Enhanced biomass production in sudangrass induced by co-treatment with copper and EDTA

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Abstract

Three-month soil experiments were conducted to examine the effects of copper (Cu^{2+}) treatment in combination with EDTA on biomass production of sudangrass [*Sorghum sudanense* (Piper) Stapf cv. Akklimat]. Cu accumulation increased in the leaves at 10^{-7} – 10^{-6} M Cu^{2+} concentrations but it did not rise further in the presence of 200 or 300 μM EDTA. Treatment with 10^{-7} M Cu^{2+} in the presence of 300 μM EDTA enhanced fresh mass accumulation and photosynthetic activity as well as increased the total sugar content in all parts of plants. The effect of higher Cu^{2+} supply (10^{-6} M) in combination with 300 μM EDTA was not so obvious; the maximal rate of CO_2 fixation was lower than in plants exposed to 10^{-7} M Cu^{2+} + 300 μM EDTA combination and the increases in stem and root biomass were not significant in these plants. Soluble sugar concentration increased with Cu^{2+} concentration and 300 μM EDTA in all plant parts. These results show that the application of Cu^{2+} fertilization in combination with suitable concentration of EDTA can be a useful strategy to increase the biomass production of sudangrass.

Key words: biomass, copper, EDTA, photosynthesis, PSI, PSII, soil culture, soluble sugars, sudangrass.
Abbreviations: A, CO_2 assimilation rate; Cu, copper; DM, dry mass; EDTA, ethylenediaminetetraacetic acid; FM, fresh mass; PPFD, photon flux density; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species.

Introduction

Sudangrass [*Sorghum sudanense* (Piper) Stapf] is a widely grown crop plant that is used for forage and silage. In addition, sudangrass has the potential to serve as an energy crop, and following fermentation of its biomass it can be used for biogas production (Mahmood, Honermeier 2012). Sudangrass and other members of the *Sorghum* genus are advantageous crops in the agriculture, because they tolerate warm and dry conditions (Bibi et al. 2010; Tari et al. 2013). In spite of the agricultural and industrial importance of sudangrass, no single study has investigated the effects of microelements, especially the effects of copper and copper chelate complex on the biomass production of this crop.

Copper (Cu) is a redox-active metal that is essential for normal plant growth and development, but in excess amounts it can be toxic for plants. Cu participes in important physiological processes, such as redox reactions in cells, photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, protein metabolism, antioxidant activity, cell wall metabolism and as a component of the ethylene receptor, in the signalling of the plant hormone ethylene. Cu is an essential cofactor of Cu/Zn superoxide dismutase, cytochrome *c* oxidase, amino-oxidase, laccase or polyphenol oxidase (Yruela 2005).

Cu^{2+} is a divalent cation that is absorbed in relatively high proportions in the organic matter of soil, carbonates, phyllosilicates and hydrous oxides of Al, Fe and Mn. It can be found in dissolved forms in soil solution as Cu^{2+} and can form organic Cu^{2+} complexes (Mengel, Kirkby 2001). It is taken up by plants as Cu^{2+} or as a copper chelate complex. The average concentration of copper in plants is 10 μg g^{-1} dry mass and the typical concentration range in the soil solution is 10^{-9} – 10^{-6} M (Yruela 2005).

Cu deficiency can disturb plant metabolism, which is well characterized in many crop plants. One of the most important effects of the copper deficiency is the inhibition of the photosynthetic activity (Baszynski et al. 1978) by strong inhibition of photosystem I (PSI) and II (PSII; Droppa et al. 1987). Under copper deficiency the leaf tips of cereals become white, the leaves become narrow and twisted, and the growth of internodes is depressed (Mengel, Kirkby 2001). However, supraoptimal concentrations of Cu also decrease photosynthesis and respiration rates, as well as inhibit growth and cause chlorotic symptoms (Prasad, Strzalka 1999; Broadly et al. 2012). Moreover, Cu^{2+} can replace metal cofactors, such as magnesium in chlorophyll molecules, which contributes to its cytotoxicity (Maksymiec 1997). High levels of Cu^{2+} cause toxic oxidative burst in plants, as Cu ions catalyze the formation of hydroxyl radicals ($OH^{\bullet}$) from the non-enzymatic chemical reaction with superoxide ($O_2^{\bullet-}$) and H_2O_2 (Halliwell, Gutteridge 1984). These reactive oxygen species (ROS) can destroy cell membranes and proteins, including structural proteins of the photosystems (Droppa, Horváth 1990).

In *Arabidopsis thaliana* cells, Cu^{2+} transport toward the cytosol is mediated by the high-affinity COPT1–COPT6 transporter family, and Cu^{2+} efflux occurs through P-type ATPases (RAN1, PAA1, PAA2, HMA5, HMA1). Moreover, metallochaperones (ATX1, CCH, CCS1) mediate Cu^{2+} delivery to specific protein targets (Penarrubia et al. 2010). Graminaceous plant species have specific hexadentate metal chelators, the phytosiderophores, which have a high affinity for complex formation with Fe(III) (Römheld, Marschner 1986). Although the synthesis of siderophores is induced by Fe deficiency, it was found that these natural chelating compounds mobilize a wide range of metals, including zinc, manganese and copper (Treeby et al. 1989).

Synthetic chelates have long been used in nutrient solutions to increase the solubility and availability of trace elements for plants. One of the most effective chelate for increasing the uptake of various metal ions including Cu^{2+} is ethylenediaminetetraacetic acid (EDTA; Thayalakumaran et al. 2003). EDTA is also one of the most effective reagents for soil remediation, as it is strong, recoverable and a relatively biostable chelating agent (Hong et al. 1999). Deram et al. (2000) found that EDTA enhanced copper content in *Arrhenatherum elatius* grass. Although EDTA and EDTA-metal complexes may be toxic for plants, it is a promising strategy to increase biomass production of crop plants by EDTA treatment. However, the potential risk of water pollution by chelated metals needs to be evaluated before the acceptance of this technology.

In our earlier work, the role of copper availability on biomass production of sudangrass was investigated in hydroponic culture (Székely et al. 2011). In the present work, the response of sudangrass to copper and copper-EDTA complex treatments was investigated by measuring the growth, photosynthetic activity and total sugar content of various plant parts to reveal the effectiveness of Cu-EDTA combinations on the biomass production of sudangrass plants grown in soil cultures.

Materials and methods

Plant material

Sudangrass [*Sorghum sudanense* (Piper) Stapf cv. Akklimat] is an exceptionally drought resistant genotype with thin stems and leaves. The seeds were germinated in darkness at 26 °C for 2 days then the seedlings were planted into pots. Two healthy plants were grown in 2.5 kg soil (Bioland Tőzegfeldolgozó Kft., Biatorbágy, Hungary) containing N (20 to 500 mg L^{-1}), P_2O_5 (200 to 500 mg L^{-1}); K_2O (300 to 600 mg L^{-1}), white peat (50%, m/v), black peat (50% m/v), and $CaCO_3$ (2 kg m^{-3}), pH 5.5 to 7.5. The plants were irrigated two times a week with 250 mL of 5×10^{-4} M $CaSO_4$ (pH 5.5) containing 10^{-7} and 10^{-6} M $CuCl_2$, or with the respective combinations of copper-containing solutions and 200 or 300 μM EDTA. The plants were grown in a greenhouse under a 12/12 h day/night cycle, at 25/20 °C day/night temperature, at 300 μmol m^{-2} s^{-1} light intensity and 55 to 60% relative humidity for 12 weeks (Guóth et al. 2010). All chemicals were from Reanal (Budapest, Hungary).

The cultivation of plants was carried out in Szeged (Hungary) during the autumn of 2010 – 2011 and in spring of 2011. The experiment presented in the article began on 26 September 2011. After harvesting the plant height, root length and fresh mass (FM) of leaves, stems and roots as well as biomass produced by 10 plants in five pots were recorded. All experiments were repeated twice.

Determination of Cu content in the soil

Three samples were taken from each treatment (at a depth of 5 to 15 cm) to assess the total Cu concentration in the soil without plants (Puskás, Farsang 2009). Dried soil samples (3 g) were crushed and sieved through a mesh of 2 mm. The samples were digested with 28 mL aqua regia (HCl/HNO_3, 3:1 solution) for 16 h and boiled for another 2 h. Samples were then cooled, diluted with double distilled water (MSZ21470-50). The measurements were performed with an atomic absorption spectrometer (Perkin Elmer 3010, Waltham MA, USA). One sample was run in triplicate. Cu contents are expressed in μg g^{-1} dry mass (DM).

Ammonium-acetate extraction in combination with EDTA has been used for a long time for the analysis of plant-available heavy metal fraction of soil in agricultural research (Lakanen, Erviö 1971). The mobilizable or plant available element fraction of 5 g soil samples (0.5 M ammonium-acetate + 0.5 M acetic acid + 0.02 M EDTA extractable fraction) was determined at the end of the experiments after harvesting.

Determination of Cu content of plant tissues

Concentrations of Cu were determined by a XSeries II ICP-MS (Thermo Scientific, Bremen, Germany). Dried plant material (100 mg) was homogenized and placed in test tube containing 6 mL concentrated nitric acid and 2 mL H_2O_2 for 20 h. The samples were digested in a microwave destructor (MarsXpress CEM, Matthews NC, USA) at 200 °C for 25 min. Samples were then cooled, diluted with 12 mL double distilled water. Cu concentration was expressed in μg g^{-1} DM.

Pigment analysis

For pigment analysis a two step extraction was applied. 250 mg leaf samples were homogenized in ice-cold 100% (v/v) acetone (1.5 mL), and extracted for 24 h. Samples were centrifuged (5 000 g for 15 min at 4 °C). The pellet was extracted again with 80% (v/v) acetone (1.5 mL) for 24 h. After spinning down (5 000 g, 15 min, 4 °C), the supernatants were collected. The pigment composition was measured by a spectrophotometer according to Lichtenthaler and Wellburn (1983).

Measurement of photosynthetic light response curves

Net photosynthetic rate (A, μmol fixed CO_2 m^{-2} s^{-1}) was measured on second, fully expanded leaves using a portable photosynthesis system (LI-6400, LI-COR, Inc.; Lincoln, NE), as described by Poór et al. (2011). Light response curves were recorded under constant conditions (25 °C, 65 ± 10% relative humidity, and controlled CO_2 supply of 360 μmol mol^{-1}) while increasing photosynthetic photon flux density (PPFD) from 0 to 1500 μmol m^{-2} s^{-1}. The A versus PPFD curves were fitted by Sigma plot 11.0 software (Systat Software Inc., Erkrath, Germany).

Determination of total sugar content

For determination of the total sugar content, 1 g of fresh plant material was frozen in liquid N_2, and homogenized. The samples in 10 mL distilled water were incubated in a 90 °C water bath for 45 min, and then centrifuged (5 000 g for 15 min at 4 °C). Supernatant (40 μL) was mixed with 400 μL 1.8% phenol and 2 mL sulfuric acid. Total sugar content was measured by a spectrophotometer at 490 nm according to Dubois (1956).

Statistical analysis

Data are presented as means of at least three independent experiments. One-way analysis of variance was carried out with Sigma plot 11.0 software (Systat Software Inc., Erkrath, Germany). When significant F-values were obtained, the individual means were compared using the Duncan's test and the differences were considered significant if $P \leq 0.05$.

Results

To investigate the role of Cu availability on biomass production in sudangrass, the plants were irrigated with 10^{-7} and 10^{-6} M Cu^{2+} alone or in combination with 200 and 300 μM EDTA. Although Cu is an essential micronutrient, in high concentrations it can be toxic for plants, but no foliar symptoms of Cu deficiency or Cu toxicity could be observed during the whole experimental period.

The copper concentrations of the irrigated soil in the absence of plants following full digestion with aqua regia (HCl/HNO_3, 3:1 solution) were 28.99 ± 1.07 μg g^{-1} dry soil in control samples, 29.08±0.03 after treatment with 10^{-7} M and 29.05±0.02 after treatment with 10^{-6} M Cu^{2+}concentration. The plant-available copper concentrations of the soil samples after harvesting the plants show that the Cu2+ content of this fraction was significantly lower in those treatments where plants were treated with EDTA during their vegetative growth. This suggests that the application of EDTA to the soil during the vegetative phase of sudangrass could increase the plant-available copper fraction, and plants used up Cu^{2+} from this fraction more efficiently (Fig. 1).

Treatments with both concentrations of Cu^{2+} caused significant increases in the Cu concentration in the leaves

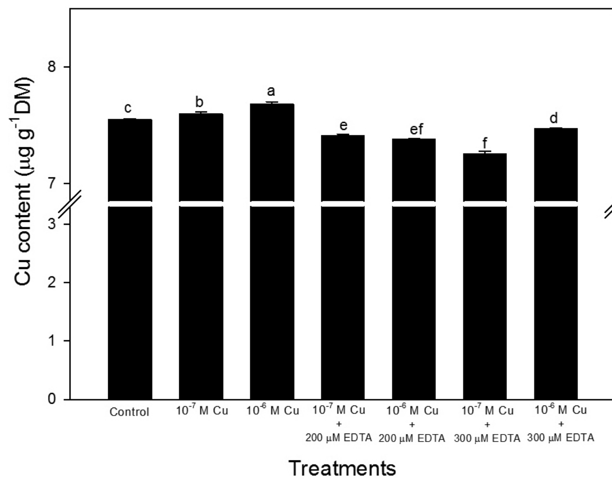

Fig. 1. Plant-available Cu^{2+} concentration in soil extracts prepared according to Lakanen and Erviö (1971) from soils after harvesting. The sudangrass plants were treated with 10^{-7} or 10^{-6} M Cu^{2+} or with copper + 200 or 300 μM EDTA combinations Results are means ± SE, n = 9). Means denoted by different letters are significantly different at $P \leq 0.05$ as determined by the Duncan's multiple range test.

(Fig. 2). In contrast, Cu^{2+} treatments did not cause significant changes in the Cu concentration of the stems but excess of copper increased significantly the Cu concentration of the roots at 10^{-7} – 10^{-6} M Cu^{2+} concentrations (Fig. 2). The application of 200 or 300 μM EDTA together with Cu^{2+} treatments did not cause any additional increase in Cu content of any plant parts but with the exception of 10-7 M CCu^{2+} + 200 μM EDTA combination, which enhanced the biomass production of plants. This means that higher plant biomass may extract higher amounts of metal from the soil solution.

Testing of the effect of Cu^{2+} in combination with various

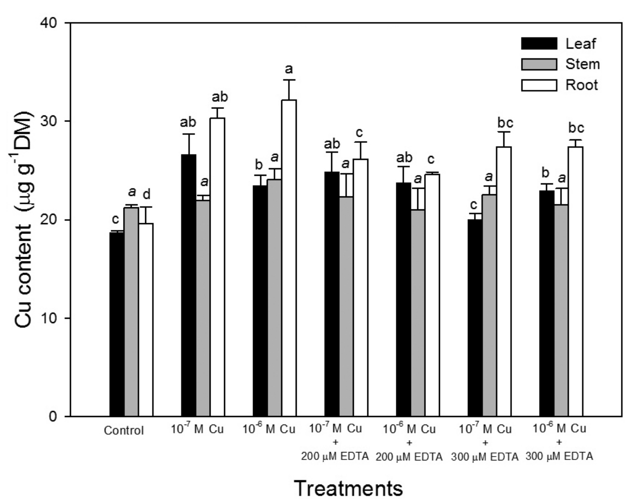

Fig. 2. Cu concentration in leaves, stems and roots of sudangrass plants exposed to 10^{-7} and 10^{-6} M Cu^{2+} in combination with 200 and 300 μM EDTA. Data are means ± SE, n = 3. Means denoted by different letters are significantly different at $P \leq 0.05$. .

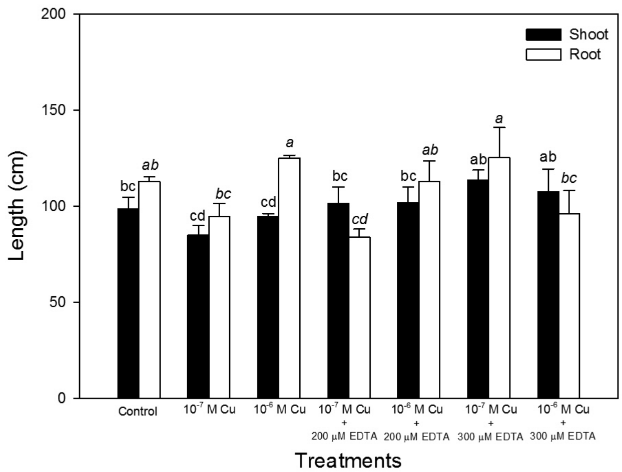

Fig. 3. The length of shoots and roots of sudangrass plants exposed to 10^{-7} and 10^{-6} M Cu^{2+} in combination with 200 and 300 μM EDTA. Data are means ± SE, n = 5. Means denoted by different letters are significantly different at $P \leq 0.05$.

concentrations of EDTA on shoot and root elongation showed that the elongation growth of plant organs was only slightly altered (Fig. 3).

Treatment with 10^{-7} M Cu^{2+} in combination with 300 μM EDTA led to significantly increased fresh mass in leaves, stems and roots suggesting that a suitable combination of Cu with EDTA may have favourable effect on plant biomass (Table 1).

Biomass production depends primarily on the photosynthetic activity of plants. Treatment with 10^{-7} M Cu^{2+} alone or 10^{-6} M Cu^{2+} in combination with 200 μM EDTA increased the chlorophyll *a* + *b* and carotenoid concentration (Fig. 4). Interestingly, the lower Cu^{2+} concentration in combination with 300 μM EDTA led to slightly reduced chlorophyll levels, which may be a consequence of increased leaf area.

Photosynthetic activity was assessed by recording the light response curves. The highest maximal net CO_2 assimilation rate was observed after treatment with 300 μM EDTA in combination with 10^{-7} M Cu^{2+} (Fig. 5). These results correlate well with the results of increased fresh mass and elongation growth caused by 10^{-7} M Cu^{2+} + 300 μM EDTA.

During the experiments, plants utilized the beneficial effects of increased availability of copper, photosynthetic activity was enhanced and as a result, more soluble sugars were accumulated in the tissues. Treatment with 10^{-7} and 10^{-6} M Cu^{2+} increased the sugar concentration in stem and root tissues (Fig. 6). This tendency was more expressed in plants exposed to 300 μM EDTA in combination with 10^{-7} or 10^{-6} M Cu^{2+} (Fig. 6)

Discussion

Copper has traditionally been used in agriculture as an antifungal agent and it is also released into the environment by human activities (Yruela 2005). Under heavy metal stress conditions, plants accumulate essential elements in excess amounts, which can be also toxic for plants (Tari et al. 2002) or for the herbivores (Bremner 1998). In contrast, Cu as an essential element is vital for normal plant growth and development (Mengel, Kirkby 2001). Maintenance of an optimal Cu concentration in plant tissues is an important task in plant biology, and addition of Cu^{2+} in combination with EDTA appears to be a promising opportunity to maximize growth and biomass yield of sudangrass.

Cu contents of the soil fractions were within the range determined by others in a similar system (Mendosa et al. 2006). Irrigation of the soil with 10^{-7} and 10^{-6} M Cu^{2+} raised Cu concentration in the leaf tissues of sudangrass to ~15 to 28 μg g^{-1} dry mass and to about 30 μg g^{-1} dry mass in the roots, which was close to the critical concentration for Cu toxicity (~30 μg g^{-1}; Broadly et al. 2012). Treatments with 10^{-7} – 10^{-6} M Cu^{2+} caused Cu accumulation in the root tissues with a slight inhibition of the elongation at 10^{-7} M and with a decrease in the fresh mass of the roots, but did not cause chlorosis in the leaves.

The fresh mass of the plants increased significantly after treatment with 10^{-7} M Cu^{2+} + 300 μM EDTA. EDTA has been extensively used for chelate-enhanced uptake (Vashegyi et al. 2013) and chelate-induced phytoextraction of various heavy metals such as Pb^{2+} (Blaylock et al. 1997)

Table 1. Changes in fresh mass of leaves, stems and roots of sudangrass supplied with 10^{-7} or 10^{-6} M $CuCl_2$ or with the combination of Cu^{2+} and 200 or 300 mM EDTA. Data are means ± SE, n = 5. Means denoted by different letters are significantly different at $P \leq 0.05$ as determined by the Duncan's multiple range test

Treatment	Fresh mass (g plant^{-1})		
	Leaves	Stems	Roots
Control	8.65 + 0.78 b	6.59 + 1.44 b	4.88 + 1.13 b
10^{-7} M Cu^{2+}	9.06 + 0.63b	7.45 + 1.70 b	4.38 + 1.32 b
10^{-6} M Cu^{2+}	8.25 + 0.12 b	7.24 + 0.77 b	4.70 + 0.39 b
10^{-7} M Cu^{2+} + 200 μM EDTA	6.70 + 1.67 b	6.63 + 2.23 b	3.80 + 0.03 b
10^{-6} M Cu^{2+} + 200 μM EDTA	8.55 + 3.91 b	8.73 + 3.94 b	4.85 + 2.95 b
10^{-7} M Cu^{2+} + 300 μM EDTA	14.73 + 1.41 a	13.82 + 1.78 a	10.71 + 1.37 a
10^{-6} M Cu^{2+} + 300 μM EDTA	7.37 + 0.01 b	7.63 + 2.11 b	7.12 + 2.17 b

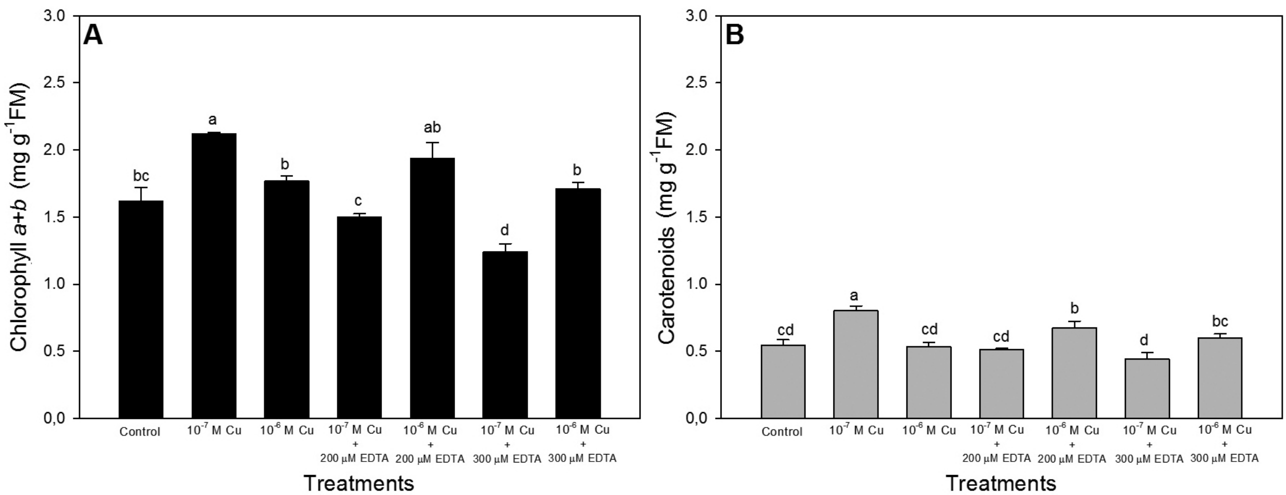

Fig. 4. Chlorophyll $a + b$ (A) and total carotenoid concentration (B) in leaves of sudangrass plants exposed to 10^{-7} and 10^{-6} M Cu^{2+} in combination with 200 and 300 μM EDTA. Data are means ± SE, n = 5. Means denoted by different letters are significantly different at $P \leq 0.05$.

or Cu^{2+} (Kim, Lee 2010). This effect of EDTA is 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. In our earlier work we found that 200 μM EDTA increased the translocation factor in the presence of Cu^{2+} in sorghum and sudangrass grown in hydroponic cultures (Székely et al. 2011). Although EDTA is effective in enhancing phytoextraction, EDTA and EDTA-heavy metal complexes at higher concentrations can be toxic both for plants and microorganisms (Grcman et al. 2001) and thus they decrease biomass production (Luo et al. 2005). It was found that EDTA can induce serious oxidative stress leading to damage to macromolecules (Geebelen et al. 2002). In contrast, Wu et al. (2004) found that EDTA can significantly enhance Cu accumulation in Indian mustard without toxicity symptoms. It can form chelates with Cu^{2+} ion and can mitigate the oxidative stress generated by Cu^{2+}. Thus, the appropriate balance between Cu^{2+} and EDTA in the soil solution is critical for successful effect of the treatment.

To clarify whether sudangrass plants growing in the presence of various Cu^{2+} concentrations in combinations with EDTA can increase their biomass production, the changes in photosynthetic activity and pigment contents of the plants were determined. Only treatment with 10^{-7} M Cu^{2+} and in combination with 200 μM EDTA increased significantly chlorophyll $a + b$ and carotenoid concentration. These results are in harmony with the findings of Jiang et al. (2012) who investigated the effects of low Cu^{2+} concentrations on *Paulownia fortunei*. Treatment of plants

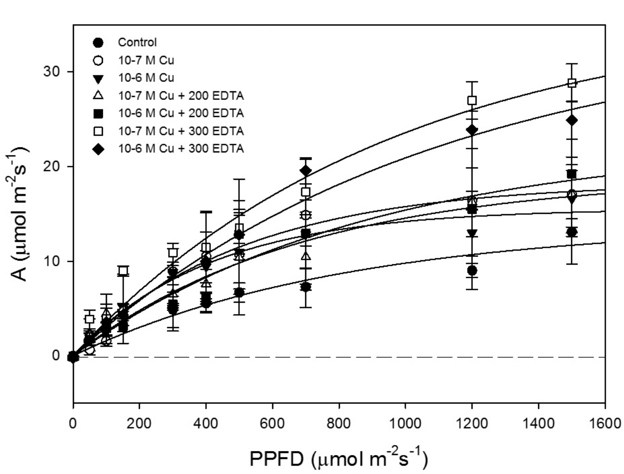

Fig. 5. Responses of CO_2 assimilation rate (A) to photosynthetic light intensity (PPFD) in leaves of sudangrass plants exposed to 10^{-7} and 10^{-6} M Cu^{2+} in combination with 200 and 300 μM EDTA. Data are means ± SE, n = 5.

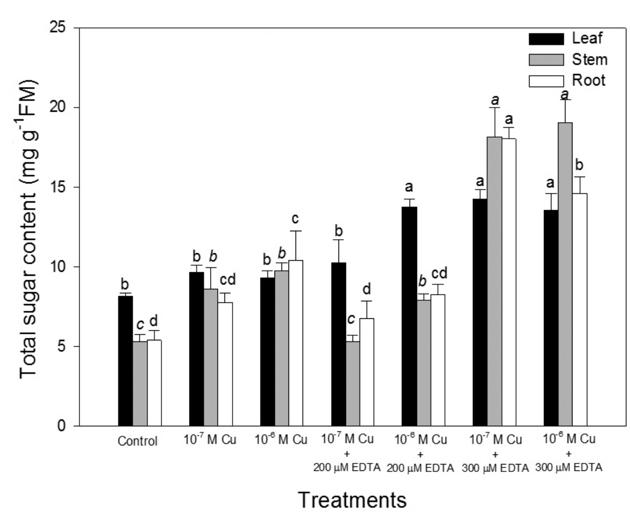

Fig. 6. Total sugar concentration in leaves, stems and roots of sudangrass plants exposed to 10^{-7} and 10^{-6} M Cu^{2+} in combination with 200 and 300 μM EDTA. Data are means ± SE, n = 5. Means denoted by different letters are significantly different at $P \leq 0.05$.

with 10^{-7} M Cu^{2+} in combination with 300 μM EDTA resulted in slightly decreased pigment concentrations. However, it is possible that the increased leaf and shoot biomass in these plants is associated with higher leaf surface, which results in a lower pigment concentration on fresh mass basis.

The stability of CO_2 assimilation rate and the chlorophyll a fluorescence induction parameters (maximum quantum use efficiency of PSII reaction centres, F_v/F_m, and relative electron transport rate) characterize healthy plants (Samsone et al. 2012) or stress tolerant species or genotypes (Andersone et al. 2012). It was found that the maximal rate of photosynthesis in response to increasing light intensity was elevated in copper-treated plants. These plants may convert light energy more efficiently in photochemical reactions and exhibit significantly higher CO_2 assimilation rates under Cu^{2+} treatments in combination with 300 μM EDTA. Treatment with 10^{-7} M Cu^{2+} + 300 μM EDTA proved to be the most effective in the stimulation of photosynthesis. Sugars, the products of photosynthesis, participate not only in plant metabolism, but also in the control of plant development. All Cu^{2+} treatments in combination with 300 μM EDTA resulted in significantly increased total sugar contents in all parts of sudangrass. Similar effects of Cu^{2+} treatments on increasing soluble sugar concentrations were reported by Jiang et al. (2012).

In conclusion, these results suggest that the beneficial effects of Cu in combination with EDTA on photosynthesis may be manifested in biomass production. EDTA can promote growth and biomass production by providing stable free [Cu^{2+}], determined by the complex stability constant of Cu^{2+}-EDTA in various plant tissues. Our results suggest that EDTA can be utilized for an efficient phytoremediation program to extract Cu from the soil and at the same time to increase biomass production of sudangrass.

Acknowledgements

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