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BRIEF COMMUNICATION

Zirconium induced physiological alterations in wheat seedlings

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Abstract

The effects of zirconium ascorbate (Zr-ASC), a water-soluble complex of Zr, were examined on wheat seedlings (*Triticum aestivum* L. cv. MV. 20). Hydroponically grown plants were exposed to 10, 33, 55, 100 and 550 μM Zr-ASC (Zr_{10}, Zr_{33} *etc*.). After 9 d of treatment inhibition of germination, retarded root and shoot growth, and increased activities of antioxidant enzymes (guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase) showed that Zr-ASC was only harmful at and over a concentration limit of 100 μM. Chlorophyll (Chl) content of plants was only decreased by Zr_{550}. Zr-ASC at lower concentrations was beneficial for plant development: Zr_{10} and Zr_{33} enhanced root elongation, Zr_{55} induced about 30 % increase in the total Chl content, while the activity of antioxidant enzymes was not elevated indicating that no oxidative stress was generated by the intracellularly accumulated Zr^{4+} ions.

Additional key words: abiotic stress, antioxidant enzymes, ascorbate peroxidase, chlorophyll, glutathione reductase, guiacol peroxidase, metal toxicity, root and shoot length, zirconium ascorbate.

Zirconium is the 20th most common element in the earth's crust, its total content in soils is deemed to be between 30 and 2000 mg kg^{-1}(dry soil), on an average 250 mg kg^{-1} (Pais and Jones 1997). Zr is present in nature in amounts higher than most trace elements. It occurs principally in inorganic insoluble compounds. Industrial utilisation of Zr in alloys, dyes, glasses, and ceramics is growing, so Zr wastes and by-products as well as radioactive Zr fallout discharged by nuclear reactors can be sources of contamination (Couture *et al.* 1989, Garnham *et al.* 1993). Its ever-growing environmental abundance has increased the importance of studies describing Zr effects on living organisms (Ghosh *et al.* 1992). Physiological effect of elements in soil always depends on the chemical form of their compounds, which determines their solubility and thence their uptake by the plants. Several factors may influence the uptake of metals. Pollution of air, natural waters, and soils among other things results in a lowered pH of the arable soils. In consequence insoluble compounds may be converted in a water-soluble form, which can enter root cells. The action of Zr on biological systems is scarcely known at present. Its toxicity was mild in young barley plants; red stems occurred as a visual symptom of Zr poisoning (Davis *et al.* 1978). Toxic effects induced by very high concentrations are non-specific in nature. Despite the presence and retention in relatively high quantities in biological systems, Zr has not yet been associated with any specific metabolic system. Apparently, the metal is neither an essential nor a toxic element in the conventional sense.

Zirconium ascorbate (Zr-ASC), a water-soluble pH-stabile chelate was formed (Fodor *et al.* 2003), which could be accumulated in a significant rate by *Chlorella* cells from the nutrient solution (Simon *et al.* 2001). This phenomenon may have practical importance in removal of Zr from contaminated aquatic environments. The intracellular quantity of several trace elements was influenced, chlorophyll (Chl) content was slightly lowered, and the Chl *a*/*b* ratio was changed in Zr-ASC treated cultures (Simon *et al.* 2001). The aim of the present study was to characterise responses of wheat seedlings treated with Zr-ASC considering whether it is a harmful pollutant or a potentially beneficial metal for higher plants.

Thirty wheat seeds (*Triticum aestivum* L. MV. 20) were imbibed for 24 h in tap water, then they were put in

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Abbreviations: AOS - active oxygen species; APX - ascorbate peroxidase; Chl - chlorophyll; GR - glutathione reductase; POD - guaiacol peroxidase; Zr-ASC - zirconium ascorbate.

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a Petri dish on a filter paper soaked by the Knop solution (Suba 1978) which was supplemented with K-ASC or different concentrations of Zr-ASC (10, 33, 55, 100 and 550 μM Zr = Zr_{10}, Zr_{33}, Zr_{55}, Zr_{100}, Zr_{550}). Zirconium was provided as Zr-ascorbate chelate. It was obtained from zirconyl chloride ($ZrOCl_2$. 8 H_2O) and L-ascorbic acid was applied as ligand. Concentrated hydrochloric acid was used to initiate complex formation, which took about one week and its process was controlled by a photometric method (Fodor *et al.* 2003). The pH was adjusted with the addition of KOH solution (10 %, m/v). All reagents were of analytical reagent grade. Plants germinated in darkness during the first 5 d and then grew at room temperature (25 °C) and natural irradiation. Number of seedlings was recorded and the percentage of germinated seeds was calculated. Shoot and root length of seedlings was measured after 9 d of Zr-ASC treatment. Nine-day-old seedlings were rinsed with distilled water, dried, and prepared for analysis. Plant material was dehydrated at 105 °C for 24 h and 0.2 g of powdered dry material was dissolved in 2 cm^3 of HNO_3 + H_2O_2 mixture (1:1, v/v). Decomposition of plant tissues was completed by incubation at high pressure and temperature.

Arsenazo III was used as a reagent to determine Zr content of the filtered solutions (Savvin 1961). This reaction is highly specific to Zr at low pH (8 M HCl). The measurement was carried out by a PC-controlled *GBC 916 UV/VIS* spectrophotometer (*GBC*, Dandenong, Australia) at 665 nm (ε = 120 $mmol^{-1}$ cm^{-1}). The acid concentration of the blank solution and the complex was adjusted to the same value. Standard Zr solution was prepared as follows: 1 cm^3 *Arsenazo III* reagent (1 g dm^{-3}), 1.25 cm^3 Zr (100 μM) in 8 M HCl was made up for a final volume of 25 cm^3. Under these conditions the measuring range is between 50 - 100 μM Zr.

Chl content was measured in an 80 % acetone extract made from 0.1 g leaf and calculated according to Arnon (1949). For enzyme analyses, plant material was homogenised with three-fold excess of buffer containing 0.1 M potassium phosphate (pH 7.8), 1 mM phenyl-methylsulfonyl fluoride, 2 mM diethylenetriamine-pentaacetic acid, 1 mM dithiothreitol and 5 mM ascorbic acid. The tissue extract was centrifuged at 10 000 *g* for 30 min. The procedure was carried out at 4 °C and the supernatant was used for further analyses. Guaiacol peroxidase (POD; E.C. 1.11.1.7) and ascorbate peroxidase (APX; E.C. 1.11.1.11.) activities were measured using guaiacol and ascorbic acid as substrates, respectively (Hegedüs *et al*. 2001). Glutathione reductase (GR; E.C. 1.6.4.2) activity was determined by using 5,5'-dithio-bis(2-nitrobenzoic acid) as substrate according to Smith *et al.* (1988).

Plants grown in Knop solution containing 5500 μM potassium ascorbate (K-ASC) were taken as control. This ascorbate concentration corresponded to that of the Zr_{550} treatment, due to the 1:10 stoichiometric proportion of the Zr-chelate. Three independent experiments were carried out and all measurements were performed with three parallels in all cases. The significance of differences between potassium ascorbate treated control and Zr-treated plants were statistically evaluated using the Student's *t*-test ($P \leq 0.05$).

K-ASC or Zr_{10} - Zr_{55} did not influence germination (Fig. 1*A*), in contrast to Zr_{100} and Zr_{550} that caused a marked concentration dependent decrease in the proportion of germinated seeds. In contrast to this, other highly toxic heavy metals as cadmium, or mercury cause significant inhibition at much lower doses (Shaw 1995).

Zr-ASC had a major impact on the root growth compared to the shoot elongation, which was practically not altered by Zr_0-Zr_{55} (Table 1). Root development was slightly enhanced by K-ASC, while Zr_{10} and Zr_{33} caused

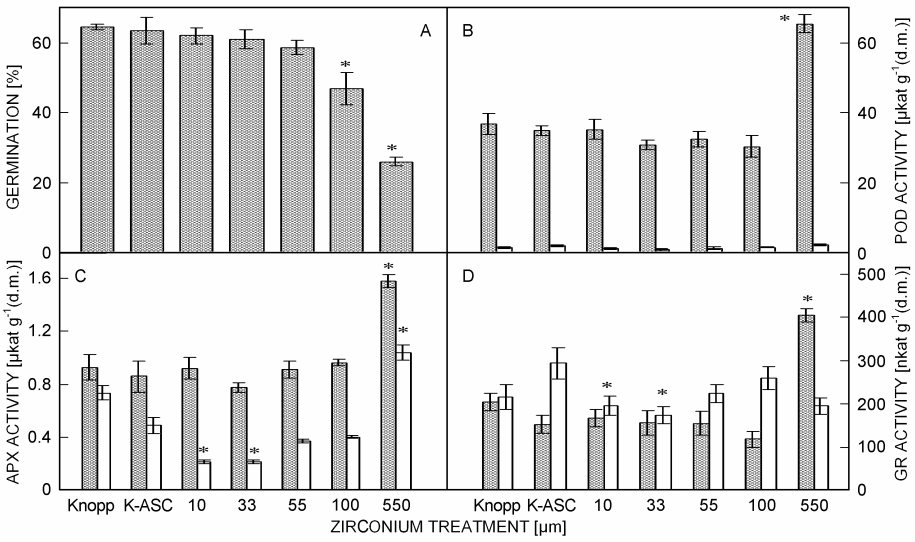

Fig. 1. Effect of Zr-ASC on germination (*A*) and on guaiacol peroxidase, POD (*B*), ascorbate peroxidase, APX (*C*), and glutathione reductase, GR (*D*) activities in roots (*filled columns*) and shoots (*white columns*) of wheat. Values of Zr-ASC treated seedlings marked with *asterisk* are significantly different at $P \leq 0.05$ from those of K-ASC grown plants.

Table 1. The effect of Zr-ASC on the root and shoot elongation; Zr accumulation in roots and shoots as well as chlorophyll content of wheat seedlings after 9 d of treatment. Values are means ± SE, $n = 3$. The mean values marked with *asterisk* are significantly different at $P \leq 0.05$ from values obtained from K-ASC treated seedlings (DL = detection limit).

	Length [cm]		Zr content [mg kg^{-1} (DM)]		Chlorophyll content
	root	shoot	root	shoot	[g kg^{-1}(d.m.)]
Knop (untreated)	6.76 ± 0.84	12.98 ± 0.75	<DL	<DL	14.1 ± 1.4
K-ASC (control)	7.55 ± 0.99	12.75 ± 0.94	<DL	<DL	14.0 ± 1.7
10 μM Zr-ASC	8.75 ± 0.77*	12.90 ± 1.01	9.7 ± 0.6	11.7 ± 0.8	14.0 ± 1.7
33 μM Zr-ASC	8.39 ± 0.98*	13.24 ± 1.15	114.0 ± 1.5	74.0 ± 5.5	17.4 ± 2.4
55 μM Zr-ASC	7.39 ± 0.52	12.63 ± 0.95	87.0 ± 4.5	117.0 ± 1.5	18.5 ± 2.4*
100 μM Zr-ASC	4.66 ± 0.48*	8.06 ± 1.38*	286.0 ± 2.5	261.0 ± 3.0	13.4 ± 1.6
550 μM Zr-ASC	0.98 ± 0.10*	4.38 ± 0.40*	1316.0 ± 2.5	233.0 ± 7.5	9.3 ± 1.2*

a further increase in the length of roots. Zr_{100} and Zr_{550} caused considerable retardation in root and shoot elongation. Growth of roots was more substantially affected by Zr than that of shoots, presumably due to the fact that roots were directly submerged in the Zr containing media.

In both roots and shoots the accumulation of Zr was more or less proportional to the Zr concentration in the medium (Table 1). This coincides with former studies obtained by other metals (Barnabás *et al.* 2000, Hegedüs *et al.* 2001). Zr content of root and shoot tissues was similar, however, at Zr_{550} roots accumulated more than five times higher amount of Zr than shoots. This may indicate a kind of defence mechanism by preventing the more sensitive shoots from accumulating hazardous quantity of Zr under a treatment well over the threshold tolerable for plants, as it was presumed by Fernandes and Henriques (1991) in case of copper.

Chlorosis is a characteristic visual symptom of the abiotic stress induced by various metals (Stoeva *et al.* 2003/4, Astolfi *et al.* 2004, Hegedüs *et al.* 2004). Chl content of leaves was only decreased at Zr_{550}. Moreover, wheat seedlings treated by Zr_{55} possessed significantly more Chl than K-ASC treated plants (Table 1). In contrast to it, Simon *et al.* (2001) experienced a slight but not significant decrease in Chl content of *Chlorella* treated by 1 - 50 μM Zr-ASC. Titanium in a similar ascorbate chelated form had also a positive effect on the Chl content of *Anacystis nidulans* (Kiss *et al.* 1985), *Chlorella pyrenoidosa* (Simon *et al.* 1988), and *Capsicum annuum* (Carvajal *et al.* 1994a). It was explained by the increase of physiologically active Fe^{2+} fraction due to low Ti^{3+}/Ti^{4+} redox potential, which in turn may stimulate pigment biosynthesis and photochemical capacity of plants (Simon *et al.* 1988, Carvajal and Alcaraz 1995). It seems to support our results, since Ti and Zr belong to the same subgroup of the periodical system and share common chemical properties conferring the possibility of a similar cellular event.

In plant cells exposed to several stress factors (*e.g.* polluting metals) oxidative stress occurs as a common consequence of the inordinate accumulation of active oxygen species (AOS) (Skórzyńska-Polit *et al.* 2003/4). Guaiacol peroxidase (POD) is one of the enzymes playing a crucial role in the elimination of harmful AOS. POD activity was much higher in roots than in shoots of wheat seedlings. Considerable increase in POD activity was only found at Zr_{550} treatment when in roots and shoots it was enhanced by 80 and 17 %, respectively (Fig. 1*B*). Similar results were obtained by Ti-ASC in *Triticum aestivum* and *Zea mays* (Pais 1983) and in *Capsicum annuum* (Carvajal *et al.* 1994b). In these analyses, ascorbate treatments did not have any effect, producing similar enzyme activities to those of the control plants, as it was also shown in our experiments. Furthermore, the increase in POD activity due to Zr_{550} treatment was accompanied by a marked decrease in the total phenol content of plant tissues (data not shown). Various phenolic compounds are used as substrates by POD isoenzymes during lignin biosynthesis (Gaspar *et al.* 1991). It suggests that isoenzymes responsible for the activity increase are those acting in the process of lignin biosynthesis, as it was previously demonstrated in case of nickel induced stress (Pandolfini *et al.* 1992). Higher lignin deposition may form a physical barrier against metal uptake (Hegedüs *et al.* 2001), nevertheless it makes cell walls rigid, which results in growth inhibition, as it was evidenced from the Zr_{100} and Zr_{550} induced growth retardation.

Ascorbate peroxidase (APX) and glutathione reductase (GR) are two key enzymes of AOS detoxification in the cytoplasm and chloroplasts where they are located (Foyer 1993). Activity of APX was unchanged in roots, while in shoots K-ASC and Zr_{10}-Zr_{100} decreased it. At Zr_{550} APX activity was about 80 and 100 % higher in roots and shoots, respectively, compared to control plants grown in K-ASC (Fig. 1*C*). The activity of GR in roots was slightly lowered by K-ASC and Zr_{10}-Zr_{100}; nevertheless, at Zr_{550} the GR activity in roots was more than 2.5-fold elevated compared to control plants (Fig. 1*D*). GR activity in shoots was more or less lowered by Zr treatments. Activities of APX and GR markedly enhanced by Zr_{550} indicated that root and shoot cells confront with a considerable oxidative challenge only at this extremely high concentration. Most of the environmental stimuli, including heavy metals (such as

Cd, Cu, or Zn) may induce even higher APX and GR activities (Chaoui *et al.* 1997, Clijsters *et al.* 1999, Šimonovičová *et al.* 2004).

Taken together, water-soluble Zr-ascorbate was only harmful for wheat seedlings at and over a concentration threshold of 100 μM. Zr-ASC at lower concentrations proved to be not harmful but beneficial for plant development: 10-33 μM enhanced root and shoot elongation, 55 μM induced a considerable increase in chlorophyll content, while no oxidative stress was generated by the intracellularly accumulated Zr^{4+} ions.

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