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Inhibition of wheat nitrate reductase activity by zinc

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Abstract

The effect of Zn^{2+} on nitrate reductase (NR, EC 1.6.6.1) activity was studied in both wheat (*Triticum aestivum* cv. Oasis) leaves and in the NR enzyme partially purified from wheat leaves. Leaf segments were floated on 0 to 5 mM $ZnSO_4$ solutions (pH 6.0) for 24 h under continuous light. Zn^{2+} at 250 μM decreased NR activity and increased membrane permeability. However, parameters of cellular oxidative damage were scarcely affected by Zn^{2+} treatments. Accordingly, the decrease of NR activity induced by Zn^{2+} was not prevented by benzoate (a scavenger of oxygen radicals). The effect of Zn^{2+} was dependent on leaf age: it decreased NR activity in mature but not in young leaves. Zn^{2+} inhibited the partially purified NR. This inhibition was not reversed by either co- or post-incubation with cysteine, and the amount of -SH groups of the purified NR was not affected by Zn^{2+} indicating that Zn^{2+} inhibition does not involve key -SH groups of the enzyme. However, *o*-phenantroline both prevented and reversed Zn^{2+}-induced NR inhibition. We concluded that the effect of Zn^{2+} on NR activity *in vivo* is not associated with an increase in active oxygen generation and involves a direct and reversible inhibition of the enzyme.

Additional key words: nitrate assimilation, *Triticum aestivum*, Zn toxicity, Cu toxicity.

Introduction

Zinc is an essential micronutrient for all living organism. At physiological concentrations (*ca.* 10^{-6} M), Zn^{2+} is an antioxidant (Bray and Betger 1990), and it is required for the maintenance of membrane integrity (Cakmak and Marschner 1988). Adequate Zn^{2+} supply plays an important role in the preservation of root cell plasmalemma structure and the ion transport across the membrane, by stabilising sulfhydryl groups of proteins (Welch and Norvell 1993) and preventing their oxidation to disulfides (Bray and Bettger 1990, Rengel 1995). However, a high concentration of Zn^{2+} is considered as one of the most important components of industrial contamination in both aquatic and terrestrial environments (Foy *et al.* 1978), causing severe damage and even the death in plant stands. Indeed, Van Assche and Clijsters (1986) demonstrated that 3.45 mM Zn^{2+} diminish the growth rate in hydroponically cultured bean plants. In algae and higher plants, Zn^{2+} inhibited photosynthetic CO_2 fixation (De Filippis 1981, Van Assche and Clijsters 1986) by affecting photosynthetic electron transport at the water-splitting site and at the plastoquinone reduction site, and photophosphorylation (Baker *et al.* 1982, Van Assche and Clijsters 1986). In consequence high concentrations of Zn^{2+} could reduce the ATP and NADPH pools and increase the generation of superoxide radical and H_2O_2, similarly as Cu^{2+} ions (Sandmann and Bögger 1980). Active oxygen species can cause the oxidative damage of a number of cellular components (Foyer *et al.* 1994).

In addition, a wide range of heavy metals including Zn^{2+}, can disrupt activities of several enzymes (Van Assche and Clijsters 1986). Within this context, an early

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Abbreviations: Chl - chlorophylls; Cys - cysteine; Cyt *b* - cytochrome *b*; DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid); FAD - flavin adenine dinucleotide; MDA - malondialdehyde; NR - nitrate reductase.
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study of Mathys (1975) showed that the activity of nitrate reductase (NR), a key enzyme of nitrate assimilation, was sensitive to high concentrations of Zn^{2+}, although to a lesser extent than to Cu^{2+}. Smarrelli and Campbell (1983) reported that Cu^{2+} and Zn^{2+} interactions with purified NR resulted in an inefficient electron transfer within the enzyme. As recently reviewed by Campbell (1996), all the known sequences of NR present one invariant and absolutely conserved Cys residue located in the Cyt *b* reductase fragment, which seems to assist electron transfer from NADH to FAD. In previous experiments (Luna *et al.* 1997) was found that Cu^{2+} inhibited NR in both wheat leaf segments and purified enzyme, probably due to the oxidative damage of essential -SH group(s). In addition, it is known that high concentrations of Zn^{2+} can decrease the amount of -SH groups in plasma membrane proteins of root cells of barley (Brune *et al.* 1995). NR activity is highly sensitive to the attack of free oxygen radicals, which can modify and even fragmentate NR protein (Luna *et al.* 1997)

In consequence, we postulate that toxic levels of Zn^{2+} could diminish NR activity of leaves *a*) indirectly, by interfering electron transfer (mainly in thylakoids) leading to an increase in the generation of active oxygen species and related free radicals; and/or *b*) directly by the effect on NR protein, probably at the level of essential -SH group(s). With the aim of achieve a better understanding of the effect of high concentrations of Zn^{2+}, like those encountered in contaminated areas, we performed experiments in which wheat leaf segments and partially purified NR were exposed to $ZnSO_4$. For comparative purposes, parallel treatments with $CuSO_4$ were also carried out in some cases.

Materials and methods

Seeds of wheat (*Triticum aestivum* L. cv. Oasis) were sown in vermiculite and seedlings were grown under continuous white light (irradiance 40 W m^{-2}) at 23 °C. Seven days after planting, 3 cm-subapical segments were cut from the first leaf of each plant and groups of ten segments were floated on $ZnSO_4$ solutions (0, 0.05, 0.10, 0.25, 0.50, 1.00, and 5.00 mM; pH 6.0) for 24 h under continuous light (40 W m^{-2}) at 23 °C. In other experiments, 6 and 12 d after planting 3 cm-subapical segments were floated on 5 mM $ZnSO_4$ for 6 h under continuous light.

The effect of free radical scavengers on Zn^{+2}-related NR activity, was studied by incubating leaf segments with either 1 or 5 mM $ZnSO_4$ solutions containing 0 or 10 mM Na-benzoate.

Nitrate reductase activity of leaf segments was assayed according to Campbell and Remmler (1986), with some modifications (Luna *et al.* 1997). The amount of nitrite produced was determined by measuring absorbance at 540 nm of the diazo compound formed from sulfanilamide (Lillo and Ruoff 1992) employing a *DU300* spectrophotometer (*Beckman*, Palo Alto, USA). One unit of NR activity is defined as that producing of 1 μmol(nitrite) min^{-1}.

For partial purification of NR, the extraction of the enzyme was followed by affinity chromatography on Blue-Sepharose (*Sigma*, Saint Louis, USA). The enzyme was purified to *ca.* 300 fold during this procedure.

Effect of Zn^{2+} was determined after incubation of NR (0.05 units) for 15 min at 25 °C with 0, 250, and 500 μM $ZnSO_4$, 0 or 0.5 mM cysteine and 0 or 0.5 mM phenantroline, in a medium containing 25 mM K-phosphate buffer (pH 7.5), 1 μM leupeptin, 1 μM FAD, and 300 mM KNO_3 (final volume 0.1 cm^3). All the reagents were degassed before use. In recovery experiments, incubations of 0.5 units of NR with 0, 250, 500 μM $ZnSO_4$ (as described, without cysteine and phenantroline), were followed by the addition of 0.01 cm^3 of water, 10 mM cysteine or 5 mM phenantroline (1 mM and 0,5 mM final concentration, respectively) and a second incubation for 10 min at 25 °C. After every treatment, NR activity was assayed as described.

-SH groups of partially purified nitrate reductase were assayed by the method of Sedlak and Lindsay (1968) with some modifications: 0.05 units of NR were incubated with 0.1, 0.5 or 1 mM $ZnSO_4$ during 30 min at 25 °C. Then, 0.05 cm^3 of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 0.89 cm^3 of 100 mM K-phosphate buffer (pH 7.5) were added. The total concentration of -SH groups was determined by measuring the absorbance at 412 nm, with glutathione as standard.

Chlorophyll (Chl) and malondialdehyde (MDA) were quantified as described previously (Luna *et al.* 1997), in ethanol extracts obtained from a parallel set of leaf treatments.

K^+ efflux was determined in the incubation media of leaf segments, using a *Corning 435* flame photometer (USA).

Protein concentration was measured with Coomassie Brilliant Blue G-250 (*Sigma*), according to Sedmark and Grossberg (1977) using bovine serum albumin as a standard.

All the experiments, in duplicates, were repeated at least five times. When appropiate, standard deviations are indicated in tables and figures.

Results and discussion

Effect of Zn^{2+} on NR activity in wheat leaf segments: An inhibitory effect of Zn^{2+} on NR activity began to be apparent at a concentration of 0.25 mM and progressively increased with the increase of Zn^{2+} concentration (Fig. 1). These results agree with the findings of Mathys (1975), who showed that NR of leaves of *Silene cucubalus* was inhibited by Zn^{2+}. A similar degree of reduction of NR activity in wheat leaves was achieved by 25 μM Cu^{2+} treatment under similar experimental conditions (Luna *et al.* 1997); indicating that, with respect to Cu^{2+}, Zn^{2+} has a lower detrimental effect on NR activity.

The decrease in NR activity provoked by Zn^{2+} treatments up to 2.5 mM was not associated with a significant oxidative damage in wheat leaf segments, as judged by the absence Chl loss and the low increase in the content of MDA, an intermediate product of membrane lipid peroxidation (Fig. 1). However, decreases in NR were accompanied by increases in membrane permeability as shown by K^+ efflux (Fig. 1). Optimal levels of Zn^{2+} protect -SH groups of membrane proteins (Bray and Bettger 1990, Rengel 1995, Welch and Norvell 1993). However, toxic concentrations of Zn^{2+} decrease the amount of protein -SH groups and induce membrane alterations (Brune *et al.* 1995). Possibly, the modification of -SH groups of membrane proteins was the one of the primary reactions induced by Zn^{2+} and lipid peroxidation was a late event (Fig. 1).

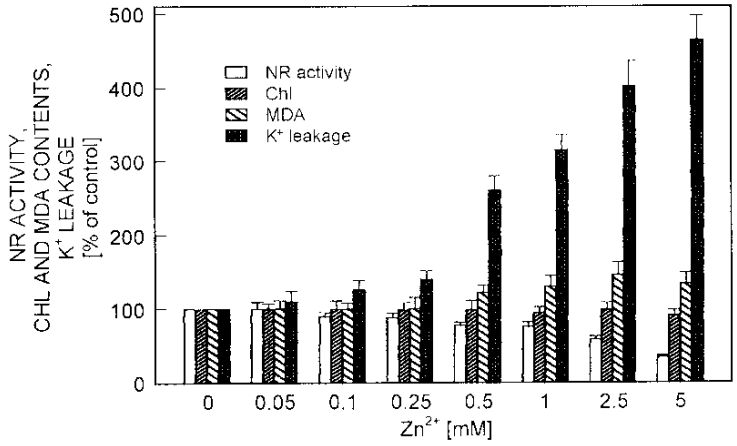

Fig. 1. Effect of Zn^{2+} on NR activity, chlorophyll and MDA contents, and K^+ efflux in 7-d-old leaf segments of wheat incubated in $ZnSO_4$ for 24 h in continuous light. Values are the results of 8 experiments expressed as % of control (0 μM Zn^{2+}): NR = 210 μmol(NO_2) segment^{-1} h^{-1}; chlorophylls = 1.07 μmol g^{-1}(f.m.); MDA = 5.21 μmol g^{-1}(f.m.), and K^+ efflux = 0.83 nmol segment^{-1} h^{-1}). For details see Materials and methods.

Table 1. Age-dependent effect of Zn^{2+} and Cu^{2+} on NR activity, MDA content and K^+ leakage. Leaf segments of 6- and 12-d-old wheat seedlings were incubated in water (control) or the heavy metals solutions and 0 or 1 mM Na-benzoate for 6 h under continuous light (40 W m^{-2}) at 23 °C. Values are expressed as % of control. Standard deviations are shown in parentheses.

Leaf age	Treatment	NR activity	K^+ leakage	MDA
6-d-old	5 mM Zn^{2+}	92 (7.0)	110 (12.0)	109 (6.8)
	5 mM Zn^{2+} + benzoate	90 (5.4)	108 (10.5)	112 (8.0)
12-d-old	5 mM Zn^{2+}	40 (5.2)	200 (16.4)	140 (12.1)
	5 mM Zn^{2+} + benzoate	35 (4.3)	186 (15.3)	120 (13.7)
6-d-old	1 mM Cu^{2+}	43 (5.0)	180 (13.4)	500 (40.2)
	1 mM Cu^{2+}+ benzoate	60 (4.2)	121 (16.0)	205 (26.3)
12-d-old	1 mM Cu^{2+}	28 (3.1)	250 (18.9)	350 (26.5)
	1 mM Cu^{2+}+ benzoate	45 (5.2)	156 (14.0)	185 (23.2)

Membrane permeability was also increased by Cu^{2+}. The effect of Cu^{2+} was more drastic than that of Zn^{2+} even though the concentration of the former was one fifth of the later (Fig. 2). While K^+ leakage increased by a 2.5 % in control and Zn^{2+} treatment after 2-h incubation, it increased by a 9 % in Cu^{2+} treatment. As the experiment

proceeded, differences between Zn^{2+} and Cu^{2+} progressively increased (Fig. 2). However, the incubation with both metals ameliorated the effect of Cu^{2+} on membrane permeability (Fig. 2). Furthermore, Cu^{2+} treatment decreased NR activity to higher extent when compared with the effect of Zn^{2+} in young and mature leaves (Table 1). The effect of Cu^{2+} on NR could be associated with an increase in the level of active oxygen, which can attack the enzyme, as previously demonstrated by Luna *et al.* (1997). The increase in lipid peroxidation (MDA) and the fact that Na benzoate, a scavenger of free OH radicals (Parups 1984), partially but consistently prevented the NR decrease induced by Cu^{2+} (Table 1) argue in favour of this hypothesis. In contrast, the decrease of NR activity induced by Zn^{2+} was not affected by the scavenger of active oxygen in either young and mature leaves treated with 5 mM Zn^{2+} for 6 h (Table 2) or young leaves treated with 1 and 5 mM Zn^{2+} for 24 h (Table 2).

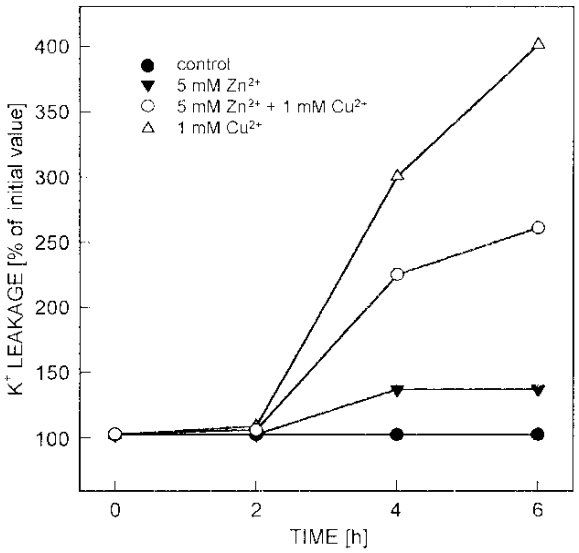

Fig. 2. Effect of Zn^{2+} and Cu^{2+} on K^+ leakage in leaf segments of *Triticum aestivum*. Ten 3-cm segments were floated on water (control), 5 mM $ZnSO_4$, 1 mM $CuSO_4$ or 5 mM $ZnSO_4$ plus 1 mM $CuSO_4$ for 6 h in continuous light (40 W m^{-2}) at 23 °C. K^+ efflux during the first hour of incubation in control (initial value) was around 0.87 nmol segment^{-1} h^{-1}.

Another distinct difference between the modes of action of Zn^{2+} and Cu^{2+} emerged from the comparison of their effects in young and mature leaves. When 6- and 12-d-old leaf segments were incubated with 5 mM Zn^{2+} for 6 h in continuous light, a significant effect of Zn^{2+} on NR could only be observed in mature leaves (Table 1). Two-fold increase in membrane permeability (K^+ efflux) and a slight increase in MDA content was also found in mature leaves, while these parameters were not affected in young leaves (Table 2). As stated above, Cu^{2+} treatments decreased NR activity and increased K^+ leakage and MDA level in both young and mature leaves, even though its effect was more pronounced in 12-day-old leaves (Table 1). In addition, the fact that NR decreases induced by Zn^{2+} were accompanied by a higher membrane permeability, as observed in young leaves treated for 24 h (Fig. 1) and in mature leaves under shorter incubations (Table 1), suggests that the intracellular effect of Zn^{2+} is dependent on the sensitivity of plasma membrane to be altered by the metal.

Table 2. Effect of Zn^{2+} and Na-benzoate on nitrate reductase activity of wheat leaf. 7-d-old leaf segments were floated on $ZnSO_4$ solutions at the indicated concentrations in the presence or absence of Na-benzoate for 24 h in continuous light (40 W m^{-2}) at 23 °C. Control NR activity was 210 μmol(NO_2^-) segment^{-1} h^{-1}. Values are expressed as % of control (incubated in water), and are mean of five independent experiments with less than 10 % standard deviation.

Zn^{2+} [mM]	0	0	1	1	5	5
Benzoate [mM]	0	10	0	10	0	10
NR activity	100	90	80	85	45	48

In summary, our results show that the *in vivo* effect of Zn^{2+} on NR activity depends on its penetration inside the leaf tissues and that this effect is not an indirect consequence of a Zn^{2+}-induced increase of active oxygens which oxidise the enzyme. A direct effect on the enzyme and the activation of some inhibitor protein are two possible mechanisms by which Zn^{2+} inactivates NR.

Effect of Zn^{2+} on the purified NR: In order to elucidate the mechanism(s) involved in the observed Zn^{2+}-induced decrease of NR activity *in vivo*, NR enzyme was partially purified from wheat leaves and then subjected to treatments with $ZnSO_4$. Zn^{2+} decreased NR activity reaching a 50 % inhibition at a concentration of 0.5 mM (Table 3). A further increase in the concentration of Zn^{2+} treatment did not increase NR inhibition (data not shown). Accordingly, Smarrelli and Campbell (1983) found that Zn^{2+} diminished the efficiency of electron transfer within the enzyme domains and finally to nitrate. On the other hand, the mechanism of Zn^{2+} inhibition of NR seems to differ from that of other heavy metals such as Cu^{2+}. Firstly, the effect of Zn^{2+} was significantly

Table 3. Zn^{2+}-induced inhibition of NR. Partially purified NR was incubated with or without $ZnSO_4$ for 15 min at 25 °C. When indicated, 500 µM cysteine or 500 µM phenantroline or an equal volume of water was added to the incubation medium (co-incubations). Then, NR incubated with or without Zn^{2+}, but with neither cysteine nor phenantroline, was post-incubated with 500 µM cysteine or 500 µM phenantroline for 10 min at 25 °C. NR activity was assayed as described in Materials and methods and expressed as % of control. Control NR activity (0 µM $ZnSO_4$, with water) was 2 U mg^{-1}(protein). Mean of six independent experiments, standard deviation less than 10 %.

Treatments	co-incubation with water	co-incubation with cysteine	post-incubation with cysteine	co-incubation with phenantroline	post-incubation with phenantroline
Control	100	100	90	100	100
250 uM Zn^{2+}	70	70	70	98	90
500 uM Zn^{2+}	50	56	56	90	80

less severe than that of Cu^{2+}, since a ten-fold concentration of the former (0.5 mM) was needed to inhibit NR at a rate similar to that observed with 0.05 mM Cu^{2+} (Luna *et al.* 1997). Secondly, Zn^{2+} inhibition does not seem to involve reaction(s) of the metal with -SH groups of the NR, since neither co- nor post-incubations with cysteine prevented NR inhibition by Zn^{2+} (Table 3).

Table 4. Effect of Zn^{2+} and Cu^{2+} on -SH groups of purified NR. Reduced SH-groups were determined in 0.05 units of NR incubated with 0 to 100 µM Cu^{2+} and 0 to 1 mM Zn^{2+} during 30 min at 25 °C (final volume 0.1 cm^3), after which 0.05 cm^3 of 10 mM DTNB and 0.89 cm^3 of 100 mM K-Pi buffer, pH 7.5, were added. Absorbance at 412 nm was recorded after incubating 10 min at room temperature. Total -SH groups of control NR (0 µM Cu^{2+} and Zn^{2+}) varied from 0.14 to 0.2 µmol of reduced glutathione equivalents per mg of enzyme. Means of seven independent experiments, standard deviation less than 5 %.

	Cu^{2+} [µM]			Zn^{2+} [µM]		
	25	50	100	100	500	1000
SH-groups [% of control]	50	40	25	100	100	100

Accordingly, the amount of-SH groups of the enzyme, available for reacting with DTNB, was not influenced by Zn^{2+} treatments (Table 4). On contrary, when NR was incubated with Cu^{2+} a marked decrease of enzymatic -SH groups was observed (Table 4). It has been demonstrated that Zn^{2+} produces an antioxidant effect through its ability to bind in the proximity of -SH groups causing conformational changes in the enzyme which protect -SH groups from oxidation (Bray and Bettger 1990). In addition, the inhibitory effect of Zn^{2+} on purified NR could be almost completely prevented by co-incubation and reversed by post-incubation with the specific chelating agent *o*-phenantroline (Table 3). Therefore, our results show that NR can be directly and reversibly inhibited by Zn^{2+} and that this effect is not related with a blockage or interaction of the metal with key -SH groups of the enzyme. The chemical nature of Zn^{2+}-NR interactions remains to be elucidated. On the basis of data of the early studies (reviewed by Vallee and Ulmer 1972), it is reasonable to propose that Zn^{2+} reacts preferably with carboxyl groups of the enzyme.

In summary, from the comparison of the effects of Zn^{2+} on leaf segments and on partially purified NR, we concluded that when plants are exposed to supraoptimal Zn^{2+} levels, nitrate assimilation decreases as a consequence of a direct inhibitory effect of the metal on NR.

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