

Role of organic acids in sunflower tolerance to heavy metals

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Abstract

Exposure of *Helianthus annuus* L. seedlings to Al^{3+} , Cd^{2+} or Zn^{2+} resulted in a marked decrease of fresh and dry masses of the shoots and the roots. The increase of Al^{3+} , Cd^{2+} or Zn^{2+} uptake was accompanied by a significant decrease of nitrate, phosphorus and K^+ uptake. There was a significant increase of malic and citric acid contents in the shoots and roots of heavy metal-treated seedlings whereas the change in fumaric acid was insignificant. Al^{3+} and Zn^{2+} alone stimulated excretion of malic and citric acids to the rhizosphere. Addition of high concentrations of malic or citric acid alleviate to some extent the inhibitory effect of Al^{3+} and Zn^{2+} on plant growth.

Additional key words: aluminium, cadmium, citric acid, fumaric acid, *Helianthus annuus*, malic acid, zinc.

Introduction

Physiological and biochemical processes such as photosynthesis, respiration and enzyme activity may be affected by external heavy metal concentrations. Concerning the effect on nutrients uptake, Al^{3+} has been shown to reduce the influx of N, K, P, Mg and Ca (Fageria and Carvalho 1982, Pettersson and Strid 1989). Bhandal and Kaur (1992) and Asp *et al.* (1994) reported that Cd^{2+} stress reduced K^+ and NO_3^- uptake as well as their translocation from roots to shoots in wheat and birch seedlings. The development of resistance strategies of plants to heavy metal stresses induce production of complexing agents to avoid and tolerate the phytotoxicity of these ions. The synthesis of phytochelatins and accumulation of organic acids in the shoots and roots of plants are participating in detoxification of excess heavy metals (Harmens *et al.* 1993, Salt and Rauser 1995, Ma *et al.* 1997). Delhaize *et al.* (1993) suggested that excretion of organic acids from the roots in the rhizosphere has been implicated in heavy metal avoidance or tolerance mechanism.

The aim of this study is to identify how plant growth, and the rate of uptake of NO_3^- , K^+ and P by sunflower plants are affected by exposure of the plants to different concentrations of Al^{3+} , Cd^{2+} or Zn^{2+} , as well as the defence mechanisms involved in the detoxification of these heavy metals.

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Materials and methods

Sunflower (*Helianthus annuus* L. cv. Miak) seeds were surface sterilized by soaking for 2 - 5 min in 70 % ethanol and then soaked in distilled water with aeration. After 24 h, the seeds were transferred to plastic pots filled with purified quartz sand. Each pot contained 20 germinating seeds. The pots were placed in the laboratory under natural light. Temperature varied from 20 - 24 °C during the day and 15 - 19 °C during night. The pots were irrigated with distilled water to keep water retaining capacity of the sand at 85 %. After 7 d, homogenous seedlings were taken carefully, washed from adhering sand and transferred to 250 cm³ bottles containing 1/4 strength Hoagland solution alone (control) or supplemented with 5 or 20 g m⁻³ of Al³⁺, Cd²⁺ or Zn²⁺ as chlorides. The solutions were initially adjusted to pH 4.0 with 0.15 M HCl and maintained at this pH by daily titration with 0.2 M NaOH. The bottles were placed in a growth chamber (day/night temperature 22 ± 2 / 17 ± 2 °C, 16-h photoperiod, irradiance of 980 μmol m⁻² s⁻¹ furnished by a bank of cool 40 W fluorescent tubes).

The fresh and dry masses of the shoots and roots of the seedlings were determined. Estimation of nitrate was carried out according to the method of Johnson and Ulrich (1950). The mixed acid digestion method of Allen *et al.* (1974) was used in preparing the samples for the determination of heavy metals. Cadmium and zinc were estimated using the atomic absorption spectrophotometer (*Perkin Elmer 2380*, Norwalk, USA). Aluminium was estimated by the method of Tawfik (1968) which depends on complexation of Al³⁺ with NaEDTA, then back titration of excess EDTA by zinc sulphate using xylenol orange as indicator. Potassium was estimated using a clinical flame photometer 410C (*Corning Science Products*, Corning, UK) and total phosphorus was estimated by the molybdenum method of Steward and Grinshaw (1974). Malic, fumaric and citric acids in the external nutrient solution and plant tissues were assayed using the enzymatic method described by Delhaize *et al.* (1993). The data of experiments were statistically analyzed using the least significant difference (LSD) as described by Steel and Torrie (1980).

Results and discussion

Exposure of sunflower seedlings to Al³⁺, Cd²⁺ or Zn²⁺ inhibits severely the fresh and dry masses of shoot and root, particularly at higher concentrations of Al³⁺ and Cd²⁺ (Fig. 1). Al³⁺, Cd²⁺, and Zn²⁺ reduced plant water content (Kastori *et al.* 1992) and consequently lowered fresh mass. Furthermore, the reduce of dry matter may be related to inhibition of photosynthesis (Greger and Orgen 1991).

The increased uptake of Al³⁺, Cd²⁺ and Zn²⁺ (Fig. 2) was accompanied by a significant decrease of the uptake of NO₃⁻, K⁺ and P and their concentrations in the shoots (Tables 1,2). Similar observations were reported with several plant species including rice (Fageria and Carvalho 1982), wheat (Pettersson and Strid 1989, Bhandal and Kaur 1992) and *Betula pendula* (Asp *et al.* 1994). The decrease in uptake and translocation of P by sunflower plants in response to Al³⁺, Cd²⁺ and Zn²⁺

may be due to interaction with PO_4^{3-} in/on the roots (Jarvis *et al.* 1976), whereas disturbance of plasma membrane integrity (Nichol *et al.* 1993, Ishikawa *et al.* 1996) may result in blocking the specific channels for NO_3^- and K^+ , hence decreased the uptake of both ions. Furthermore, inhibition of respiration (Strickland and Chaney 1979, Collier *et al.* 1993) may result in a decrease of the energy required for the active absorption of NO_3^- , P and K^+ .

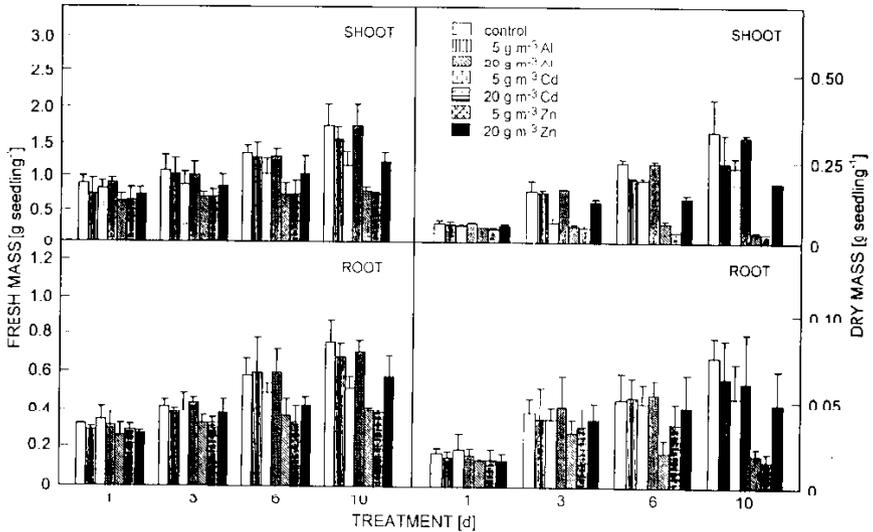


Fig. 1. Changes in the fresh and dry masses of the shoots and roots of sunflower seedlings grown in nutrient solution alone (control) or supplemented with 5 or 20 g m^{-3} of Al^{3+} , Cd^{2+} , or Zn^{2+} . Means of four replicates \pm SE.

Al^{3+} and Zn^{2+} stimulated the excretion of malic and citric acids, whereas the amount of fumaric acid released in the rhizosphere was lower compared to that of untreated plants. On the other hand, only little amounts of malic, citric and fumaric acids were excreted from Cd^{2+} -treated seedlings (Fig. 3). Miyasaka *et al.* (1991) and Pellet *et al.* (1996) reported that excretion of citric and malic acids from snapbeans and wheat roots has been implicated as tolerance mechanism. Therefore, if organic acids have a role in heavy metal tolerance, they should be able to chelate these metals in solutions and render them nonphytotoxic. The results in Fig. 4 demonstrated that adding higher concentrations of malic or citric acid to the nutrient solution of sunflower plants containing 20 g m^{-3} of Al^{3+} or Zn^{2+} resulted in an increase of fresh mass, which was restored to the control level. A decrease of Al^{3+} and Zn^{2+} taken up by the seedlings was also noted (Table 4). These results indicate that excretion of malic and citric acid by sunflower roots may be a tolerance mechanism for Al^{3+} and Zn^{2+} . However, fumaric acid seems to be a poor chelator for Al^{3+} and Zn^{2+} . In addition it is unlikely that excretion of malic, citric and fumaric acids contribute to Cd^{2+} tolerance of sunflower plants under the prevailing experimental conditions.

Table 1. Nitrate, P and K uptake [mg seedling⁻¹] by sunflower seedlings grown in the nutrient solution alone or supplemented with 5 or 20 $\mu\text{g m}^{-2}$ Al^{3+} , Cd^{2+} or Zn^{2+} for 1, 3, 6 and 10 d. Means of four replicates, significant differences (*) and highly significant difference (**) from control.

Treatment	NO_3^- uptake				P uptake				K uptake			
	1 d	3 d	6 d	10 d	1 d	3 d	6 d	10 d	1 d	3 d	6 d	10 d
Control	0.621	1.303	2.815	3.590	0.236	0.349	0.478	0.508	0.772	1.466	2.448	2.831
5 $\mu\text{g m}^{-2}$ Al^{3+}	0.490	0.896*	0.785**	0.751**	0.270	0.350	0.540	0.648	0.065**	0.345**	1.025**	1.665**
20 $\mu\text{g m}^{-2}$ Al^{3+}	0.207**	0.413**	0.41***	0.380**	0.210	0.390*	0.300*	0.320**	0.065**	0.130**	0.205**	0.492**
LSD at 5 %	0.162	0.311	0.658	1.380	0.067	0.010	0.082	0.068	0.418	0.419	1.008	0.740
LSD at 1 %	0.364	0.599	0.346	1.905	0.091	0.049	0.1185	0.095	0.505	0.856	1.008	1.119
5 $\mu\text{g m}^{-2}$ Cd^{2+}	0.589	1.140*	1.41***	1.392**	0.250	0.270*	0.380**	0.290**	0.275**	0.625**	1.705**	2.165*
20 $\mu\text{g m}^{-2}$ Cd^{2+}	0.553	0.799**	7.10***	0.591**	0.170*	0.170**	0.140**	0.110**	0.205**	0.485**	0.625**	1.085**
LSD at 5 %	0.057	0.115	1.168	1.002	0.055	0.035	0.102	0.144	0.201	0.683	0.122	0.521
LSD at 1 %	0.089	0.216	1.253	1.165	0.081	0.092	0.178	0.175	0.401	0.795	0.514	0.793
5 $\mu\text{g m}^{-2}$ Zn^{2+}	0.805	1.022*	2.309*	2.215**	0.188	0.332	0.457*	0.473*	0.725	1.045*	2.005*	2.505*
20 $\mu\text{g m}^{-2}$ Zn^{2+}	0.433	0.895*	1.503**	1.791**	0.190	0.350	0.364*	0.336*	0.715	1.125*	1.145**	1.665**
LSD at 5 %	0.190	0.154	0.389	0.817	0.063	0.028	0.021	0.014	0.063	0.198	0.108	0.219
LSD at 1 %	0.252	0.415	0.803	0.959	0.078	0.039	0.233	0.179	0.069	0.566	0.899	0.746

Table 2. Nitrate, P and K concentrations [mg g^{-1} (d.m.)] in shoots and roots of sunflower seedlings grown in the nutrient solution alone or supplemented with 5 or 20 g m^{-3} Al^{3+} , Cd^{2+} or Zn^{2+} for 3 and 10 d. Means of four replicates significant difference (*) and highly significant difference (**) from control.

Treatment	NO ₃ ⁻ concentration		P concentration				K concentration			
	3 d	10 d	3 d	10 d	3 d	10 d	3 d	10 d	3 d	10 d
	shoot	root	shoot	root	shoot	root	shoot	root	shoot	root
Control	1.53	1.44	11.06	8.27	14.88	13.17	19.37	10.74	31.02	20.47
5 g m^{-3} Al^{3+}	0.46*	1.40	0.20**	10.38*	10.85*	17.20*	16.12*	13.77*	19.37*	25.22*
20 g m^{-3} Al^{3+}	0.31**	0.70*	5.50**	10.02*	6.00**	17.55*	9.87**	12.03*	13.50**	22.08**
LSD at 5%	0.73	0.35	2.81	1.7-	2.91	3.66	2.83	1.09	5.21	1.48
LSD at 1%	0.91	0.80	4.59	3.5-	5.38	5.21	6.0	3.51	6.80	3.14
5 g m^{-3} Cd^{2+}	0.87	1.87*	6.58**	4.76*	5.08**	9.24*	6.37**	13.20**	11.87**	29.18**
20 g m^{-3} Cd^{2+}	0.71*	1.74*	1.86**	2.62**	3.05**	4.59**	4.87**	13.22**	5.12**	15.93*
LSD at 5%	0.51	0.21	3.51	3.22	1.70	2.81	3.38	0.85	3.70	3.21
LSD at 1%	0.66	0.55	4.40	5.1	4.83	5.16	4.0	2.15	6.18	4.88
5 g m^{-3} Zn^{2+}	1.21	2.74*	9.60	8.10	9.00**	14.12	7.70**	9.34	16.08**	24.28*
20 g m^{-3} Zn^{2+}	0.86*	2.94**	7.57*	7.56**	5.00**	7.31**	6.87**	8.51*	7.83**	22.32*
LSD at 5%	0.35	0.88	2.89	0.48	2.85	2.81	3.81	1.46	4.22	1.61
LSD at 1%	0.62	1.44	4.33	0.63	4.17	5.03	4.76	2.80	6.77	3.87

Table 3. Changes of malic, citric and fumaric acid content [mg g^{-1} (d.m.)] of shoots and roots of sunflower seedlings grown in the nutrient solution alone or supplemented with 5 or $20 \text{ g m}^{-3} \text{ Al}^{3+}$, Cd^{2+} or Zn^{2+} for 3 and 10 d. Means of four replicates, significant difference (*) and highly significant difference (**) from control

Treatment	Malic acid		Citric acid				Fumaric acid					
	3 d shoot	root	10 d shoot	root	3 d shoot	root	10 d shoot	root	3 d shoot	root	10 d shoot	root
Control	0.36	0.15	0.20	0.20	0.15	0.9	0.13	0.91	1.30	1.21	1.36	1.38
$5 \text{ g m}^{-3} \text{ Al}^{3+}$	0.93*	0.30	1.53**	0.36*	0.30*	1.9*	1.33*	1.43**	1.30	1.29	1.39	1.32
$20 \text{ g m}^{-3} \text{ Al}^{3+}$	1.44**	0.92**	1.88**	0.62**	0.33*	1.33*	1.50*	1.46**	1.33	1.37	1.43	1.41
LSD at 5%	0.50	0.19	0.77	0.11	0.09	0.92	1.15	1.05	0.09	0.21	0.15	0.25
LSD at 1%	0.91	0.43	0.95	0.32	0.19	1.28	1.38	1.19	1.21	0.38	0.44	0.28
$5 \text{ g m}^{-3} \text{ Cd}^{2+}$	1.62*	0.25*	2.97**	0.40*	0.81*	1.80*	1.67*	1.69*	1.05	1.26	1.39	1.51
$20 \text{ g m}^{-3} \text{ Cd}^{2+}$	2.85**	0.46*	3.96**	2.01**	0.93*	1.96*	1.68*	1.97**	1.49	1.24	1.45	1.48
LSD at 5%	1.01	0.07	1.58	0.19	0.46	0.60	1.09	1.38	0.27	0.11	0.31	0.18
LSD at 1%	1.95	0.33	2.07	0.98	0.84	1.88	1.78	1.67	0.34	0.17	0.50	0.40
$5 \text{ g m}^{-3} \text{ Zn}^{2+}$	0.95*	0.22*	2.35**	1.73**	1.60*	1.64**	2.16**	1.99*	1.71	1.57	1.92	2.31
$20 \text{ g m}^{-3} \text{ Zn}^{2+}$	1.86*	0.50**	2.62**	2.88**	1.44*	2.18**	2.35**	4.82**	1.73	1.81	1.95	2.35
LSD at 5%	0.44	0.03	1.77	0.80	1.12	1.38	1.10	1.58	0.50	0.64	0.66	1.10
LSD at 1%	1.77	0.18	3.20	1.44	1.68	1.50	1.74	2.50	0.73	0.73	0.99	1.19

Table 4. Effect of different concentrations of malic and citric acid on uptake of Al^{3+} , Cd^{2+} or Zn^{2+} by sunflower seedlings. Ten-day-old seedlings were transferred to nutrient solutions supplemented with 20 g m^{-3} Al^{3+} , Cd^{2+} , or Zn^{2+} or nutrient solutions that contained 50, 200 or 400 mM malic or citric acid for 10 d. Means of four replicates (** - highly significant differences from control).

Treatment	Uptake [$\mu\text{g seedling}^{-1}$]		
	Al^{3+}	Cd^{2+}	Zn^{2+}
Control	16.96	14.09	56.02
50 mM malic acid	16.49	13.53	52.07
200 mM malic acid	11.74**	13.45	30.20**
400 mM malic acid	10.75**	13.69	28.58**
1.SD at 5%	3.44	3.72	5.33
1.SD at 1%	4.26	3.89	7.33
50 mM citric acid	16.58	13.99	51.73
200 mM citric acid	10.15**	13.60	39.18**
400 mM citric acid	8.79**	13.59	38.39**
1.SD at 5%	3.59	2.19	6.15
1.SD at 1%	6.60	3.20	10.71

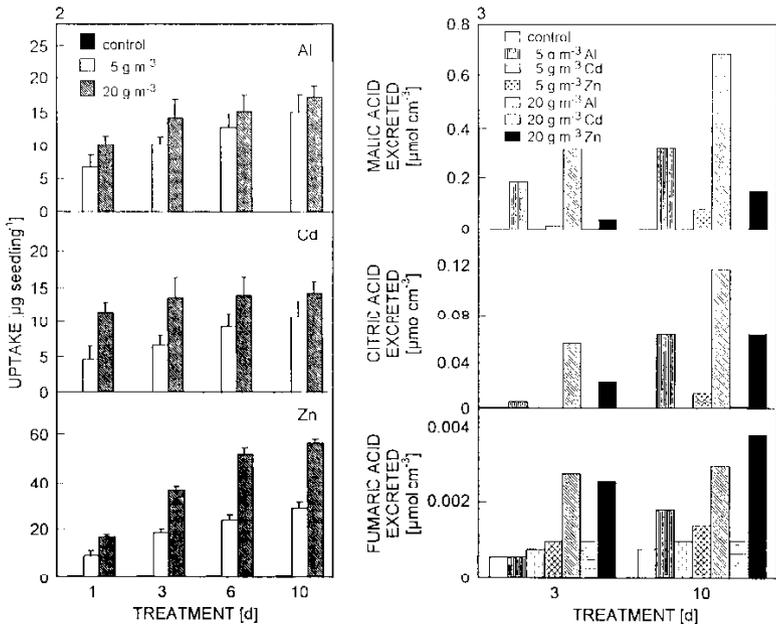


Fig. 2. Uptake of Al^{3+} , Cd^{2+} , or Zn^{2+} by sunflower seedlings grown in nutrient solution alone (control) or supplemented with 5 or 20 g m^{-3} of Al^{3+} , Cd^{2+} , or Zn^{2+} . Means of four replicates \pm S.E.

Fig. 3. Excretion of malic, citric and fumaric acids by sunflower seedlings grown in nutrient solution alone (control) or supplemented with 5 or 20 g m^{-3} of Al^{3+} , Cd^{2+} , or Zn^{2+} .

A significant increase of malic and citric acid contents was recorded in the shoots and roots of sunflower seedlings exposed to Al^{3+} , Cd^{2+} and Zn^{2+} , whereas the change in fumaric acid content was insignificant (Table 3). These results are in agreement with the results obtained by Mathys (1977), Subayda and Haug (1986), Kortz *et al.* (1989) and Harmens *et al.* (1993), and denoting that malic and citric acids may play a prominent role in the mechanism of heavy metal tolerance in sunflower. One can propose from the above mentioned results that tolerance mechanisms in sunflower include: firstly, exclusion (external) tolerance through excretion of organic acids in the rhizosphere reducing the uptake of these heavy metals and hence their deleterious effects on plant growth and secondly, internal tolerance via chelation of these heavy metals with organic in the cytoplasm, preventing their inhibitory effects on metabolic activities of the cells and increasing the rate of diffusion of these chelates to vacuole resulting in their accumulation.

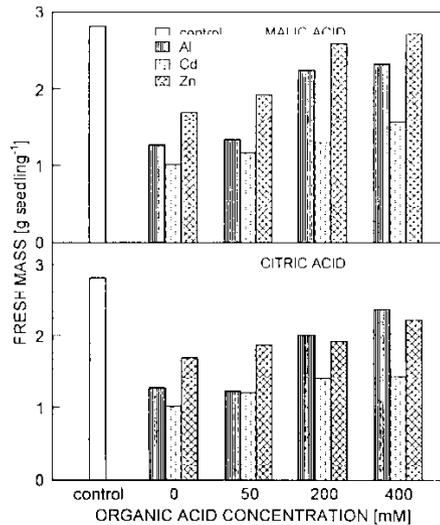


Fig. 4. Effect of addition of malic and citric acids on the fresh mass of sunflower seedlings. Ten-day-old seedlings were transferred to the nutrient solution contained 20 g m^{-3} of Al^{3+} , Cd^{2+} , or Zn^{2+} alone or supplemented with 50, 200, or 400 mM malic or citric acid for further 10 d. The control consisted of seedlings grown in nutrient solution alone.

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