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Interaction between Copper and Zinc and Their Uptake by *Halimione portulacoides* (L.) Aellen

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The interaction between two heavy metals (essential or not) and the way it affects plant growth have been previously investigated. Agarwala *et al.* (1977) observed that an excess of Mn, Cu, Zn, Co and Ni reduced Fe uptake by barley plants and affected its distribution in roots and shoots. Polar and Kuçukcezzar (1986) verified a considerable Mn decrease in *Lemna gibba* (L.) fronds as a result of increasing concentrations of Cd in the culture medium.

In the Sado River estuary (South of Portugal), the halophyte *Halimione portulacoides* (L.) Aellen often colonizes the banks of creeks (silty-loam soils), or constitutes a buffer between terrestrial and estuarine habitats (sandy soils) with a pH ranging between 7.2 and 7.95. This species accumulates high levels of Cu and Zn in spite of the low levels in the substratum (Reboredo 1988; 1993).

The pattern of accumulation of different concentrations of Cu and Zn by *H.portulacoides* cultivated *in vitro* was studied by Reboredo (1991). The effects of each concentration of Cu on the Zn content of the species and *vice-versa*, as well as on Fe and chlorophyll a and b levels were also investigated, although not included in the above-mentioned work. The present study describes those results using an analysis of variance to examine the differences of metal levels between exposure times, organs and tested concentrations.

MATERIALS AND METHODS

Cuttings used for *in vitro* experiments (approximately 20cm long and at a similar degree of development) were obtained from *Halimione* plants collected in the marshes of the Sado River estuary. Copper and zinc levels extracted from the soil from the marsh with double distilled water (minimum of availability) ranged from 0.7 to 4.5 $\mu g.g^{-1}$ for Cu and 18.0 to 149.8 $\mu g.g^{-1}$ for Zn, on a dry weight basis. The maximum levels of total Cu and Zn in the soil were 44.6 and 296.8 $\mu g.g^{-1}$, respectively (Reboredo 1988), leading to the choice of the concentrations used in experiments.

For the first two weeks, cultures (with normally one plant per pot) were kept in a greenhouse under daylight, and received only distilled water. Soil mixture contained 10% organic matter, 11% total carbonates, 24% sand and 55% silt-clay; the pH was 6.5 and the Eh was 411.3mV.

At least 12 plants were exposed (the test solution was renewed each day) to each of the test concentrations of Cu and Zn used separately - 100 ml of seawater (35‰ salinity) containing 5, 25 and 50 $\mu g.ml^{-1}$ of Cu as $CuCl_2$, and 50, 100 and 150 $\mu g.ml^{-1}$ of Zn as $ZnCl_2$ (the pH was adjusted with HCl in each case, to prevent precipitation). Controls were treated daily with an equal volume of seawater without either Cu or Zn.

Plants were harvested in triplicate from separate pots after 2, 4 and 8 weeks of treatment, and also when toxicity symptoms were observed. Samples were divided into roots, stems and leaves, carefully rinsed with distilled water, and dried at 70°C to constant weight.

Each sample of dry plant (1 g) (stems and roots were previously ground in an agate mortar), was digested with concentrated HNO_3-$HClO_4$(4:1) according to Agemian and Chau (1976). Copper, iron and zinc were determined by atomic absorption spectrophotometry using a Perkin-Elmer model 5000 fitted with a deuterium background corrector.

Determination of the content of chlorophylls a and b in the leaves was carried out in parallel with the collection of the samples for Cu, Fe and Zn analysis. Chlorophyll was extracted in 80% (v/v) aqueous acetone and the absorption measured in a Shimadzu UV-160 spectrophotometer according to Arnon (1949).

Analytical grade reagents were used. Analytical accuracy was verified using replicate determinations and blanks. An analysis of variance, F-test (Davis 1986) was used to examine for differences between metal levels for exposure times, organs and tested concentrations. A value of $P<0.05$ was considered to be significant. Metal (n=3) and chlorophyll (n=5) concentrations were also analyzed for variance by the F-test.

RESULTS AND DISCUSSION

Copper at levels of 5 $\mu g.ml^{-1}$ did not affect Zn concentrations in the roots or leaves (Table 1). Treated plants contained 101.4 and 82.3 $\mu g.g^{-1}$ Zn in their roots and leaves, respectively, while controls contained 125.3 and 87.9 $\mu g.g^{-1}$ Zn in the same organs.

Cu levels of 25 and 50 $\mu g.ml^{-1}$ resulted in decreased Zn levels in the roots and leaves from the 2^{nd} to the 5^{th} week. During the fifth week, foliar necrosis of mature leaves and withering of most of the young shoot leaves were observed. These effects coincided with a remarkable decrease of Chl. a, b and Fe content (Tables 1 and 3). In control plants Zn and Fe levels increased in all the organs during the treatment (Table 1).

After 2 weeks of treatment with 5 $\mu g.ml^{-1}$ Cu, the Fe and Chl a and b levels of control and treated leaves were not significantly different ($P<0.05$), whereas two weeks later the differences were significant ($P<0.05$). After 8 weeks, stems and leaves of control plants had 1.2 and 2.7x more Fe, respectively, than the plants exposed to 5 $\mu g.ml^{-1}$ Cu. The Chl. a content of these leaves was also 1.3 times higher than exposed plants (Tables 1 and 3).

Table 1. Levels of Fe and Zn in plants exposed to different levels of copper (5, 25 and 50 $\mu g.ml^{-1}$) and diferent exposure times (2, 4, 5 and 8 weeks)

		Root		Stem		Leaf	
		Fe*	Zn*	Fe*	Zn*	Fe*	Zn*
2	5	$527^{a}\pm98.7$	$50.8^{a}\pm7.4$	$147^{b}\pm12.9$	$34.9^{b}\pm7.9$	$225^{a}\pm64.5$	$52.5^{b}\pm6.7$
	25	$421^{a}\pm60.1$	$64.6^{b}\pm11.0$	$146^{b}\pm16.7$	$27.8^{a}\pm7.6$	$280^{b}\pm41.9$	$65.2^{c}\pm5.5$
	50	$374^{a}\pm82.8$	$72.5^{c}\pm4.4$	$140^{b}\pm46.3$	$23.6^{a}\pm5.4$	$280^{b}\pm36.1$	$55.5^{b}\pm5.6$
	Control	$471^{a}\pm83.5$	$59.0^{b}\pm6.7$	$81.3^{a}\pm11.7$	$20.5^{a}\pm2.3$	$216^{a}\pm48.6$	$39.5^{a}\pm2.5$
4	5	$1288^{c}\pm257$	$82.6^{b}\pm12$	$122^{a}\pm21.6$	$38.8^{b}\pm5.8$	$190^{ab}\pm23.4$	$63.2^{b}\pm6.1$
	25	$374^{a}\pm87.4$	$47.6^{a}\pm7.8$	$138^{a}\pm24.8$	$34.7^{b}\pm3.8$	$157^{a}\pm29.7$	$64.4^{b}\pm4.9$
	50	$341^{a}\pm43.4$	$44.8^{a}\pm9.2$	$124^{a}\pm30.5$	$36.0^{b}\pm7.5$	$219^{b}\pm39.3$	$64.9^{b}\pm13.0$
	Control	$523^{b}\pm76.7$	$71.5^{b}\pm3.7$	$110^{a}\pm28.0$	$28.4^{a}\pm6.6$	$288^{c}\pm58.3$	$52.3^{a}\pm10.2$
5	25	$367^{b}\pm101$	$50.2^{a}\pm11.8$	$91.3^{a}\pm18.5$	$32.5^{a}\pm3.5$	$141^{c}\pm20.9$	$57.2^{a}\pm3.9$
	50	$366^{b}\pm68.5$	$45.1^{a}\pm12.0$	$110^{a}\pm33.1$	$29.1^{a}\pm9.1$	$255^{b}\pm42.2$	$40.5^{b}\pm3.4$
	Control	$603^{a}\pm118$	$82.3^{b}\pm5.6$	$119^{a}\pm18.7$	$33.1^{a}\pm7.0$	$331^{a}\pm53.0$	$67.4^{a}\pm10.0$
8	5	$1423^{b}\pm430$	$101^{a}\pm13.0$	$116^{b}\pm34.0$	$52.5^{a}\pm9.7$	$151^{b}\pm35.9$	$82.3^{a}\pm8.8$
	Control	$750^{a}\pm87.0$	$125^{a}\pm11.4$	$146^{a}\pm16.4$	$41.9^{a}\pm5.8$	$405^{a}\pm58.5$	$87.9^{a}\pm12.0$

Table 2. Levels of Cu and Fe in plants exposed to different levels of zinc (50, 100 and 150 $\mu g.ml^{-1}$) and different exposure times (2, 4 and 8 weeks).

		Root		Stem		Leaf	
		Cu*	Fe*	Cu*	Fe*	Cu*	Fe*
2	50	$14.7^{a}\pm3.6$	$1236^{c}\pm316$	$6.5^{a}\pm1.8$	$450^{c}\pm101$	$11.7^{b}\pm4.1$	$254^{ab}\pm68.8$
	100	$12.4^{a}\pm2.8$	$417^{a}\pm139$	$6.1^{a}\pm0.7$	$85.5^{a}\pm28.6$	$6.2^{a}\pm1.4$	$215^{a}\pm33.5$
	150	$10.8^{a}\pm3.8$	$742^{b}\pm284$	$6.7^{a}\pm2.0$	$309^{b}\pm41.1$	$8.4^{ab}\pm1.8$	$288^{b}\pm83.7$
	Control	$15.3^{a}\pm3.2$	$471^{a}\pm83.5$	$7.6^{a}\pm1.1$	$81.3^{a}\pm11.7$	$12.1^{b}\pm4.1$	$216^{a}\pm48.6$
4	50	$24.2^{a}\pm4.1$	$1746^{d}\pm348$	$5.2^{a}\pm1.2$	$631^{c}\pm175$	$13.4^{a}\pm1.9$	$316^{b}\pm73.1$
	100	$24.6^{a}\pm4.0$	$833^{b}\pm226$	$6.9^{a}\pm2.1$	$243^{b}\pm86.9$	$15.7^{a}\pm4.1$	$167^{a}\pm49.5$
	150	$20.2^{a}\pm4.4$	$1213^{c}\pm308$	$7.9^{a}\pm2.9$	$286^{b}\pm27.1$	$15.3^{a}\pm4.2$	$192^{a}\pm27.9$
	Control	$26.2^{a}\pm3.6$	$523^{a}\pm155$	$9.6^{b}\pm1.7$	$110^{a}\pm28.0$	$18.5^{a}\pm4.8$	$288^{b}\pm58.3$
8	50	$29.8^{b}\pm5.0$	$1984^{c}\pm390$	$7.0^{a}\pm1.3$	$484^{c}\pm159$	$22.3^{a}\pm5.4$	$512^{c}\pm161$
	100	$26.0^{a}\pm6.1$	$770^{a}\pm240$	$8.2^{a}\pm0.8$	$203^{a}\pm47.4$	$26.3^{a}\pm2.0$	$150^{a}\pm37.3$
	150	$26.3^{a}\pm6.7$	$855^{b}\pm87.4$	$8.5^{a}\pm1.9$	$277^{b}\pm26.7$	$26.2^{a}\pm6.9$	$172^{a}\pm21.1$
	Control	$36.7^{b}\pm5.0$	$750^{a}\pm87.0$	$12^{b}\pm1.5$	$146^{a}\pm16.4$	$31.7^{a}\pm6.0$	$405^{b}\pm58.5$

(*) - Means expressed as $\mu g.g^{-1}$ dry weight ± Standard deviation (n=3). Means not followed by a common letter are significantly different at the 0.05 significance level.

Table 3. Chlorophyll levels in the leaves of *Halimione* plants treated with different concentrations of Zn (50, 100 and 150 $\mu g.ml^{-1}$) and Cu (5, 25 and 50 $\mu g.ml^{-1}$), after different exposure times (2, 4 and 8 weeks).

	Weeks	Concentration ($\mu g.ml^{-1}$)	Mean* (Chl. a)	Mean* (Chl. b)
Zn	2	50	$350^{b}\pm24.8$	$152^{b}\pm16.5$
		100	$280^{a}\pm45.2$	$124^{a}\pm19.0$
		150	$298^{a}\pm26.6$	$114^{a}\pm13.2$
		Control	$273^{a}\pm25.8$	$118^{a}\pm12.8$
	4	50	$367^{c}\pm29.1$	$152^{c}\pm19.2$
		100	$251^{a}\pm20.1$	$120^{a}\pm6.17$
		150	$270^{ab}\pm43.7$	$125^{ab}\pm14.6$
		Control	$308^{b}\pm25.2$	$133^{bc}\pm9.90$
	8	50	$377^{b}\pm13.8$	$157^{b}\pm10.3$
		100	$249^{a}\pm18.8$	$120^{a}\pm9.03$
		150	$258^{a}\pm25.8$	$122^{a}\pm5.71$
		Control	$271^{a}\pm22.5$	$116^{a}\pm8.82$
Cu	2	5	$255^{ab}\pm37.2$	$111^{a}\pm14.0$
		25	$230^{a}\pm25.5$	$111^{a}\pm9.11$
		50	$217^{a}\pm25.9$	$109^{a}\pm12.0$
		Control	$273^{b}\pm25.8$	$118^{a}\pm12.8$
	4	5	$214^{a}\pm33.8$	$102^{a}\pm11.5$
		25	$216^{a}\pm29.4$	$116^{a}\pm11.7$
		50	$203^{a}\pm17.0$	$107^{a}\pm6.72$
		Control	$308^{b}\pm25.2$	$133^{b}\pm9.90$
	5	25	$200^{a}\pm26.3$	$108^{a}\pm7.29$
		50	$190^{a}\pm18.0$	$103^{a}\pm9.81$
		Control	$294^{b}\pm22.7$	$128^{b}\pm12.6$
	8	5	$203^{a}\pm19.3$	$105^{a}\pm8.00$
		Control	$271^{b}\pm22.5$	$116^{a}\pm8.82$

(*) Means expressed as µg/g wet weight ± Standard Deviation (n=5). Means not followed by a common letter are significantly different at the 0.05 significance level.

Although 5 $\mu g.ml^{-1}$ Cu did not hinder the accumulation of Fe by roots (Table 1), it reduced considerably translocation to the above-ground organs. Discolored white-greenish spots and a general wrinkling of the young leaves became evident during week 8.

Concerning the Fe and Zn levels in Cu-treated plants, the interactions among exposure time, organ and concentration were highly significant ($P<0.001$), indicating a very strong inter-dependence (Tables 4 and 5). In both cases the interaction between exposure time and organ was not significant at the 0.05 significance level, although each one of these factors can act independently ($P<0.001$).

In controls and Zn-treated plants the levels of Cu in the different organs increased during the experiment, although the levels in the controls were always higher (Table 2). Roots and leaves of plants treated with 50 $\mu g.ml^{-1}$ Zn contained 29.8 and 22.3 $\mu g.g^{-1}$ Cu, respectively, after 8 weeks, while plants treated with 150 $\mu g.ml^{-1}$ Zn contained 26.3 and 26.2 $\mu g.g^{-1}$ Cu in the same organs.

Plants treated with 50 $\mu g.ml^{-1}$ Zn accumulated Fe throughout the experiment (Table 2); this level enhanced the uptake of Fe by the roots. Although the roots probably acted as an effective barrier to the translocation of Fe to the leaves (containing 25.8% of the total Fe root content), they did not limit leaf concentrations (512.1 $\mu g.g^{-1}$ Fe), in comparison to that of the control leaves (405.1 $\mu g.g^{-1}$ Fe).

Chlorophyll a levels of the leaves of plants treated with 50 $\mu g.ml^{-1}$ Zn increased throughout the experiment, while the Chl. b levels remained approximately constant (Table 3). Leaves of control plants had 2.7 and 2.3x more Fe than the leaves of plants treated with 100 and 150 $\mu g.ml^{-1}$ Zn, respectively, but not more Chl. a and b (treatment and control means were not significantly different - $P<0.05$) - Tables 2 and 3.

In Zn-treated plants the interactions among the different factors (exposure time, organ and concentration) were generally highly significant ($P<0.001$), indicating a very strong inter-dependence. However, the interaction between concentration and exposure time was not significant at the 0.05 significance level, although each one of these factors can act independently ($P<0.001$) - Tables 6 and 7.

The lower concentration of Cu did not reduce the levels of Zn in the different organs of *Halimione*, although it did significantly reduce the levels of Fe and Chl. a in the leaves (Tables 1 and 3), probably due to the competition between Cu and Fe rather than an excess of Cu in the plant.

Leaves of plants treated with 5 $\mu g.ml^{-1}$ Cu contained 21.6 $\mu g.g^{-1}$ Cu, as compared to 31.7 $\mu g.g^{-1}$ Cu in the controls (Reboredo 1991). This discrepancy can be related to the analysis of older leaves in controls than in treatments.

Davis (1982) stated that in some cases toxicity can take the form of induced deficiency, often of Fe. Agarwala *et al.* (1977) demonstrated that excess Cu reduced Fe uptake by barley plants *Hordeum vulgare* (L.),

Table 4. Iron levels in Cu-treated plants

Factors and Interactions	F-Test
Exposure time	12.765***
Organ	146.392***
Exp. time x Organ	1.129†
Concentration	13.554***
Exp. time x Concentr.	4.872***
Organ x Concentr.	7.320***

Table 5. Zinc levels in Cu-treated plants

Factors and Interactions	F-Test
Exposure time	102.337***
Organ	136.584***
Exp. time x Organ	2.739†
Concentration	2.591†
Exp. time x Concentr.	5.142*
Organ x Concentr.	6.047**

Table 6. Copper levels in Zn-treated plants

Factors and Interactions	F-Test
Exposure time	91.065***
Organ	142.022***
Exp. time x Organ	4.786**
Concentration	8.358***
Exp. time x Concentr.	1.438†
Organ x Concentr.	1.138†

Table 7. Iron levels in Zn-treated plants

Factors and Interactions	F-Test
Exposure time	9.386***
Organ	184.442***
Exp. time x Organ	13.992***
Concentration	50.119***
Exp. time x Concentr.	2.075†
Organ x Concentr.	14.928***

(***) P<0.001; (**) P<0.05; (*) P<0.01; (†) Not significant at 0.05 significance level.

causing a significant decrease in the soluble protein and chlorophyll contents. Terry (1980) verified a positive correlation between levels of chlorophyll and Fe in leaves of sugar beets under Fe stress.

The injurious effects of the higher levels of Cu were probably due to the large concentrations absorbed and translocated to the leaves. Control leaves had 20.0 $\mu g.g^{-1}$ Cu, while leaves of plants treated with 25 and 50 $\mu g.ml^{-1}$ Cu had 36.5 and 53.8 $\mu g.g^{-1}$ Cu, respectively (Reboredo 1991).

The reduction in leaf elongation and mild chlorosis in *Typha latifolia* (L.) was detected at approximately 80 $\mu g.g^{-1}$ Cu (Taylor and Crowder 1983), while the background and upper critical concentrations of young barley in sand culture were 8 and 20 $\mu g.g^{-1}$ Cu, respectively (Davis 1982). This indicates that Cu tolerance varies according to species.

The presence of excess Cu decreased the levels of Zn, Fe and Chl. a in the plants, although it is not known whether degradation or inhibition of the Chl. a synthesis occurred. It seems that both processes may coexist; from the 2nd to the 5th week the Chl. a content was reduced 13% and 12% in plants treated with 25 and 50 $\mu g.ml^{-1}$ Cu, respectively (Table 3), probably due to degradation. Copper was recognized as acting on the

enzyme-substrate affinity of ALA-dehydratase in corn seedlings (Scarponi and Perucci 1984), one of the key enzymes in both haem and chlorophyll synthesis, and Fe is also involved in this pathway, showing that inhibition may have occurred.

The uptake and accumulation of Cu by *Halimione* was independent of Zn levels (Table 2). This is consistent with the findings of Lepp and Eardley (1978) who concluded that excess Zn did not affect the uptake of Cu by roots of *Acer pseudoplatanus* (L.).

Agarwala *et al.* (1977) observed that chlorophyll and soluble protein contents of *Hordeum vulgare* (L.) were not reduced by excess Zn, although the Fe content of the roots decreased. Brauwers (1982) observed a decrease of 4, 13, 25 and 45% of the chlorophyll content of *Chlorella pyrenoidosa* (L.) after 1 day of treatment with 1, 2, 5 and 10 $\mu g.ml^{-1}$ Zn, respectively, while 50 and 300 $\mu g.ml^{-1}$ Zn induced reduction rates similar to that observed for 10 $\mu g.ml^{-1}$ Zn.

High levels of Zn strongly affected Fe concentrations in *Halimione* leaves, but not the contents of chlorophyll meaning that plants may accumulate Fe far above their metabolic requirements. This is particularly true for plants treated with lower concentrations of Zn which showed an Fe and Chl. a enrichment in their leaves as compared with controls (Tables 2 and 3).

The results for *Halimione* are in contrast to those of Rosen *et al.* (1977), who observed that high Zn levels exerted a strong negative influence on chlorophyll production of *Zea mays* (L.) in hydroponic culture without lowering the Fe content. In this experiment, competition between Zn and Fe may have occurred. However, the decrease of Fe cannot be related only to the concentrations of Zn in the leaves after week 8. For example, control leaves after week 8 had 88 $\mu g.g^{-1}$ Zn, while leaves of plants treated with 100 $\mu g.ml^{-1}$ Zn had 108 $\mu g.g^{-1}$ Zn (Reboredo 1991).

Soil pH increased during the experiment from 6.5 in the beginning to 8.1-8.4 at the end. Alkaline pH values constitute a strong impediment to root absorption due to the low solubility (mainly oxides and hydroxides) of almost all micronutrients and particularly Cu, Fe, Zn and Mn (Donahue *et al.* 1977), inducing a reduced growth. Thus, an obvious question is, how long should an experiment such as this be carried out?

The divergent and sometimes contradictory results agree with Wong and Beaver (1981), who stated "simulations of metal interactions in laboratories are not likely to produce similar toxic effects to those observed in the field". Nevertheless, information about metal uptake and interactions under controlled conditions are needed for interpretation or prediction of effects of contamination in the natural habitat, although as previously stated by Reboredo (1991), "all the extrapolations derived from plant behaviour *in vitro* must be avoided or done with great care".

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