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Effects of nanomolar copper on water plants—Comparison of biochemical and biophysical mechanisms of deficiency and sublethal toxicity under environmentally relevant conditions

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ABSTRACT

Toxicity and deficiency of essential trace elements like Cu are major global problems. Here, environmentally relevant sub-micromolar concentrations of Cu (supplied as $CuSO_4$) and simulations of natural light- and temperature cycles were applied to the aquatic macrophyte *Ceratophyllum demersum*. Growth was optimal at 10 nM Cu, while PSII activity (F_v/F_m) was maximal around 2 nM Cu. Damage to the PSII reaction centre was the first target of Cu toxicity, followed by disturbed regulation of heat dissipation (NPQ). Only after that, electron transport through PSII (Φ_{PSII}) was inhibited, and finally chlorophylls decreased. Copper accumulation in the plants was stable until 10 nM Cu in solution, but strongly increased at higher concentrations. The vein was the main storage site for Cu up to physiological concentrations (10 nM). At toxic levels it was also sequestered to the epidermis and mesophyll until export from the vein became inhibited, accompanied by inhibition of Zn uptake. Copper deficiency led to a complete stop of growth at "0" nM Cu after 6 weeks. This was accompanied by high starch accumulation although electron flow through PSII (Φ_{PSII}) decreased from 2 weeks, followed by decrease in pigments and increase of non photochemical quenching (NPQ). Release of Cu from the plants below 10 nM Cu supply in the nutrient solution indicated lack of high-affinity Cu transporters, and on the tissue level copper deficiency led to a re-distribution of zinc.

1. Introduction

Plant micronutrients include the metals copper, iron, molybdenum, nickel and zinc belonging to the first and second row transition-elements. The availability, acquisition and distribution of these elements within the plants are prime targets of research as they have a major role in the proper physiological functioning of plants.

One of the major metals which has been studied is copper. Copper has a particularly narrow beneficial range for the growth and development of the plant and becomes toxic after a particular concentration and causes deficiency effects on the plants when below the beneficial range. It has a major role in the physiology of plants mainly because of its multiple oxidation state existence in vivo, Cu^+ and Cu^{2+}. Its role as a micronutrient has been known in plants for a long time (Sommer, 1931; Lipman and McKinney, 1931). Copper is mainly required at least in six locations in a plant cell which include the cytosol, the endoplasmic reticulum (ER), the inner membrane of the mitochondria, the stroma of the chloroplast, the thylakoid lumen and the apoplast (Marschner, 1995), because of its function as the active centre of various enzymes. Copper plays an important role in photosynthetic electron transport, where the transfer of electrons takes place through plastocyanin (the most abundant of Cu protein in green tissue (Yamasaki et al., 2008)), which gets reduced and oxidized as the electron is transferred from the cytochrome b_6f complex to the PSI reaction centre. Another important function of the Cu (in the +1 oxidation state) is to bind to small molecules like O_2 as a ligand. Thus these ions act

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as a cofactor for various enzymes like Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, etc. (Küpper and Kroneck, 2005).

The concentration of Cu is less than 32 nM in natural waters (Baccini, 1985) but these values reach up to 32 μM in polluted conditions, resulting in the creation of copper toxicity for plants living in such environments (Moore and Ramamoorthy, 1984). The increase of copper levels in the environment is mainly a result of anthropogenic activities, which include the industrial (metal plating, steelworks, refineries) and domestic waste emissions, application of fertilizers, sewage sludge, and pesticides (Yamamoto et al., 1985; Zhang et al., 2003).

The greatest damage caused by copper in photosynthetic organisms results from the inhibition of photosynthesis, mainly of the light reactions (review by Küpper and Kroneck, 2005). Here the substitution of Mg^{2+} in the chlorophyll (Chl) molecule by heavy metal ions leading to the formation of a heavy metal substituted chlorophylls ([hms]-Chls) (Küpper et al., 1996, 2002) is an important mechanism of damage at environmentally relevant Cu concentrations. [Hms]-Chls are unsuitable for photosynthesis unlike [Mg]-Chls, because of their less stable singlet excited state and lower tendency to bind axial ligands (Küpper et al., 2006). The excited energy from these altered chlorophylls may be accidentally transferred to oxygen resulting in the production of singlet oxygen, one of the reactive oxygen species (ROS), which causes oxidative damage (Pinto et al., 2003). Potential participation of Cu in Fenton reaction would also result in ROS production (Halliwell and Gutteridge, 1984), although it has never been shown to be relevant in vivo. A degradation of the grana stacking, the stroma lamellae, increase in the number and size of the plastoglobuli and alteration in the PSII membrane fluidity was found as indirect effects of the Cu toxicity (Quartacci et al., 2000), which would further decrease the activity of the photosystems (Lidon and Henriques, 1991; Ouzounidou et al., 1992). Excess Cu is also known to induce deficiency of essential ions (Mn^{2+}, Zn^{2+}, etc.) as there is a competition between the various metals according to the Irving–William series (Frausto da Silva and Williams, 2001).

Cu deficiency changes the chloroplast's thylakoid membranes (Droppa et al., 1987), decreases the pigments (chlorophyll and carotenoids) and affects the PSII activity. Like in the case of toxicity, damage to the photosynthetic apparatus will divert absorbed light energy towards different processes, finally resulting in oxidative stress. When there is deficiency in Cu there is no proper functioning of Cu/Zn SOD, causing further rise of oxidative stress (Marschner, 1995; Küpper and Kroneck, 2005). Further, Cu-deficient plants substitute Cu-proteins with proteins of similar or overlapping function but different central ion (Puig et al., 2007).

Most of the Cu toxicity studies were carried out at higher (up to 500 μM) Cu concentrations (Tsay et al., 1995, Baryla et al., 2000), which are much above the range of even the most polluted environments (Moore and Ramamoorthy, 1984). This causes a decrease in the specificity of any inhibition – as soon as all high-affinity binding sites are saturated with Cu, further Cu will bind to low-affinity binding sites that would not be a target of copper binding at environmentally relevant toxic Cu concentrations. Additionally, in earlier studies chelating agents were used to achieve Cu deficiency, which bind to other heavy metals (incl. essential nutrients) and reduce their bioavailability. Moreover, unnatural light conditions, which include continuous light or rectangular switch-on – switch-off, were used in the older studies although it is known that for the extent and symptoms of the heavy metal induced damage light intensity and dark phase are important (e.g. Cedeno-Maldonado et al., 1972; Küpper et al., 1996, 2002). Because of these reasons, it remained unknown which of the mechanism(s) of inhibition by copper toxicity are actually relevant in environmentally relevant conditions. Further, even though all the mechanisms stated above have been studied, an interdependence of these mechanisms has not been explained till now.

Thus, the kinetic pattern and the concentration thresholds of the occurrence of different damage mechanisms were the main focus of the current paper. We used the model plant *Ceratophyllum demersum* L., which is an aquatic submerged macrophyte sensitive to heavy metal stress. Since it has no roots, all nutrients are taken up over a large surface area of the entire shoot. Because of its ability to grow without a solid substrate it has been used for environment control and life support system studies in space through the successful spaceflight projects of CEBAS/Aquarack (Blüm et al., 1994) and the currently undergoing OMEGAHAB-B1 projects of the DLR.

2. Materials and methods

2.1. Plant material and cultivation

The submerged, rootless macrophyte *C. demersum* L. was used for the experiments. Plants were cultivated in an optimized nutrient solution for submerged macrophytes and water plants (SMNS, Table S1, pH 7.8). Since 2005 the strain was continuously cultivated in hydroponic cultures under 12 h day/12 h night light conditions with two FLUORA® fluorescent and two warm white fluorescent tubes (Osram, München, Germany) and a temperature cycle from 18 °C at 6 a.m., over 20 °C at 9 a.m., to a maximum of 22 °C at 3 p.m., back over 20 °C at 9 p.m. to 18 °C again at 6 a.m. The high light culture unit had a slightly different set up with "daylight" fluorescent tubes (Dulux L 55 W/12 950, Osram, München, Germany), 12 h sinusoidal light cycle with maximal irradiances at 500–650 μE inside the media and 12 h night. The temperature was 19 °C at 6 a.m., 21.5 at 9 a.m., 24 at 3 p.m., 23 at 9 p.m. and 19 at 6 a.m.

For each copper treatment ("0", 0.5, 1, 2, 5, 10, 20, 50, 100, 200 nM prepared by $CuSO_4$) around 2 g of plants were placed into an aquarium containing 2 l of continuously aerated medium to secure a low biomass to water volume ratio. A continuous exchange of nutrient solution (flow rate 0.5 $l.day^{-1}$) was set up to ensure that the metal uptake into the plants was limited only by the concentration, but not by the amount of nutrient solution available. The increase in growth was measured at the end of each week after the plants were cleaned. The experiment was carried out for 6 weeks at the end of which the plants were harvested. Young tissues being 4 cm from the apex and 2 cm from the apex of side branches, old tissues 8 cm from the stem end and the rest of the side branches were separated. Remaining SMNS was removed by shaking, the plants were frozen in liquid nitrogen and stored at −80 °C until further analyses.

2.2. Photosynthesis biophysics

To study the physiological changes in the plants induced by metals, two-dimensional (imaging) microscopic measurements using the Chl fluorescence kinetic microscope (Küpper et al., 2007a) were performed. One leaf from the 5th nodium, counted from the apex of the plant, was fixed in the measuring chamber with the help of cellophane. There was a continuous flow of the culture medium (but without micronutrients as they caused background in peroxide measurements) in the chamber (Küpper et al., 2008) that was used for the kinetics measurement. An area (approximate size of 1.1 μm × 1.1 μm) just before the last leaf branching point was measured. A detailed description of the microscope and the used protocols can be found in Küpper et al., 2007a; all photosynthetic parameters analysed in the current study are explained and referenced in Table S2. Values are given as means of five different experiments with two technical replicates each.