). After 42 days less than 1% of the added *Bradyrhizobium japonicum* cells were recovered from the soil with sewage sludge application. The loss was also attributed to the presence of heavy metals in the sludge (REDDY et al., 1983).

According to the permissible limits, only Ni had no toxic effect on the rhizobial population or growth of clover in the U.K. Zn and Cd were the most toxic metals to *Rhizobium leguminosarum* bv. *trifolii* (CHAUDRY et al., 1992) and to other beneficial microbes (BIRÓ et al., 1995). Copper was also toxic, but to a lesser extent, because it was strongly bound by organic and inorganic matter, which moderated the harmful effects (ANALITA & PICKERING, 1987).

Among microelements, Zn, Cu, Ni, Co and Cr are essential or beneficial micronutrients for plants, animals and microorganisms (ALLOWAY, 1990). Others, such as Cd, Hg and Pb have no known biological and/or physiological functions (KÁDÁR, 1991). All of these metals, however, may be toxic at higher concentrations (BIRÓ et al., 1995).

As a consequence of sewage sludge application on agricultural lands soil becomes contaminated with more than one metal, making it difficult to determine which metals are responsible for the observed toxic effects (CHAUDRY et al., 1992). The large surplus of mineralizable N and other nutrients in the fertilizers and sewage sludge may also supress both nodulation (N_2 fixation) and the AMF colonization by legumes (JOHNSTON et al., 1982; BIRÓ et al., 1993), which makes it difficult to realize the real metal effects. Therefore only long-term experiments (Luddington, U. K.; Nagyhörcsök, Hungary) using sewage sludge-free metal salts can give the opportunity to study the real toxic effects of each heavy metal on the beneficial soil microbes along a time scale.

In the present paper experiments are reported in which pure metal salts were used and made it possible to determine which metals and what concentrations were toxic to the beneficial microsymbionts, such as the colonization by endomycorrhizal fungi and the indigenous population of *Rhizobium leguminosarum* bv *trifolii* or R. *meliloti* and for their symbiosis or nitrogen fixing ability with alfalfa and red clover.

Impact of microsymbionts on metal toxicity

Microsymbionts, such as *Rhizobium* bacteria and AM fungi are well known colonizers of the root systems of leguminous crops. In a free living stage they are considered to be especially sensitive to toxic metals, applied both *in vitro* or *in situ* (REDDY et al., 1983). Once having the symbiotic relations, however, they are less sensitive to metal contamination. Despite of this fact colonization by arbuscular endomycorrhizal fungi (AMF) on clover was still delayed by long-term sewage sludge application (KOOMEN et al., 1990). On the other hand, there was no real decrease in the frequency of AM fungi (F %), when there was enough time left for the natural selection of metal tolerant strains (BIRÓ et al., 1996). For more details see VÖRÖS et al. (1998) in this issue of Agrokémia és Talajtan.

Regarding the element uptake of mycotroph-host systems, there is a great discrepancy in the literature. Increase of the uptake of some mineral elements (N, P, K, Ca, Cu and Zn), was shown, especially when these elements are in low concentrations (GEORGE et al., 1992; GILDON & TINKER, 1983). Direct (increased hyphal surface) and indirect effects (i. e. hormonal balance) can influence the nutrient or water mobilization and uptake in various soil–plant systems. At high metal rates a plant protective role of AM fungi is frequently mentioned (WEISSENHORN et al., 1995; LEYVAL et al., 1991).

Materials and Methods

The calcareous loamy chernozem soil used originated from the ploughed layer of a long-term field experiment at Nagyhörcsök Experimental Station (Hungary) and had the following characteristics: about 25% clay, 3% humus, 5% $CaCO_3$ content and a pH of 7.0.

Suspensions of 13 microelement salts (Al, As, Ba, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Se, Sr, Zn) were applied separately in 1991 on 4 levels (0, 90, 270 and 810 kg ha^{-1} in soluble salts, which is equal to 0, 30, 90 and 270 mg kg^{-1} soil in the ploughed layer) in the experiment arranged in split-plot design (21 m^2 area of each). 100 kg ha^{-1} N, P_2O_5 and K_2O were also given yearly, with the exception of leguminous crops.

Among the metals 6 were selected (using only the lowest and highest concentration levels) for preliminary pot experiments with alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) so as to compare the metal effect on the nodulation and endomycorrhizal (AMF) colonization in 1995 (four years after the metal application). The soil was dried and subdivided into 200 g portions. Alfalfa and red clover were grown (approximately 10 plants per pot) for 2 months under greenhouse conditions. The number of root nodules by the indigenous *Rhizobium* populations and the shoot and root biomass (fresh and dry weight) were assessed. Acetylene reduction activity (ARA) of the washed roots after 2 hours of incubation (using 10% acetylene in each bottle) were also measured (HARDY et al., 1973; VINCENT, 1970).

Colonization of arbuscular endomycorrhizal fungi was checked by the method of KRJUGER et al. (1968) and PHILIPS & HAYMAN (1970), using a dissecting stereo-microscope. Two parameters of AMF colonization (frequency and intensity of AM infection – F and M – two for calculating the arbuscularity – a and A) were estimated after an anilin-blue staining of the cleared, washed and boiled root samples.

Available metal contents of the original plots were analyzed by the NH_4-Ac+EDTA method of LAKANEN & ERVIÖ (1971). Plant material of the pot experiment was digested in teflon bombs, using cc. $HNO_3 + H_2O_2$ and measured by ICP technique.

Statistical analysis. – All chemical measurements are given as the means of duplicate analyses of soil or plant samples. Dry weight, nodulation and acetylene fixation activities were measured in triplicates. Colonization by AM fungi was estimated on 30 root segments. Least significant differences were calculated and are represented at 5% level.

Results and Discussion

Permissible amounts of metals

In Table 1 permissible metal limits as „total" concentrations in the ploughed layer are shown, which are used for arable fields with permanently applied sewage sludge. Among these, German limits are more strict as compared to others, as a function of soil pH. Limits of Zn for instance vary between 200 and 300 mg kg^{-1}, that is a tremendous difference with respect to soil biological processes (GILLER et al., 1989; GILLER & MCGRATH, 1988). Most probable number counts of the free living *Rhizobium* bacteria were found to be especially sensitive beyond the 200 mg kg^{-1} rates, therefore a more economic legislation policy is urgently highlighted in the literature. Permissible limits in Hungary have a certain plasticity and are dependent partly on the cation exchange capacity, the organic matter content and the actual crops of the agricultural fields.

Table 1

Permissible limits for total metals (mg kg^{-1}) in agricultural soils permanently receiving sewage sludge (ploughed layer)

Metals in soils	United Kingdom [1]			Germany [2]		Hungary [3]		
	pH			pH		CEC*		
	6-7	5.5 - 6	5 - 5.5	>6	5-6	5-15	15-25	25-35
Cr	400	400	400	100	100	75	100	100
Pb	300	300	300	100	100	100	100	100
Zn	300	250	200	200	150	200	250	300
Cu	135	100	80	60	60	74	100	100
Ni	75	60	50	50	50	50	50	50
Cd	3	3	3	1.5	1	1	2	3

[1] Department of the Environment (1989); [2] Federal Ministry of the Environment (1992); [3] Ministry of Agriculture (1990); *CEC - meq. 100 g $soil^{-1}$ (ALLOWAY, 1990; KÁDÁR, 1991)
Note: Permissible limits in Hungary are: As 7-15, Se 10 mg kg^{-1}

In Table 2, the „available" metal contents in our experimental soil are shown in the ploughed layer in 1992. The analytical data reflect that in the 810 kg ha^{-1} (e. g. 270 mg kg^{-1} soil) treatment even the available contents of Cd, Se, As, Ni and Cu exceed the Hungarian limits given for the total pool. More exactly, in the fourth year of the experiment, mobile Cd was 82, Se 9, As 6 and Cu 2 times higher in the ploughed layer than the permissible limit given for total concentrations.

Table 2

Effect of treatments on the available metal content (NH_4 - acetate + EDTA, mg kg^{-1}) in the ploughed layer of the calcareous chernozem soil (Nagyhörcsök, 1994)

Metals used	Chemical forms used	Applied elements (mg kg^{-1})			$LSD_{5\%}$
		0	30	270	
As	As_2O_3: $NaAsO_2$	0.1	4	80	3
Cd	$CdSO_4 \cdot 8\backslash 3\ H_2O$	0.2	14	164	13
Cu	$CuSO_4 \cdot 5H_2O$	90	23	192	12
Ni	$NiSO_4 \cdot 7H_2O$	5.0	22	86	4
Zn	$ZnSO_4 \cdot 7H_2O$	2.0	19	144	11
Se	Na_2SeO_3	0.1	8	89	11

Effect of toxic metals on plant growth and N_2 fixing ability

Table 3 presents the root nodulation of alfalfa and red clover by the indigenous *Rhizobium* population of the heavy metal contaminated soil. As, Se (and Cd for clover) were found extremely toxic to the plants. The effect of Zn, especially at the lowest level was not significant as compared to the control

Table 3

Number of root nodules and N_2 fixing ability (ARA) of clover and alfalfa grown in heavy metal contaminated soil in 1995

Metals applied (mg kg^{-1})		Alfalfa		Clover	
		Nodules (piece/plant)	ARA (ppb/2hr/plant)	Nodules (piece/plant)	ARA (ppb/2hr/plant)
Control		7.30	534	4.90	729
As –	30	3.45	405	2.50	686
	270	3.15	303	0.38	516
Cd –	30	5.55	668	1.95	204
	270	3.80	648	1.35	220
Cu –	30	3.85	468	3.80	143
	270	3.92	285	4.25	135
Ni –	30	3.50	646	2.13	101
	270	2.75	210	3.21	163
Zn –	30	7.05	683	4.05	297
	270	5.50	420	3.52	185
Se –	30	3.00	276	0.55	390
	270	0.90	71	0.00	0
$LSD_{5\%}$		2.35	145	2.62	282

plants. Copper was not toxic either to red clover nodulation. This calcareous loamy chernozem has low available Zn and Cu concentrations, therefore crops often show some deficiency in these elements (KÁDÁR, 1991).

According to the N_2 fixing potential of the two tested crops, however, a high reduction was found with all added metals. Alfalfa on the other hand was resistant to the Zn 30 and Cd 30 and 270 mg kg^{-1} rates. The lowest acetylene-reduction capacity was found for the Se concentrations with both hosts. There were only small and white ineffective nodules in case of these metals, which were found to be phytotoxic, as well.

No positive direct effect was found for the correlation between nodulation and the nitrogen fixing ability. In case of As (30 kg and clover), Zn (30 kg for alfalfa) and Cd (at both concentrations, alfalfa) an increased acetylene fixation activity (ARA) suggested an enhanced rhizobial diversity as a tendency.

Effects of toxic metals on the colonization of arbuscular endomycorrhizal fungi

Among the 6 metals only three (Cd, Ni and Zn) were subjected to colonization measurements of AM-host system even at the lowest concentrations (Table 4). There was only one significant difference found by using the frequency parameter (F %) for estimating the metal effects. The highest amount of Zn caused a reduction of AM frequency in case of alfalfa. The high AM infection frequency suggests that the 4-year metal contamination period possibly allowed the selection of some appropriately infective metal tolerant mycorrhizal fungi in the field experiment.

Table 4

Colonization of clover and alfalfa by arbuscular endomycorrhizal fungi in heavy metal contaminated pots in 1995 (soil originating from a field experiment, Nagyhörcsök, contaminated in 1991, 4 years prior the pot experiment)

Metals applied mg kg^{-1}		Alfalfa		Clover	
		F %	A %	F %	A %
Control		93	10	98	23
Cd –	30	82	6	58	13
	270	83	30	73	4
Ni –	30	67	34	97	11
	270	83	3	83	30
Zn –	30	93	7	90	15
	270	52	18	95	46
$LSD_{5\%}$		25	22	26	25

F %: frequency in the root; A %: arbuscularity

In an earlier study the number of arbuscules, on the other hand, decreased dramatically when the metal was toxic to the plant–microsymbiont system. Preceding the toxic concentrations, the functionality (the number of arbuscules) increased to a certain point. The highest number of arbuscules were found at Ni 270 and Zn 270 mg kg^{-1} concentrations in clover and Cd 270, Zn 270 mg kg^{-1} rates in the alfalfa. The quantity of arbuscules was significantly higher at those metal concentrations as compared to the control plants. The same intensification of arbusculum content was found in an other soil–plant–toxic metal system, using undisturbed soil monoliths in an earlier study (BIRÓ et al., 1994).

There was always an optimal rate of metals found when the functionality of AM fungi was the highest. This certain point, however differed as a function of the host, the metals and their rates among some various soil–plant systems.

Regarding the four parameters of endomycorrhizal colonization, arbuscularity (A %) seems to be a reliable indicator of the environmental (heavy metal) pollution. This fact is in agreement with earlier results (BIRÓ et al., 1994), and with some new findings presented by VÖRÖS et al. (1998).

Tendency of metal uptake by AM fungi

Table 5 demonstrates the metal content of the alfalfa and clover shoot grown in the metal contaminated soil. Plant concentrations of the three metals in the control shoots differed greatly: plant concentrations of Zn being the highest and Cd the lowest. The accumulation of metals in the shoots of clover and alfalfa depended on the level of soil contamination. According to the metal loadings, the metal uptake of crops increased concomitant by the applied doses of metals. Alfalfa, however, as a tendency, accumulated only about half of the metal amount that clover had. Phosphorous content, on the other hand, was found in

Table 5

Tendency of metal uptake of clover and alfalfa shoot affected by the various metal loadings of Cd, Zn and Ni (Pot experiment, 1995)

Metals applied (mg kg^{-1})	Clover				Alfalfa			
	Cd (ppm)	Ni (ppm)	Zn (ppm)	P (%)	Cd (ppm)	Ni (ppm)	Zn (ppm)	P (%)
Control	0.6	11	57	0.32	0.3	3.3	35	0.33
Cd - 30	**18.2**	10	45	0.33	**13.9**	3.4	23	0.33
Cd - 270	**95.0**	11	71	0.29	**56.9**	5.0	43	0.32
Ni - 30	0.6	**20**	59	0.33	0.5	**9.7**	30	0.40
Ni - 270	0.8	**62**	50	0.34	0.5	**47.2**	34	0.33
Zn - 30	0.4	10	**79**	0.34	0.9	3.7	**48**	0.40
Zn - 270	0.3	10	**162**	0.30	0.2	4.6	**113**	0.32
$LSD_{5\%}$	only one average sample was analyzed							

the same level in both crops and did not change significantly with the various concentrations. Although AM fungi are known mainly to increase the P content of their hosts (MARSCHNER, 1995), this was not really pronounced in this study. This fact was an obvious consequence of the regular (overloaded) N, P, K fertilization of the field experiment.

Effect of treatment on dry weight of clover and alfalfa

Figure 1 and Table 6 show the effect of selected metal salts on the dry weight of alfalfa and red clover. Biomass production was decreased by As, Cd

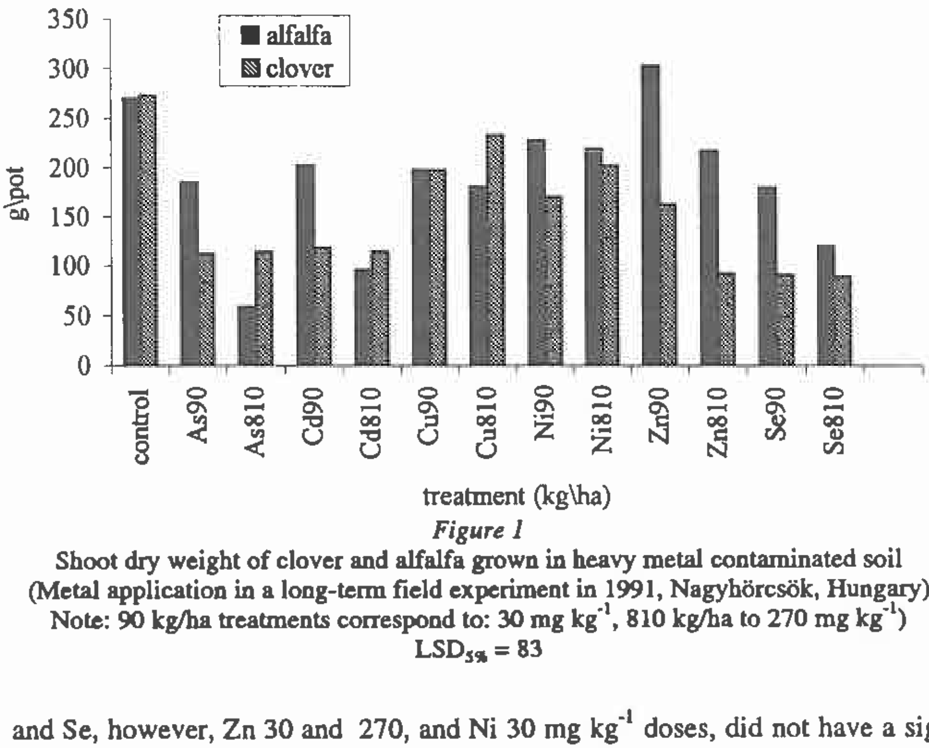

Figure 1

Shoot dry weight of clover and alfalfa grown in heavy metal contaminated soil (Metal application in a long-term field experiment in 1991, Nagyhörcsök, Hungary) Note: 90 kg/ha treatments correspond to: 30 mg kg^{-1}, 810 kg/ha to 270 mg kg^{-1})
$LSD_{5\%}$ = 83

and Se, however, Zn 30 and 270, and Ni 30 mg kg^{-1} doses, did not have a significant effect in the case of alfalfa. Red clover (*Trifolium pratense* L.) seems to be more sensitive to heavy metal contamination, where the highest Zn load also induced shoot biomass loss.

Results have demonstrated that low concentrations of heavy metals (below the European statutory limits) in soil can cause reductions not only in the number of rhizobia, but also in the dry matter production of plants. All these effects were observed at metal concentrations very near to the permissible amounts for Zn, Cu and Ni. The same was found by GILLER et al. (1993)

Table 6

Effect of treatments on dry weight of clover and alfalfa grown in heavy metal contaminated soil in pot experiment, 1995
(Metal application in 1991 in a field trial on calcareous chernozem)

Metals used mg kg^{-1}	Alfalfa g pot^{-1}	Red clover g pot^{-1}	Metals used mg kg^{-1}	Alfalfa g pot^{-1}	Red clover g pot^{-1}
Control	285	273	Control	285	273
As – 30	182	116	Ni – 30	228	183
As – 270	69	107	Ni – 270	219	233
Cd – 30	203	119	Zn – 30	318	162
Cd – 270	96	116	Zn – 270	217	92
Cu – 30	198	170	Se – 30	169	90
Cu – 270	182	202	Se – 270	112	86
$LSD_{5\%}$	83	74	$LSD_{5\%}$	83	74

Conclusions

Two parameters of symbiotic functionality (namely, the acetylene reduction activity of nodulated legumes, and/or the arbuscular endomycorrhizal colonization) were found to be especially sensitive indicators of heavy metal pollutions.

Further research is necessary, however, to establish the maximum heavy metal loading rates for agricultural soils that will have no immediate or long-term effect on soil biological processes. In particular, the effects on symbiotic nitrogen fixation and on the indigenous AM fungi – which appeared to be especially sensitive indicators of metal contamination – warrants further attention.

Summary

The nodulation, acetylene reduction and arbuscular endomycorrhizal (AMF) colonization of alfalfa (*Medicago sativa L.*) and red clover (*Trifolium pratense L.*) were examined. Calcareous chernozem soil used, was artificially contaminated with heavy metal salts in a long-term field experiment, four years prior to the pot experiment (in 1991). Doses of the selected 6 heavy metals (As, Cd, Cu, Ni, Zn and Se) were 90 and 810 kg ha^{-1}, added to the soil as soluble salts. After 2 months of cultivation alfalfa and red clover plants were harvested, the AMF colonization and the native *Rhizobium* effectiveness were estimated.

Among the selected metals As and Se proved to be especially toxic both to the plants and to the microsymbionts. Although low number of nodulation occurred, the nodules found however, were white and small, ineffective pseudo-nodules. Cd was also toxic, but to a lesser extent, although its concentration was 216 times higher than the actual legislated limits. Cu and Zn on the other

hand – being below the statutory concentration – had no visible effect on nodulation.

Intensification of the arbuscular number was developed as a strategy for the crops to decrease heavy metal uptake, when their concentration became toxic. Regarding the dry weight of shoot biomass, red clover was found to be more sensitive as compared to alfalfa. The harmful effect of metals therefore highly depends on the host.

It is concluded that microsymbionts are indicators sensitive enough to predict the toxicity limits of heavy metals, as a function of various environmental conditions.

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