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Effect of EDTA on the growth and copper accumulation of sweet sorghum and sudangrass seedlings

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ABSTRACT   The effect of EDTA on the growth parameters, copper accumulation and the translocation of Cu from root to shoot tissues was analyzed in sweet sorghum (*Sorghum bicolor* var. *saccharatum* (L.) Mohlenbr. Róna1 genotype) and sudangrass (*Sorghum sudanense* (Piper) Stapf cv. Akklimat). Although treatment with 200 µM EDTA enhanced the growth parameters and the translocation factor for Cu in both species, it did not increase the total accumulation of copper in plants grown in the presence of 10^{-6} M Cu^{2+} concentration.
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Sweet sorghum (*Sorghum bicolor* L. Moench) is a widely used crop plant that provides grain and stem for feeding and industrial utilization and can be used to produce concentrated syrup, sugar or alcohol, forage as well as silage for animal feed. After the development of an efficient biomass gasifier the bagasse from sweet sorghum can be used for thermal energy production. This biomass gasification system including an efficient gas combustion unit and a furnace, can also use other low-density biomass like residues of grasses, such as sudangrass or 'Hungarian energy grass' cv. Szarvasi-1 originated from Hungarian populations of tall wheatgrass (*Elymus elongatus* subsp. *ponticus*) (Bagi and Székely 2006). In addition to heat production, these gasification systems can be used for electricity generation, thus great efforts have been devoted to improve the biomass yield of these energy crops.

Copper (Cu) is an essential redox reactive transition metal that is involved in a range of physiological processes, such as photosynthesis, mitochondrial electron transport chain, cell wall metabolism or ethylene signalling. Cu acts as a cofactor of various enzymes, such as Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, amino oxidase, laccase or polyphenol oxidase (Yruela 2005). Plants are able to maintain Cu concentrations in the cells at low level (about 10 µg g^{-1} dry mass), below these values they show typical symptoms of Cu deficiency, chlorotic tissues at the leaf tips and malformed leaves (Marschner 1995). Cu deficiency reduces both photosystem I (PS I) (Baszynski et al. 1978) and PS II activity (Droppa et al. 1987). However, supraoptimal concentration of copper can become extremely toxic, because Cu can catalyze the formation of hydroxyl radicals (OH·) from the non-enzymatic chemical reaction between superoxide ($O_2^{·}$) and hydrogen peroxide (H_2O_2) (Halliwell

and Gutteridge 1984), which initiates oxidative stress leading to severe stunting of root growth (Tari et al. 2010). The most characteristic toxicity symptoms of excess copper are chlorosis, necrosis and inhibition of root growth (van Assche and Clijsters 1990; Tari et al. 2002).

Several transport mechanisms were identified which facilitate the entrance of Cu into plant cells and cell compartments. Five members of COPT proteins have been found in *Arabidopsis thaliana*, and the cDNA of COPT1 was able to functionally complement a *Saccharomyces cerevisiae* mutant defective in high-affinity Cu uptake (Kampfenkel et al. 1995). A sub-group of P-type ATP-ases, the 1B ATP-ases have been identified as heavy metal ATP-ases and they are implicated in the transport of various heavy metal ions, such as Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} across cell membranes. 1B heavy metal-transporting P-ATP-ases transport Cu(I) rather than Cu(II) (Voskoboinik et al. 2002). The highly conserved *N-ramp* gene family has also been shown to mediate the uptake of metal ions such as Fe(II) in rice (Belouchi et al. 1997), *Arabidopsis* (Curie et al. 2000) and soybean (Kaiser et al. 2003), and their possible function as a Cu transporter cannot be excluded.

Graminaceous species can enhance iron acquisition by release of phytosiderophores (PS), small Fe(III) chelating molecules into the rhizosphere. The rate of Fe(III)PS uptake of roots in graminaceous species increases by a factor of about 5 under Fe deficiency (Römheld and Marschner, 1990). Phytosiderophores can chelate not only Fe, but other positively charged heavy metal ions, such as Cu^{2+} or Zn^{2+} (von Wirén et al. 2000). In contrast to monocots, this specific uptake system is absent in dicots. However, among monocots sorghum is characterized by a low rate of PS release into the root surroundings in the early seedling stage (Römheld and Marschner 1990). Bioavailability of metals may be enhanced not only by natural chelators, such as PS, organic acids and

phenolics, but also by synthetic chelates. Metal-chelate complexes are expected to be less phytotoxic than free metal ions themselves, and synthetic chelate-metal complexes can be transported in the xylem sap as well as they can increase shoot metal concentration. Addition of ethylenediaminetet-raacetic acid (EDTA) to the soil led to higher amount of Cd, Zn and Pb in the shoot of *Brachiaria decumbens* (Santos et al. 2006), and other synthetic chelates can also be used to increase of the capacity of plants to translocate metals from root to shoot.

Although EDTA and the formed EDTA-metal complexes may be toxic for plants, it is of interest to know, whether EDTA affects the growth, biomass production, Cu(II) up-take and translocation in graminaceous species with low PS release. In this work we investigated the response of sweet sorghum and sudangrass to increasing copper supply in order to reveal the role of copper availability on biomass production and we also determined the effect of EDTA on the transloca-tion of copper from root to shoot tissues.

Materials and Methods

Plant material

Seeds of sweet sorghum (*Sorghum bicolor* var. s*accharatum* (L.) Mohlenbr. Róna1 genotype) and sudangrass (*Sorghum sudanense* (Piper) Stapf cv. Akklimat) were germinated in darkness at 26°C for 2 days. Then they were grown in hydro-ponic cultures, in minimal culture medium of $5x10^{-4}$ M $CaSO_4$ containing 10^{-8}-10^{-5} M $CuCl_2$, or 200, 250 and 300 µM EDTA or a combination of these treatments. The pH was adjusted to 7.0 every day. The plants were grown under a 12-h day/ and 12-h night cycle, at 25/20°C day/night temperature, at 300 µmol m^{-2} s^{-1} light intensity and 55-60% relative humid-ity for 10 days (Guóth et al. 2010). After harvest, the plant height and root length as well as the biomass production were recorded.

Pigment analysis

For pigment analysis a two-step extraction was applied. Leaf tissues were homogenized in ice-cold 100% (v/v %) acetone (1.5 ml for 250 mg sample), and were extracted for 24 hours. Samples were centrifuged at 5000 g for 15 minutes at 4°C. The pellet was extracted again with 80% (v/v %) acetone (1.5 ml for 250 mg sample) for 24 hours. After centrifuging (5000 g, 15 minutes, 4°C), the supernatants were collected. The pigment composition was measured by spectrophotometer according to Lichtenthaler and Wellburn (1983).

Determination of Cu content by atomic absorption spectrometry

Concentrations of copper was determined by a Zeeman polar-ized atomic absorption spectrometer (Hitachi Z-8200, Tokyo, Japan) with the application of air-acetilene flame (Trivedi and
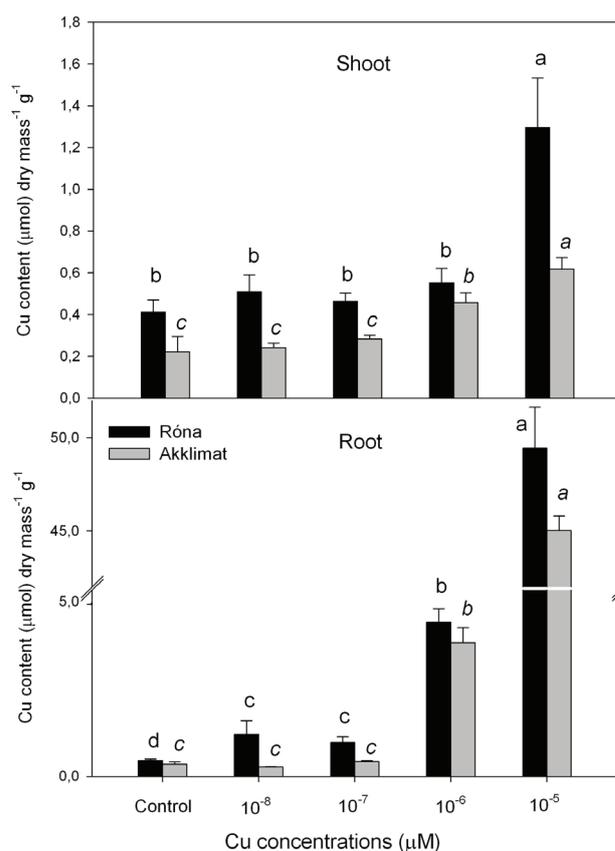

Figure 1. Cu contents in shoots and roots of 12-day-old sweet sorghum (*Sorghum bicolor* var. *saccharatum* (L.) Mohlenbr. Róna1 genotype) and sudangrass (*Sorghum sudanense* (Piper) Stapf cv. Akklimat) plants supplied with 10^{-8}-10^{-5} M Cu^{2+}. Means±SE, n=3. Means followed by the same letter are not significantly different at a probability level of 0.05 as determined by Duncan's multiple range test performed separately within species.

Erdei 1992). Cu content is expressed in µmol g^{-1} dry mass. 100 milligram of dried plant material was homogenized and placed in test tubes containing 5 ml cc. HNO_3 and 4 ml H_2O_2 at 200°C. The resulted colorless or pale samples were diluted with La-Cs solution dissolved in HNO_3.

Statistical analysis

Data presented in the figures are means of three to five repli-cations ± SE. Data were analyzed by Student's t-test or after analysis of variance by Duncan's test using a SigmaStat 3.1 software. Means denoted by *, ** or *** were significant at P < 0.05, P < 0.01 and P < 0.001 levels, respectively.

Results

Cu is an essential micronutrient, and the concentration range used in our experiments (0-10^{-5} M) enables the detection of Cu deficiency or the symptoms of Cu excess. Although sorghum is highly susceptible to Fe deficiency (to lime-

Table 1. The elongation growth of shoots and roots as well as chlorophyll (*a+b)* and carotenoid contents in µg g^{-1} FW in the youngest mature leaves of 12-day-old sweet sorghum and sudangrass plants exposed to 10^{-8}-10^{-5} M Cu^{2+}, 200 µM EDTA or 10^{-6} M Cu^{2+} in combination with 200 µM EDTA (Means±SE, n=10). Means denoted by *, ** or *** were significant at P < 0.05, P < 0.01 and P < 0.001 levels, respectively.

	Chlorophyll (*a+b*) content (mg g^{-1} fresh mass)	Carotenoid content (mg g^{-1} fresh mass)	Shoot length (cm)	Root length (cm)
Róna				
Control	0.984±0.01	0.320±0.01	13.41±0.41	17.29±0.65
10^{-8} M Cu^{2+}	1.140±0.05*	0.367±0.01*	12.20±0.42*	16.57±0.56
10^{-7} M Cu^{2+}	1.110±0.11	0.350±0.04	12.60±0.46	19.12±0.73
10^{-6} M Cu^{2+}	1.014±0.03	0.317±0.01	12.87±0.49	13.75±0.54***
10^{-5} M Cu^{2+}	0.956±0.03	0.301±0.01	9.93±0.23***	1.94±0.06***
200 µM EDTA	0.459±0.03***	0.157±0.01***	15.12±0.54*	19.10±0.55*
10^{-7} M Cu^{2+}+200 µM EDTA	0.401±0.03***	0.177±0.03**	16.79±0.57***	17.61±0.61
Akklimat				
Control	1.233±0.03	0.380±0.01	11.63±0.33	14.77±0.70
10^{-8} M Cu^{2+}	1.364±0.02*	0.426±0.01**	11.95±0.30	16.57±0.60
10^{-7} M Cu^{2+}	1.302±0.03	0.367±0.03	12.32±0.43	17.64±0.70**
10^{-6} M Cu^{2+}	1.293±0.08	0.393±0.03	11.89±0.30	11.35±0.72**
10^{-5} M Cu^{2+}	1.030±0.14	0.326±0.05	8.25±0.36***	1.29±0.14***
200 µM EDTA	0.690±0.03***	0.243±0.02**	11.86±0.57	20.03±0.84***
10^{-7} M Cu^{2+}+200 µM EDTA	0.736±0.03**	0.241±0.02**	12.95±0.47*	17.98±0.60**

chlorosis), no foliar symptoms of Cu deficiency or Cu toxicity could be observed during the experimental period. The elongation growth of the two genotypes, Róna and Akklimat displayed similar tendencies as a function of increasing Cu concentration. The root growth was more sensitive to excess of copper, and it was significantly inhibited from 10^{-6} M Cu^{2+} concentration, while at 10^{-5} M the growth of both shoots and roots was significantly decreased. There were no significant changes in total chlorophyll and carotenoid levels even at the highest concentration, moreover at 10^{-8} M small increases in pigment contents could be detected (Table 1).

There was little increase in Cu concentration of shoot tissues up to 10^{-6} M Cu^{2+} concentration in the culture medium, while Cu content of roots increased more steeply with solution Cu. Tissues of sweet sorghum accumulated more Cu both in the roots and shoots than those of sudangrass (Fig. 1).
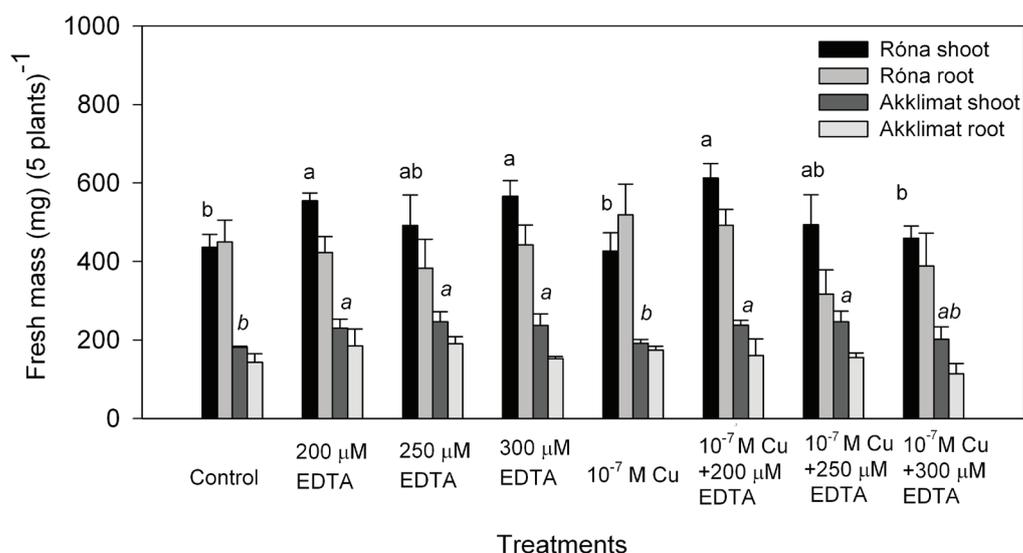

Figure 2. The effect of 200, 250 and 300 µM EDTA on shoot and root mass of 12-day-old sweet sorghum and sudangrass plants. (Means±SE, n=10). Means denoted by different letters are significantly different at P < 0.05 as determined by Duncan's multiple range test.
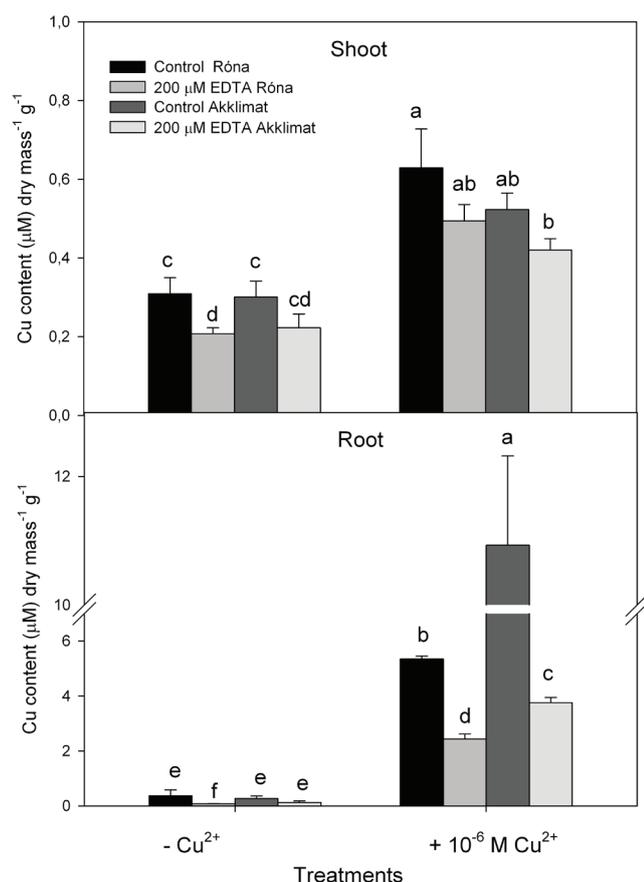
Figure 3. The effect of 200 µM EDTA on the Cu accumulation of sweet sorghum and sudangrass plants under control condition or in the presence of 10^{-6} M Cu^{2+}. Means±SE, n=3. Means followed by the same letter are not significantly different at a probability level of 0.05 as determined by Duncan's multiple range test performed separately within species.

We determined the effect of various concentrations of EDTA on the growth of plants and found that unexpectedly, the chelator induced a significant increase in shoot fresh mass of both species while the fresh mass of roots was unaffected (Fig 2). We were also interested in how EDTA affects the growth of plants in combination with copper treatment. It was found that 200 µM EDTA in combination with 10^{-7} M Cu^{2+} concentration retained the promoting effect on fresh mass accumulation, while at higher EDTA concentrations the fresh mass of plants declined to the control level (Fig 2). Although 10^{-7} M Cu^{2+} concentration in the presence of 200 µM EDTA increased the shoot length of both sweet sorghum and sudangrass, EDTA treatments resulted in a reduced chlorophyll (*a+b*) and carotenoid contents (Table 1) suggesting that EDTA may cause chlorosis in treated plants.

The effect of EDTA on the accumulation and on the transport of Cu from root to shoot was also investigated in sweet sorghum and sudangrass. It was found that 200 µM EDTA was not effective in the promotion of Cu^{2+} accumulation in the shoot, in contrast, it caused a small decline in the shoot and had a very significant inhibitory effect on the accumulation of copper in the root tissues (Fig. 3). The translocation factor, the relationship between shoot and root metal concentration has been introduced by several authors to evaluate the capacity of plants to translocate metals from root to shoot (Santos et al. 2006). It was found, that 200 µM EDTA increased the translocation factor in Róna genotype grown in 10^{-6} M Cu^{2+} from 0.12 to 0.203, and in cv. Akklimat from 0.048 to 0.112.

Discussion

EDTA has been extensively used for chelate-enhanced phytoextraction of various heavy metals such as Pb, Cd, Zn or Cu. This effect of EDTA was due to the enhanced bioavailability of metal ions in polluted soils. At the same time, EDTA may enhance the transport of heavy metals from roots to shoots, because it may increase the translocation factor in treated plants.

EDTA was found to induce the accumulation of Pb, Zn and Cu from heavy metal polluted soil in *Brassica juncea*, whereas Cd and Ni accumulation was not stimulated (Baylock et al. 1997). It also enhanced Zn accumulation in *B. juncea*, but had no effect on the Zn content of oat and barley (Ebbs and Kochian 1998).

At 10^{-6} M Cu^{2+} concentration in the culture medium the concentration of Cu in the shoot tissues of sweet sorghum and sudangrass plants was ~32 and 28.5 µg g^{-1}, respectively, which is in a range of the critical concentration of Cu toxicity (20-30 µg g^{-1}) suggested by Marschner (1995). However, it seems likely that both species are able to cope with this degree of heavy metal loading because this concentration of Cu had no influence on the growth parameters and pigment content of plants. 200 µM EDTA alone or in combination with copper treatment increased the biomass accumulation of plants, on the other hand the pigment content expressed on fresh mass basis declined in the leaf tissues. It is possible that the increased shoot biomass in these plants is associated with higher leaf surface, which results in a lower pigment content on fresh mass basis.

Degradation of photosynthetic pigments can be induced by oxidative stress generated by EDTA, but EDTA may also affect the chlorophyll organization by chelating Mg^{2+} and thus decreasing the availability of the central metal ion for chlorophyll biosynthesis. It was found that the greening process of seedlings was slower in the presence of EDTA than in control plants, but the seedlings were able to acclimate to the chelator.

The level of photosynthetic pigments can vary in sorghum with genotype (Surwenshi et al. 2010), developmental phases and environmental conditions (Oliveira Neto et al. 2009) or with the supply of fertilizers (Amujoyegbe et al. 2007). We found that toxic concentration of copper (10^{-5} M) had no

influence on the accumulation of photosynthetic pigments in the early phase of seedling growth in sweet sorghum and sudangrass.

The main question of these experiments was whether EDTA facilitates the translocation of copper from roots to shoot. Isermann (1979) placed the chelates in the following order of chelate stability for EDTA: $Fe^{3+} \rightarrow Cu^{2+} \rightarrow Zn^{2+} \rightarrow Fe^{2+} \rightarrow Mn^{2+} \rightarrow Ca^{2+} \rightarrow Mg^{2+}$, this means that Cu^{2+}-EDTA chelate is very stable among heavy metal-EDTA complexes. It was found that Cu can form stable complexes and can be transported in the xylem sap bound to citrate (Mullins et al. 1986), asparagine and histidine in soybean exudate (White et al. 1981) or nicotianamine in tomato (Pich and Scholz 1996). In *Brassica carinata* histidine and proline were the most important candidates for Cu binding at supraoptimal Cu concentrations, while the concentration of nicotianamine, a non-proteinogenic amino acid increased in the xylem sap under Cu deficiency (Irtelli et al. 2009). It was found that both Cu-histidine and Cu-nicotianamine complexes have very high stability constants, $\log K_{st} = 17.5$ for His-Cu and $\log K_{st} = 18.6$ for nicotianamine-Cu complexes, however, as far as the stability constant is concerned, EDTA could compete with them as a ligand for Cu ($\log K_{st} = 18.8$ for Cu-EDTA). Although in our experiments 200 μM EDTA increased the growth of sweet sorghum and sudangrass, the plants exhibited adverse symptoms such as mild chlorosis. Thus the treatment did not enhance the accumulation of Cu but it increased the translocation factor in the presence of supraoptimal Cu concentration.

Acknowledgements

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