

Modulation of Cu^{2+} accumulation by (aminoethoxyvinyl)glycine and methylglyoxal bis(guanyldihydrazone), the inhibitors of stress ethylene and polyamine synthesis in wheat genotypes

Irma Tari, Jolán Csiszár, Katalin Gémes and Ágnes Szepesi

Department of Plant Physiology, University of Szeged, Egyetem u. 2. POBox 654, H-6701 Szeged Hungary

Corresponding author: tari@bio.u-szeged.hu

Summary

The effectiveness of Cu^{2+} accumulation was investigated in three wheat cultivars with different copper sensitivity (*Triticum aestivum* cv. GK Tiszatáj, GK Kata and GK Óthalom). Supraoptimal Cu^{2+} concentrations result in toxicity symptoms in the sensitive genotype and increase the production of the stress hormone, ethylene both in the leaves and root tissues of wheat seedlings. The sensitive cultivar, cv. Óthalom produced less ethylene than the tolerant genotypes (cvs Tiszatáj and Kata) in the roots whether the ethylene measurements were done over the 6-h period after Cu^{2+} exposure. Levels of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, did not change characteristically during this period in either the tolerant or sensitive seedlings. The biosynthesis of ethylene has a common intermediate, S-adenosylmethionine (SAM) with the synthesis of the polyamine spermidine and 2'-deoxymugineic acid, a Fe^{3+} -solubilizing and transporting wheat phytosiderophore. These chelating substances also mediate the transport of different bivalent cations, such as Cu^{2+} . The biosynthetic pathways of ethylene and polyamines, spermidine and spermine may compete for SAM with the phytosiderophore synthesis. Simultaneous inhibition of SAM decarboxylase by 0.5 M methylglyoxal bis(guanyldihydrazone) (MGBG), and 1-aminocyclopropane-1-carboxylic acid synthase by 10 μM (2-aminoethoxy-vinyl)glycine (AVG), significantly increased the Cu^{2+} accumulation in root tissues of the wheat cultivars independently of their sensitivities. MGBG alone resulted in an enhanced copper content but AVG proved to be ineffective. This suggests that the amount of SAM allocated for polyamine formation may limit the phytosiderophore synthesis or spermidine (spermine) in itself may control the uptake of Cu^{2+} .

Key words: (2-aminoethoxyvinyl)glycine, copper accumulation, ethylene production, methylglyoxal bis(guanyldihydrazone), phytosiderophores, spermidine synthesis

Introduction

Copper is an essential microelement for plants and its deficiency affects both the vegetative growth and grain formation of cereals. However, elevated concentrations of essential heavy metals in the soil or in the hydroponic culture solution can lead to toxicity symptoms, which may be due to a range of molecular, biochemical and physiological interactions such as the plasma membrane damage and oxidative stress (De Vos and Schat 1991), ion transport (Demidchik *et al.* 1997), photosynthesis (Ciscato *et al.* 1997), cell division (Jiang *et al.* 2001) or root growth (Tari *et al.* 2002). For this reason, the selection of genotypes with efficient copper uptake and translocation or investigation of the mechanisms of copper tolerance are challenging tasks for breeders.

Graminaceous species (Strategy II plants) such as wheat or barley solubilize Fe by the secretion of mugineic acid-type phytosiderophores (PSs) into the rhizosphere. The uptake through the plasmalemma occurs as a phytosiderophore-ferric complex without reduction of Fe^{3+} . PS release from the roots increases under Fe-deficiency and exhibits a diurnal pattern. Methionine, a precursor of PSs is converted to mugineic acid derivatives through S-adenosylmethionine (SAM) and nicotianamine (NA) intermediates (Mori and Nishizawa 1987). NA has been found in all plants so far surveyed and the crucial step in the biosynthesis of mugineic acid-like phytosiderophores that distinguishes graminaceous plants from other species, is the amino-group transfer of NA by nicotianamine aminotransferases. The type of PS is characteristic of species, e.g. hexaploid, tetraploid or diploid wheat lines secrete 2'-deoxymugineic acid while barley synthesizes 3-epihydroxymugineic acid and mugineic acid (Ma *et al.* 1999). However, mugineic acid derivatives are not specific for Fe^{3+} or Fe^{2+} ion, they bind and transport other bivalent cations including Cu^{2+} . Metal analyses of wheat plants revealed that Fe deficiency increased Cu uptake and translocation suggesting that Fe stress-induced PS release led coincidental accumulation of Cu^{2+} ion (Shenker *et al.* 2001).

Supraoptimal Cu^{2+} concentrations are effective inducers of the stress ethylene synthesis and they also interfere with polyamine metabolism (Turano *et al.* 1997).

SAM is a common intermediate of the ethylene and polyamine (PA) synthesis and a possible competition between polyamine and ethylene metabolisms was studied by several authors (Quan *et al.* 2002). SAM is readily converted to the immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, which can be blocked by (2-aminoethoxyvinyl)glycine (AVG), a pyridoxal phosphate enzyme inhibitor (Bregoli *et al.* 2002). After decarboxylation, the aminopropyl group of SAM may be donated for the synthesis of spermidine and spermine in the polyamine biosynthetic pathway. Methylglyoxal bis(guanyldrazone) (MGBG), an effective inhibitor of SAM decarboxylase is generally used to counteract the physiological effects of spermidine (Mader 2004). Although labelled methionine incorporation was two orders of magnitude larger into spermidine than into ethylene (Even-Chen *et al.* 1982), the two biosynthetic pathways regulate each other mutually. In soybean leaves Turano *et al.* (1997) demonstrated that the polyamine titer was increased by AVG and the ethylene production was elevated by MGBG. Ethylene was shown to inhibit the activity of SAM decarboxylase and polyamines decreased the transcript accumulation and activity of ACC synthase (Li *et al.* 1992), but the results were often contradictory (Quan *et al.* 2002).

The aim of our work was to investigate the effect of excess copper on the ethylene production and on the accumulation of ethylene precursor, ACC in wheat seedlings with different copper sensitivity and to relate the ethylene production rate of cultivars to their sensitivities. We supposed if the conversion of SAM into the ethylene precursor ACC or the decarboxylation of SAM and as a consequence, spermidine and/or spermine synthesis were blocked, this might generate an enhanced Cu^{2+} accumulation in wheat plants.

Materials and Methods

Wheat caryopses (*Triticum aestivum* cv. GK Tiszatáj, GK Kata and GK Óthalom) were surface-sterilized for 30 min with 1% NaOCl, and were germinated in a dark thermostat for 24 h at 24 °C. The seedlings were grown hydroponically for 4 days in $5 \cdot 10^{-4}$ M CaSO_4

solution (pH 6.5) in a Conviron growth chamber at 22/19 °C day/night temperatures under 14/10 h light/dark period, at 75% relative humidity. On the 5th day, they were transferred for 24 hs into a fresh CaSO_4 solution (control) or were treated with 10 μM CuSO_4 in the low-salt solution. In half of the samples S-adenosylmethionine decarboxylase was inhibited by 0.5 M MGBG and/or the synthesis of the ethylene was blocked by 10 μM AVG. The treatments with Cu^{2+} began at one o'clock p.m., and the samples were harvested after 24 hs.

For ethylene determination the excised intact roots were sealed in 5.5 cm^3 tubes and ethylene was collected above the tissues for 1 h. At this stage, the roots had no branches. The ethylene production and ACC contents of the samples was determined with a Hewlett-Packard gas chromatograph, as described earlier (Tari and Mihalik 1998). ACC determinations occurred at the end of the ethylene measurements. The samples consisted of five replicates, and were repeated twice.

The roots of seedlings were washed with 5 mM $\text{Pb}(\text{NO}_3)_2$ for 30 min in order to remove the surface adhering Cu^{2+} ions. Concentrations of Cu^{2+} and K^+ were determined after wet digestion of 100 mg dried plant material in a mixture of nitric acid and 30% H_2O_2 (5:4; v/v) at 200 °C for at least 3 hs with a Hitachi Z-8200 atomic absorption spectrophotometer.

Results

Wheat cultivars used in our experiments had different sensitivity to supraoptimal copper concentrations. On the basis of root growth test, K^+/Na^+ ratio in the roots and glutathione metabolism, two of the investigated cultivars GK Tiszatáj and Kata exhibited high and medium tolerance, respectively, and cv. GK Öthalom proved to be sensitive (Tari *et al.* 2002).

Evolution of ethylene by the shoots and roots of wheat seedlings were compared over a period of 24 hours. Ethylene production rate in the root tissues increased earlier and exhibited much higher values in the roots of the two tolerant genotypes, cv. Tiszatáj and Kata than in the sensitive cv. Öthalom during the first 6 hours after 10 μM Cu^{2+} treatment. The increases in ethylene production due to copper stress were not so high in the shoots of the tolerant lines, and the sensitive genotype, cv. Öthalom synthesized slightly less ethylene during the first period of the acclimation process (Fig. 1).

The ACC contents of the root and shoot tissues did not change much during the first 24 hours after 10 μM Cu^{2+} treatment. In the roots of cv. Kata, however, significantly enhanced ACC contents could be detected 24 hours after copper treatment. The shoot of the same cultivar contained only trace amount of ACC (Fig. 2).

Simultaneous inhibition of spermidine synthesis by 0.5 mM MGBG and that of ethylene by 10 μM AVG significantly increased Cu^{2+} uptake by the roots in 10 μM CuSO_4 solution but did not promote translocation of copper into the shoots of seedlings (Table 1.) There were no significant differences in the effect of inhibitors between genotypes.

In the roots, however, there was no similar accumulation of potassium which could originate exclusively from the grains on a CaSO_4 -containing culture medium but under copper stress a small decrease could be observed both in the shoot and root potassium contents in the presence of the inhibitors.

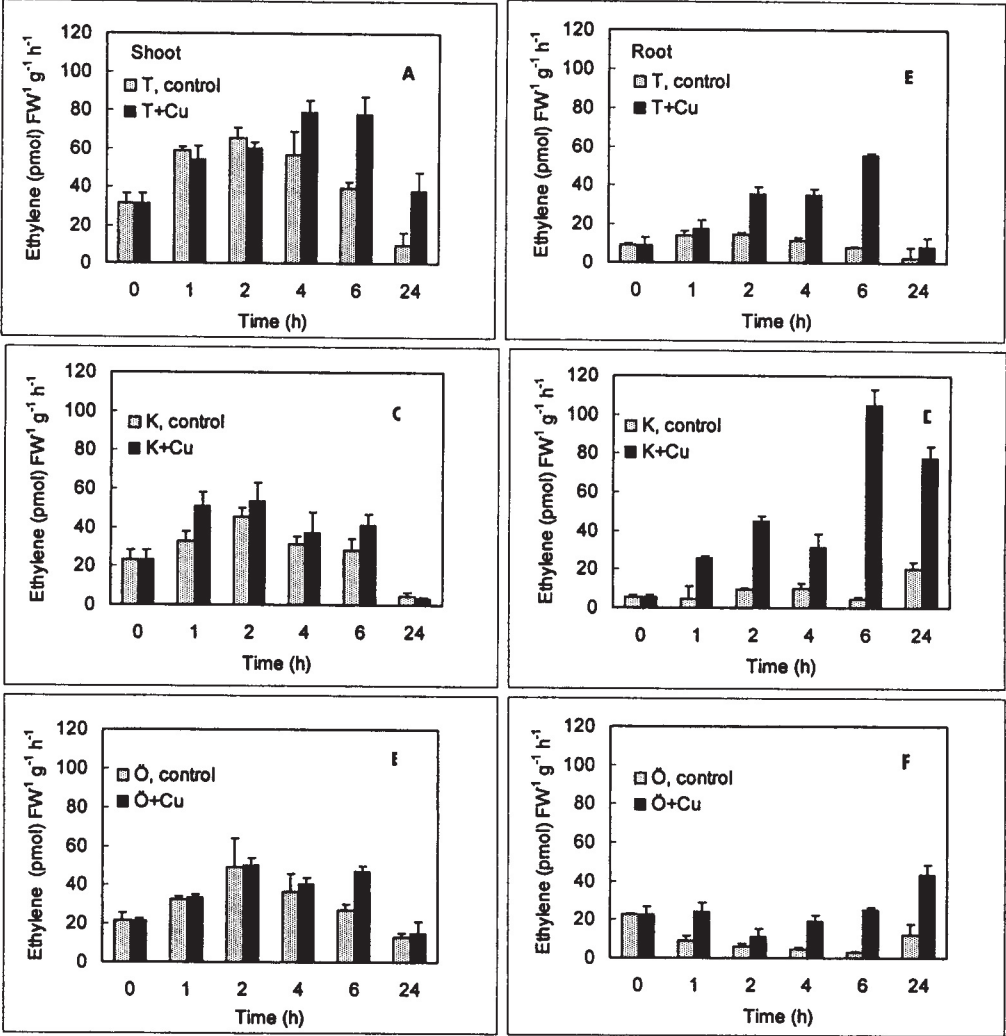


Fig. 1. Ethylene production as a function of time by the shoots and roots of 5-day-old wheat seedlings after 10 μ M CuSO₄ treatment. A: *Triticum aestivum* cv. Tiszatáj, shoot; B: cv. Tiszatáj, root; C: cv. Kata, shoot; D: cv. Kata, root; E: cv. Öthalom, shoot; F: cv. Öthalom, root. Means $\pm$ SE, n=5.

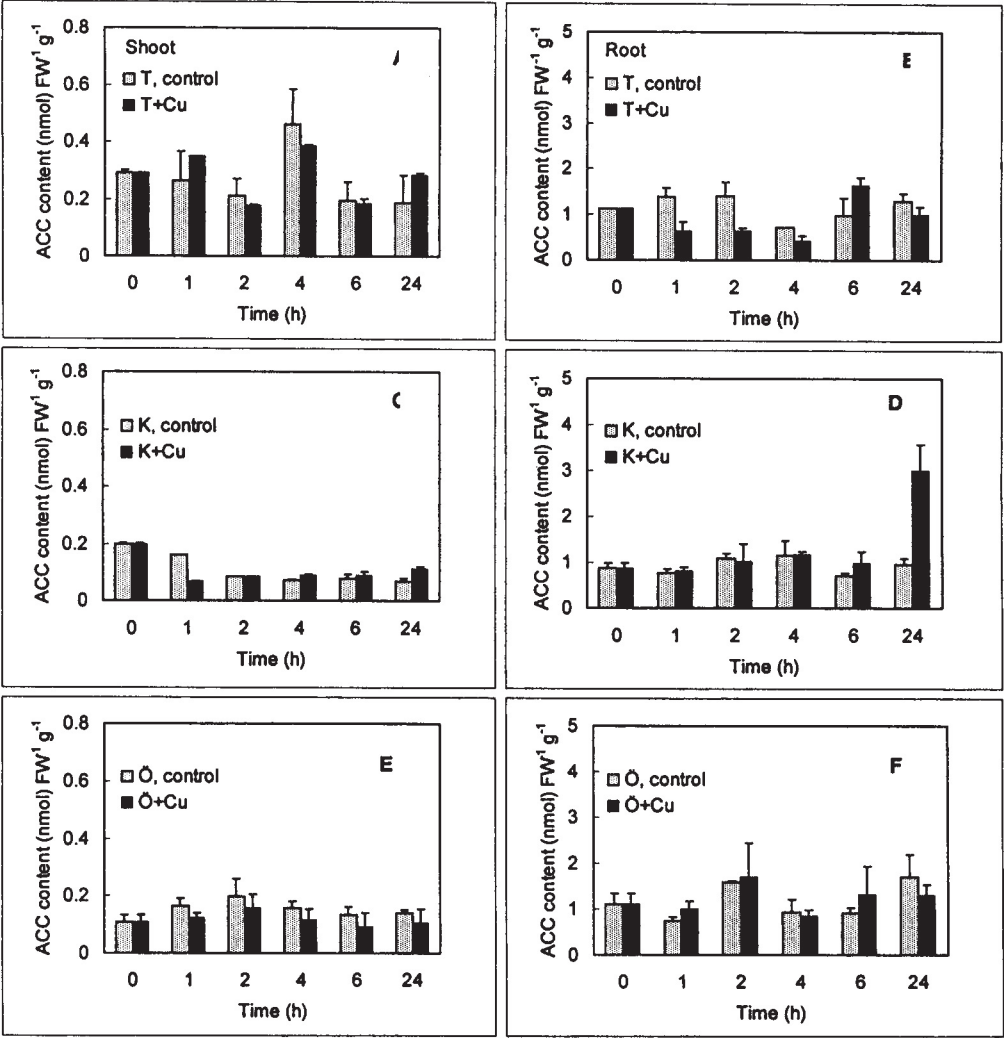


Fig. 2. 1-aminocyclopropane-1-carboxylic acid (ACC) contents as a function of time in the shoots and roots of 5-day-old wheat seedlings after 10 μ M CuSO_4 treatment. A: *Triticum aestivum* cv. Tiszatáj, shoot; B: cv. Tiszatáj, root; C: cv. Kata, shoot; D: cv. Kata, root; E: cv. Öthalom, shoot; F: cv. Öthalom, root. Means $\pm$ SE, n=5.

Table 1. Effect of a 24-hour Cu^{2+} treatment in the presence or absence of 0.5 mM methylglyoxal *bis*(guanylhydrazone) and 10 μM (2-aminoethoxyvinyl)glycine (I: inhibitors) on Cu^{2+} and K^+ accumulation in three wheat cultivars (*Triticum aestivum* L. cv. Öthalom, Kata or Tiszatáj). C: control; I: inhibitors; Means $\pm$ SE of three independent experiments. Means denoted by different letters indicate a significant difference between the treatments and plant parts of a cultivar ($P \leq 0.05$, Duncan's test).

Cultivars	cv. Öthalom	cv. Kata	cv. Tiszatáj
Cu^{2+} content [$\mu\text{mol g}^{-1}$ (d.m.)]			
Root			
C	0.14 $\pm$ 0.01c	0.30 $\pm$ 0.01c	0.10 $\pm$ 0.01c
C + I	0.36 $\pm$ 0.10c	0.27 $\pm$ 0.01c	0.28 $\pm$ 0.01c
10 μM Cu^{2+}	6.33 $\pm$ 0.30b	8.78 $\pm$ 0.40b	5.80 $\pm$ 0.20b
10 μM Cu^{2+} + I	8.74 $\pm$ 0.20a	10.5 $\pm$ 0.20a	9.12 $\pm$ 0.30a
Shoot			
C	0.17 $\pm$ 0.02c	0.14 $\pm$ 0.02c	0.14 $\pm$ 0.01c
C + I	0.16 $\pm$ 0.03c	0.10 $\pm$ 0.01c	0.11 $\pm$ 0.01c
10 μM Cu^{2+}	0.20 $\pm$ 0.01c	0.23 $\pm$ 0.02c	0.18 $\pm$ 0.00c
10 μM Cu^{2+} + I	0.19 $\pm$ 0.01c	0.20 $\pm$ 0.01c	0.14 $\pm$ 0.01c
K^+ content [$\mu\text{mol g}^{-1}$ (d.m.)]			
Root			
C	148.1 $\pm$ 4.7d	129.54 $\pm$ 7.8d	159.84 $\pm$ 8.1c
C + I	175.7 $\pm$ 1.5d	122.58 $\pm$ 12.9d	163.51 $\pm$ 6.5c
10 μM Cu^{2+}	167.4 $\pm$ 11.8d	236.31 $\pm$ 5.5c	185.94 $\pm$ 11.5c
10 μM Cu^{2+} + I	140.3 $\pm$ 3.0d	137.96 $\pm$ 2.5d	155.33 $\pm$ 15.3c
Shoot			
C	401.40 $\pm$ 25.4a	406.41 $\pm$ 15.2ab	393.25 $\pm$ 7.8a
C + I	357.48 $\pm$ 22.1b	364.55 $\pm$ 17.3b	361.34 $\pm$ 6.2b
10 μM Cu^{2+}	321.63 $\pm$ 3.4b	422.42 $\pm$ 20.6a	355.22 $\pm$ 4.9b
10 μM Cu^{2+} + I	236.98 $\pm$ 14.5c	376.10 $\pm$ 11.9b	338.10 $\pm$ 6.9b

In those experiments where the inhibitors were applied separately in the hydroponic culture medium of cv. Öthalom, similar tendencies could be observed for 0.5 mM MGBG treatment. MGBG significantly stimulated Cu^{2+} accumulation in the roots but only slightly increased the copper content of shoots in the presence of 10 μM Cu^{2+} . In the presence of AVG, however, Cu^{2+} contents of plant parts were not different from those of the Cu^{2+} -treated samples (Fig. 3.).

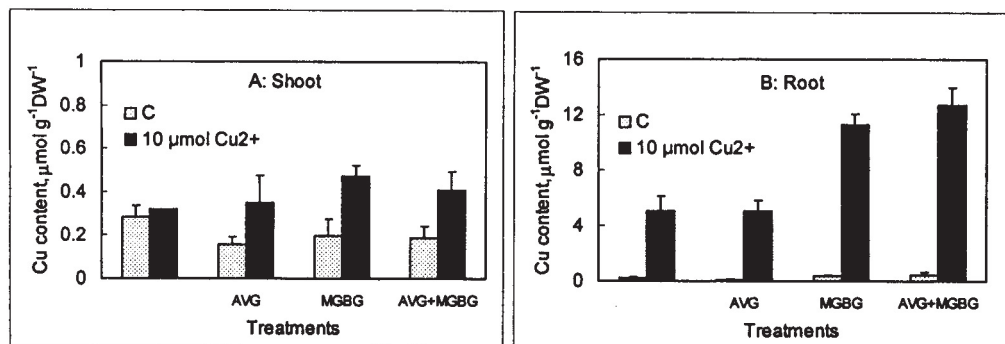


Fig. 3. Effects of 10.0 μM AVG, 0.5 mM MGBG or AVG + MGBG on Cu^{2+} contents of 5-day-old wheat seedlings (*Triticum aestivum* L. cv. Öthalom) treated with 10.0 μM CuSO_4 for 24 h. A: shoot; B: root.

Discussion

The ethylene production during the first 24 hours after copper exposure differed significantly in the tolerant and sensitive cultivars. The more tolerant genotypes produced significantly more ethylene and increased the ethylene evolution more efficiently from the root cells under the exposure of 10 μM CuSO_4 . Thus, the higher stress ethylene production in the alarm phase of the copper stress seems to be related to a better stress tolerance. The enhanced ethylene production may induce different stress-related acclimatization mechanisms, e.g. induction of the enzymes involved in the antioxidative defence and detoxification, such as peroxidases (Ievinsh *et al.* 1995) or glutathione S-transferases (Marrs, 1996).

The original hypothesis, stating that inhibition of ethylene and polyamine synthesis presumably would channel SAM towards phytosiderophore production has to be applied for that of polyamines. Thus, the presence of MGBG, an inhibitor of SAM decarboxylase in an *in vivo* system may indeed result in an endogenous surplus of Cu^{2+} . In the increased uptake, however, the Cu^{2+} chelating effect of MGBG cannot be excluded. In case of AVG similar effect could not be observed, that is, the enhanced consumption of SAM by ethylene synthesis did not cause significant changes in Cu contents. We can conclude that the cellular pool of SAM in wheat roots is probably large enough to satisfy the demand of ethylene synthesis, but the spermidine and spermine formation may compete with the synthesis of 2'-deoxymugineic acid. However, polyamines may also influence the uptake and intracellular transport of Cu^{2+} . The experimental proof of this hypothesis needs further experiments.

These results may add further information to the effect of MGBG *in vivo*, namely, this compound may modify the Cu^{2+} content, and as a consequence, the ethylene production of tissues. High affinity ethylene binding in *Arabidopsis* is mediated by an ethylene receptor protein (ETR1), which contains a copper co-factor. Thus, the MGBG effect on tissue Cu^{2+} concentration may interfere not only with ethylene production but also with the ethylene signal transduction.

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