IN VITRO ANTIBACTERIAL ACTIVITY OF SINGLE, DOUBLE AND TRIPLE COMBINATIONS OF METAL SALTS ON *RHIZOBIUM* AND *ESCHERICHIA* STRAINS

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Abstract
The antibacterial effect of Ag, Hg, Cd, Co, Cu, Fe, Ni, Mo, Mn, Pb, Zn and Se mineral salts and two fungicides Cineb and Mancozeb were investigated on *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *trifolii, R. leguminosarum* bv. *phaseoli, R. leguminosarum* bv. *viciae, Bradyrhizobium japonicum, Bradyrhizobium* sp. (*Acacia*) and *Escherichia coli* strains with brothdilution method. The strains showed different sensitivity to inorganic and organic metal salts. All strains were sensitive to toxic effect of Ag and Hg metal salts, the minimum inhibitory concentrations (MICs) were 2-20 µM. In contrast all strains showed high level resistance to Se, Fe, Mn, Pb salts and Cineb (MIC > 1000 µM). The toxic effect of metal salts alone better-inhibited growth of bacterial strains *in vitro* than double or triple combinations of metal salts. In the presence of 10 µM Fe^{2+} or 10 µM Fe^{2+} plus 1 µM Mo^{6+} and Mg^{2+} (30 or 50 µM) ions decreased the antibacterial effects Co^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+} and two fungicides (Cineb and Mancozeb) on the *E. coli*, *Bradyrhizobium* and *Rhizobium* strains.

Introduction

Efflux-pumps mediated by P-glycopeptide increases the level of resistance metal retention in the cells by inhibiting the efflux-pump mechanism of bacteria cells (Li *et al*. 1998). The divalent cations of Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} are essential nutrients for bacteria, required as trace elements at nanomolar concentrations. However, at micro- or millimolar concentrations of Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} are toxic. These cations are transported into the cell by constitutively expressed divalent cation uptake systems of broad specificity i.e. basically Mg^{2+} transport systems. In the case of heavy metal stress, as a response to the resulting metal toxicity, metal resistance determinants evolved which is mostly plasmid encoded in bacteria. Reduced accumulation based on an active efflux of the cation is the primary mechanism developed in prokaryotes and *Saccharomyces cerevisiae*. All bacterial cation efflux systems characterized to date are plasmid encoded (Nies 1992). Zn^{2+} is a component of many DNA and RNA polymerases and in recent years many eucaryotic and some prokaryotic gene regulatory proteins were found to contain a motif called a "zinc finger" for nucleic acid recognition (Hake *et al*. 1998). Trace elements such as Zn^{2+}, Co^{2+} and Ni^{2+} must be transported into cells against concentration gradients i.e., trace concentrations outside and substantial amounts within the cells. High- affinity and highly specific Ni^{2+} uptake systems were found in *Alcaligenes eutrophus* (Lohmayer and Friedrich 1987), *Anabaena cylindrica* (Campbell and Smith 1986), *Clostridium thermoaceticum* (Lundie *et al*. 1988), and probably *Bradyrhizobium japonicum* (Stults *et al*. 1987). The specific Ni^{2+} transporter in *A. eutrophus* was identified as part of the plasmid encoded hydrogenase gene cluster on the 450 kb megaplasmid pHGI (Eberz *et al.* 1989) and it is a membrane-bound protein with a molecular mass of 33 kDa (Eitinger and Friedrich 1991). Zn^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+} in *A. eutrophus* are transported into the cell by the Mg^{2+} uptake system (Nies and Silver 1989). In *Escherichia coli* and other enterobacteria, Co^{2+} and Ni^{2+} are also transported into the cell by a constitutive Mg^{2+} transport system. In contrast to this situation, Cd^{2+} is transported into cells of gram-positive bacteria via the Mn^{2+} uptake system, e.g. in *Staphylococcus aureus* (Nies 1992). Heavy metal resistances have been frequently found on plasmids of gram negative and gram positive eubacteria (Silver and Mirsa 1988). The possible mechanisms of resistance include only complexation and active efflux of Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}. Methylation and other covalent modifications of these metals are also not candidates for resistance mechanisms because the resulting organometallic compounds are unstable, mutagenic, and more toxic than divalent cations (Nies 1992). Generally, eukaryotes produce binding factors and prokaryotes prevent accumulation of Cd^{2+}, Zn^{2+}, Co^{2+}, or Ni^{2+} by active cation efflux (Nies 1995). For plasmid-determined Cu resistance, a complex set of mechanisms involves bioaccumulation sequestration by periplasmic and intracellular copper-binding proteins, and finally lowered intracellular levels due to copper efflux. Chromosomal and plasmid genes work together to result in copper resistance (Trajanovska 1997). Here we examined antibacterial effect of metal salts alone and in combination with 10 μM Fe^{2+} or 10 μM Fe^{2+} plus 1 μM Mo^{6+} and Mg^{2+} (30 or 50 μM) ions on *Sinorhizobium, Rhizobium, Bradyrhizobium* and *Escherichia coli* strains.

Materials and Methods

Bacterial strains: *Sinorhizobium meliloti* (11), *S. meliloti* (13), *Rhizobium leguminosarum* bv. *phaseoli* (Bab 5/3), *R. leguminosarum* bv. *phaseoli* (6), *R. leguminosarum* bv. *trifolii* (Ló 133/64), *R. leguminosarum* bv. *viciae* (Bükköny 75/4), *R. leguminosarum* bv. *viciae* (HB 3841), *Bradyrhizobium* sp. (*Acacia*) (*Acacia* 4), *B. japonicum* (Soya 1), *B. japonicum* (Soya 2), *Escherichia coli* (W1 Azid^{r})*, E. coli* (R144 drd^{+}), *E. coli* (K12 F' lac).

Compounds: $AgNO_3$, $CdSO_4$ x 4 H_2O, $MnCl_2$ x 4 H_2O, $ZnCl_2$ and Na_2SeO_3, $CoCl_2$ x $6H_2O$, $CuSO_4$ x $5H_2O$, $CuCl_2$ x $2H_2O$, $HgCl_2$, $FeSO_4$ x 7 H_2O, $NiSO_4$ x 4 H_2O, $Pb(NO_3)_2$ (Reanal, Budapest, Hungary). Promethazine chlorate (Pipolphen), and imipramine chlorate (Melipramine) were obtained from EGIS, Budapest, Hungary.

Pesticides: Cineb (Zineb 80 WP), (Zn-ethylene-bis-dithiocarbamate), Agria- Plovdiv, Bulgaria. Mancozeb (Dithane M-45), (Mn+Zn-ethylene-bis-dithiocarbamate), Rohm Haas France.

Media: Cultures of *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* strains were maintained on yeast extract mannitol (YEM) medium according to Kleczkowska *et al*. (1968). For culturing *E. coli* strains, Tryptone yeast extract (MTY) broth medium was prepared according to Alföldi *et al*. (1968).

Antibacterial effect: An overnight 16 h pre-culture of *E. coli, Bradyrhizobium*, *Sinorhizobium* and *Rhizobium* strains at aliquots of 50 µl (approx. 1-2x 10^5 CFU /ml) was transferred to 5 ml of MTY or YMB broth containing the compounds to be examined at different concentrations. After incubation at 37°C for 24 h, (*E. coli*), or 28 °C for 48 h, (*Rhizobium*, *Sinorhizobium, Bradyrhizobium*), the optical densities were measured in the Bausch and Lomb Spectronic 20 photometer at 620 nm. The lowest amount of the compound causing complete inhibition of growth was regarded as the minimum inhibitory concentrations (MIC). The MIC of metal salts were determined at 1, 2, 5, 20, 40, 80, 160, 320, 640, 1250, 2500, 5000 and 10000 µM concentrations on bacteria strain.

Results and Discussion

Effect of inorganic toxic metal salts and fungicidal organic compounds (Cineb and Mancozeb) were examined on *Sinorhizobium meliloti Rhizobium, Bradyrhizobium* and *E. coli* strains. The strains showed varied tolerance against metal salts and organic compounds (Tables 1 and 2).

The Ag^+ ions were most toxic for all strains. The strains of *Bradyrhizobium* and *R. leguminosarum* (MIC = 2-5 µM) were more sensitive to Ag^+ ions than *S. meliloti* strains (MIC = 5 µM) and *E. coli* strains (MIC = 10-20 µM). The strains were more resistance to Hg^{2+} ions than to Ag^+ ions, and *E. coli* (W1 Azi^r) strain was the most tolerant (MIC = 20 µM) to Ag^{2+}. *R. leguminosarum* and *Bradyrhizobium* strains showed moderately tolerance (MIC = 320 - 640 µM), where *E. coli* strains (MIC = 640 - 1250 µM) were the most resistant to Cd^{2+} ion. *S. meliloti* strains showed lowest level of resistance to Cd^{2+} ions (MIC = 160 µM) as well as to Co^{2+} ion (MIC = 80 - 160 µM). *R. leguminosarum*, *Bradyrhizobium* and *E. coli* (W1 Azi^r) strains were have middle level of resistance to Co^{2+} ion (MIC = 320-640 µM) and only *E. coli* (R144 drd$^+$) strain showed high level of resistance (MIC = 2500 µM) to Co^{2+}.

Table 1. Effect of metal salts on *Bradyrhizobium, Rhizobium*, *Sinorhizobium* and *Escherichia* strains

Bacterial strains	**Minimum inhibitory concentrations (MICs) μM**						
	$AgNO_3$	$HgCl_2$	$CdSO_4$	$CoCl_2$	$CuCl_2$	$CuSO_4$	$FeSO_4$
S. meliloti (11)	5	20	160	80	320	640	2500
S. meliloti (13)	5	20	160	160	320	320	5000
Bab 5/3	2	10	640	320	320	640	5000
R. phaseoli (6)	5	5	320	320	160	320	5000
Ló 133/64	2	10	320	160	320	320	5000
Bükköny 75/4	2	10	320	320	320	320	5000
HB 3841	5	10	320	320	320	640	10000
Acacia 4	2	2	320	640	640	1250	5000
Soya 1	5	10	640	160	320	640	5000
Soya 2	2	2	320	160	160	320	5000
W1 Azi^r	20	2	1250	640	640	1250	1250
K12 R 144 drd^+	10	2	1250	2500	2500	5000	2500
K12 LE 140 F'lac	10	2	640	1250	640	640	1250

Table 2. Effect of mineral salts and pesticides on *Bradyrhizobium, Sinorhizobium and Escherichia strains*

Bacterial strains	Minimum inhibitory concentrations (MICs) μM						
	$NiSO_4$	$MnCl_2$	$Pb(NO_3)_2$	$ZnCl_2$	Na_2SeO_3	Cineb	Mancozeb
S. meliloti (11)	160	2500	640	320	5000	640	320
S. meliloti (13)	320	2500	1250	640	5000	1250	640
Bab 5/3	320	2500	5000	640	10000	1250	320
R. phaseoli (6)	320	5000	5000	640	10000	2500	1250
Ló 133/64	160	2500	1250	1250	10000	2500	1250
Bükköny (75/4)	160	5000	1250	640	10000	1250	320
HB 3841	320	5000	5000	1250	5000	1250	320
Acacia 4	640	2500	5000	1250	10000	2500	1250
Soya 1	320	5000	5000	1250	5000	2500	1250
Soya 2	160	2500	1250	640	5000	1250	640
W1 Azi^r	640	2500	10000	640	2500	1250	640
K12 R144 drd^+	2500	5000	10000	1250	10000	1250	640
K12 LE 140 F'lac	320	2500	2500	160	2500	1250	640

Antibacterial effect of Cu^{2+} metal salts was influenced under anions too. The Cl^- anion was more toxic to the bacterial strains than SO_4^{2-} anion except in case of the strains *S. meliloti* (13), *R. leguminosarum* bv. *trifolii* (Ló 133/64), *R. leguminosarum* bv. *viciae* (Bükköny 75/4), and *E. coli* (K12 LE 140 F'lac) when the effect was similar in both anions. *E. coli* (K12 R 144 drd$^+$) strain showed high level resistance to Cu^{2+} ions at both anions. *S. meliloti* (11), *Rhizobium leguminosarum* bv. *trifolii* (Ló 133/64), *R. leguminosarum* bv. *viciae* (Bükköny 75/4), and *B. japonicum* (Soya 2), showed the same level of resistance to Ni^{2+} ions (MIC = 160 µM), and *E. coli* (K12 R 144 drd$^+$) was the most resistant strain (MIC = 2500 µM). All strains showed high level of resistance to Fe^{2+} ions (MIC = 1250-10000 µM), and *R. leguminosarum* bv. *viciae* (HB 3841) was the most resistant (MIC = 10000 µM) strain. *E. coli* (K12 R 144 drd$^+$), *R. leguminosarum* bv. *viciae* (HB 3841), *R. leguminosarum* bv. *trifolii* (Ló 133/64) and *B.* sp. *Acacia* (Acacia 4) and *B. japonicum* (Soya 1) were have the highest level of resistance to Zn^{2+} ions (MIC = 1250 µM), but *E. coli* (K12 R 144 drd$^+$) strain was the most sensitive one (MIC = 160 µM). All strains were shown the highest largest resistance to Se^{4+} ions (MIC = 2500-10000 µM). All strains were more sensitive to Mancozeb (MIC = 320-1250 µM) than to Cineb (MIC = 640 - 2500 µM). Antibacterial effect of Mancozeb was higher than of inorganic Zn^{2+} or Mn^{2+} ions but all strains were resistance to Cineb than to Zn^{2+} ions. *S. meliloti* (11) strain was the most sensitive (MIC = 650) Pb^{2+} while *E. coli* (K11 Az^r and K12 R 144 drd$^+$) were the most tolerant (MIC = 10000 µM) strains (Tables 3 and 4).

Table 3. Antibacterial activity of alone, double and triple combinations of metal salts on *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Escherichia* strains

Bacterial strains	**Minimum Inhibitory Concentrations (MICs) µM**								
	$CoCl_2$			**$CuCl_2$**			**$NiSO_4$**		
	alone	10µM Fe^{2+}	10µM Fe^{2+}+ 1µM Mo^{6+}	alone	10µM Fe^{2+}	10µMFe^{2+}+ 1µMMo^{6+}	alone	10µM Fe^{2+}	10µMFe^{2+}+ 1µM Mo^{6+}
S. meliloti (11)	80	160	320	320	640	640	160	320	320
Ló 133/64	160	320	640	320	640	640	160	320	320
R. phaseoli (6)	320	640	640	160	320	320	320	640	640
Soya 1	160	320	640	320	640	640	320	640	640
Soya 2	160	320	320	160	320	320	160	320	320
E. coli (F'lac)	1250	2500	2500	640	1250	1250	320	640	640
Km^r R144 drd$^+$	2500	2500	2500	2500	2500	2500	2500	2500	2500
E. coli (Azi^r)	640	1250	1250	640	1250	1250	640	1250	1250

Table 4. Antibacterial activity of alone, double and triple combinations of metal salts and pesticides on *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium* and *Escherichia* strains

Bacterial strains	Minimum Inhibitory Concentrations (MICs) μM								
	$ZnCl_2$			Cineb			Mancozeb		
	alone	10μM Fe^{2+}	10μM Fe^{2+}+ 1μM Mo^{6+}	alone	10μM Fe^{2+}	10μM Fe^{2+} + 1μMo^{6+}	alone	10μM Fe^{2+}	10μM Fe^{2+}+ 1μM Mo^{6+}
S. meliloti (11)	320	640	640	640	1250	1250	320	640	640
Ló 133/64	1250	2500	2500	2500	5000	5000	1250	2500	2500
R. phaseoli (6)	640	1250	1250	2500	5000	5000	1250	2500	2500
Soya 1	1250	2500	2500	2500	5000	5000	640	1250	1250
Soya 2	640	1250	1250	1250	2500	2500	640	1250	1250
E. coli (F'lac)	160	320	320	1250	2500	2500	640	1250	1250
Km^r R144 drd^+	1250	2500	2500	1250	2500	2500	640	1250	1250
E. coli (Azi^r)	640	1250	1250	1250	2500	2500	640	1250	1250

MICs in combinations of Co, Cu, Ni, Zn, Cineb and Mancozeb with 10 μM Fe^{2+}, or 10 μM Fe^{2+} plus 1 μM Mo^{6+} ions were determined. All strains in the tests showed decreased toxic effect of metal salts in the double and triple combinations. The toxic effect of cobalt salt in combination with 10 μM Fe^{2+} plus 1μM Mo^{6+} ions showed similar results to the combination of 10 μM Fe^{2+}. The *E. coli* (drd^+ Km^r) strain was the most resistant against Co, Cu, Ni metal salts (MIC = 2500 μM), and toxic effect of double, and triple combinations of metal salts did not change values. In contrast Zn, Cineb and Mancozeb combination with 10 μM Fe^{2+} and 10 μM Fe^{2+} plus 1 μM Mo^{6+} ions, the MICs were at larger concentrations. The following toxicity ranking was obtained on artificial nutrient medium Ag > Hg > Ni > Co >Cu > Cd > Zn > Pb > Mn > Fe > Se at test bacterial strains *in vitro.* The MIC of the strains in Cu, Ni, Zn, and Mancozeb in combination with 10 μM Fe^{2+} plus 1 μM Mo^{6+} was changeless. The antibacterial effects of heavy metal salts ($CdSO_4$, $CoCl_2$, $CuSO_4$ and $NiSO_4$) was tested in the presence of different concentration of Mg^{2+} on *R. leguminosarum* bv. *phaseoli* (6) and *E. coli* (K12 LE 140) strains. In the present study the toxic effect of mineral salts for several of *Rhizobium, Sinorhizobium, Bradyrhizobium* and *E. coli* strains was investigated. The Ag^+ and Hg^{2+} ions were the most toxic to all strains, but *E. coli* strains were more tolerant to Ag^+ ions than other strains. In contrast, *S. meliloti* strains were more tolerant to Hg^{2+} ions. The Ni^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} ions were more toxic to all stains than Pb^{2+}, Fe^{2+}, Mn^{2+}, Se^{4+} and the two fungicides. Our results were similar to other search data carried out with *R. leguminosarum* bv. *trifolii* by Chaudri *et al.* (1992a). Our *Rhizobium strains* more tolerated Zn^{2+} than Cd^{2+} ions and not agree with establishments data of Chaudri *et al*. (1992b). Obbard *et al*. (1990) examined toxic effect of metal ions to *Rhizobium* strains and found decreasing order of toxicity Zn > Cu > Ni that agreed with results of Chaudri *et al.* (1992b). Derylo and Skopruska (1991) found that Zn^{2+}, Mn^{2+} and Co^{2+} inhibited activity of iron uptake genes. In contrast with our results Bhattacharya and Basu (1992) established that $CuSO_4$ more inhibited growth of *Bradyrhizobium* sp. (Acacia 4) than Ni^{2+} or Zn^{2+} ions. The Fe, Mn and Mo are essential elements for endosymbionts in soil-borne bacteria. The Ni, Co, Cd and Cu metals in certain concentration are toxic to bacteria, but other microelements such as Fe^{2+} and Mo^{6+} ions could decrease the toxic effect of these metals. The presence of the Fe^{2+} (10 μM) and Fe^{2+} plus Mo^{6+} (1 μM) combination decreased the antibacterial effects of Co^{2+}, Cu^{2+}, Cd^{2+} and Ni^{2+} on the *E. coli* and *Rhizobium* strains.

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