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Copper Effects on Photosynthetic Activity and Membrane Leakage of *Azolla filiculoides* and *A. caroliniana*

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ABSTRACT

The toxic effects of copper on the photosynthetic apparatus and cell membrane stability of *Azolla* species were evaluated by means of the chlorophyll fluorescence, leakage of electrolytes and total phenolic compounds. Different collects from *Azolla filiculoides* (Xalapa & Ensenada) and *A. caroliniana* (CEG) were incubated with different Cu^{2+} concentrations (0.02, 0.2, 1 & 2 mM) for 36 h in a plant growth chamber. The results showed that treatments with higher Cu concentration (1 & 2 mM) presented clear signs of disrupted photosystem II, since the potential photochemical yields (Fv/Fm) were lower than the control responses. However, membrane permeability (leakage of electrolytes) of both species significantly enhanced with the increase in Cu concentration after 12 h of exposure. Additionally, total phenolic constituents of both species did not differ significantly by Cu concentrations excepting for lower doses of Cu (0.02 mM) after 36 h of exposure. In conclusion, chlorophyll fluorescence analysis can be used as a useful physiological tool to assess early changes in photosynthetic performance of *Azolla* in response to heavy metal pollution. © 2010 Friends Science Publishers

Key Words: Photosynthetic; *Azolla;* Chlorophyll

INTRODUCTION

The Mexicali Valley is located in northeastern Baja California, south of the Imperial Valley in California. Approximately 70% of the cultivated land in Mexicali is irrigated with water from the Colorado River by which elevated concentrations of heavy metals in agricultural drain waters might be expected. Additionally, the Hardy River also receives brine waste with potentially high concentrations of heavy metals from a geothermal energy plant located at Cerro Prieto in the middle of the agricultural valley, approximately 30 km south of the city of Mexicali. Due to their occurrence and persistence in aquatic pollution, metals are one group of contaminants commonly assessed (Johnston *et al*., 2009). Copper (Cu^{+2}) is an essential plant micronutrient and often occurs in high concentrations in the aquatic ecosystems (Chen *et al*., 2007; Rauf *et al*., 2009). In addition, Cu has become a widespread contaminant due to its use as a mineral pesticide in agriculture (e.g., $CuSO_4$ in Bordeaux mixture) (Ahmad *et al*., 2008). On the other hand some plants have been demonstrated to accumulate heavy metals and other nutrients from contaminated waters and they may be directed to phytoremediation processes (Mkandawire *et al*., 2004). However, selection of plant species for removing metal ions from polluted water will also depend on their growth rate, level of tolerance to heavy metals and concentration of metals in the environment (Garbisu *et al*., 2002). In this sense, the aquatic fern *Azolla* has been reported to accumulate high concentration of heavy metals and metalloids (3-4 mgL^{-1}) from aqueous media (Khosravi *et al*., 2005; Rai *et al*., 2009). *Azolla* ferns are worldwide distributed and grow in all kinds of fresh and wastewaters (Scharpenseel & Knuth, 1987). In this regard *Azolla* may detoxify aquatic contaminated with heavy metals, specifically in the region of the valley of Mexicali, Baja California (Mexico).

However, even though, it is known that small amounts of metals are accumulated in frond of *Azolla* species, their tolerance and physiological responses have been scarcely studied. The aim of this work was to investigate the characteristics of PSII photochemistry induced by Cu^{+2} and also to evaluate the use of chlorophyll fluorescence, leakage of electrolytes and total phenolic compounds as possible indicators for heavy metal stress in *Azolla* ferns.

MATERIALS AND METHODS

Biological material and experimental conditions: The Mexican accessions of *Azolla filiculoides* (Xalapa), *A. filiculoides* (Ensenada) and *A. caroliniana* (CEG), were

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utilized for this experiment. The accessions, belonged to the Azolla collection Azollatum of Colegio de Posgraduados (Zimmerman *et al*., 1993). One-gram of fresh weight of *Azolla* species were placed into plastic containers with 150 mL of Yoshida´s nutrient solution was applied (Yoshida *et al*., 1971; Castro-Carrillo *et al*., 2008). The pH initial of nutrient solution was 5.5 and electrical conductivity of 876 µS m^{-1}. In addition, a set of increased $CuSO_4$ concentrations were applied to the nutrient solution (0.02, 0.2, 1 & 2 mM) and a non-contaminated nutrient solution was used as control. There were five treatments with three replicates for each *Azolla* species. A completely randomized experiment was established under growth chamber conditions (26°C, 12 h photoperiod, light 280 µmol cm^{-2} s^{-1} & 80% HR; Lab-Line Biotronette ®).

Estimation of membrane permeability (electrolyte leakage): Electrolyte leakage was determined as described by Gonzalez-Mendoza *et al*. (2009). The initial electrical conductivity (C_1) of the medium with *Azolla* biomass were measured at time zero and variable electrical conductivity (C_n) were measured at 12, 24 and 36 h, respectively. Finally, the samples were autoclaved at 121°C for 20 min to release all electrolytes; cooled to 25°C and the final electrical conductivity (C_F) was measured. Three replicates were measured for each copper treatment and for the controls control. Degree of electrical leakage (E_T) was calculated from the following equation:

$$E_T = (C_n - C_1)/C_F)\times 100$$

Chlorophyll fluorescence: Chlorophyll fluorescence was measured by a Plant Efficiency Analyser (PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk PE32 1JL, UK) according to Strasser *et al*. (1995). Readings were taken at 0, 12, 24 and 36 h after exposure to copper using three single fronds per treatment. The randomly selected fronds were subjected to a 5 min period of adaptation to darkness to induce the complete oxidation of the reaction centers. The potential photochemical yield (Fv/Fm) was calculated according to the method of Küpper *et al*. (2002).

Analysis of phenolic compounds: Total phenolic content for the three *Azolla* accessions treated with copper was evaluated by the Folin-Ciocalteu reagent assay utilizing chlorogenic acid as standard, at 36 h (Soong & Barlow, 2004). In brief, 100 mg of fronds were placed in a eppendorf tube, with 1.5 mL of methanol (80%), grinded at 4°C and centrifuged at 14000 x *g* for 15 min.

Reaction mixture consisted of mixing 30 µL of the extract added with 90 µL of Na_2CO_3 and 150 µL of Folin-Ciocalteau reagent in a 96-well microplate. After 30 min, absorbance readings (725 nm) were taken in a KC-4 spectrophotometer (Biotek 2 ® Instruments, Inc. Winooski, Vt.).

Statistical analysis: Data were analyzed with analyses of variance (ANOVA) and mean were comparison test (Tukey's α = 0.05) was performed (Statistical Package version 5.5, Statsoft, USA). Significant differences were accepted if $p < 0.05$ and data was expressed as mean ± Standard error.

RESULTS

Electrolyte leakage: The effect of Cu^{+2} doses on *Azolla* fronds resulted in significant (p < 0.001) changes in electrolyte leakage as metal concentrations increased (Fig. 1). After 12 h of exposure, to either 0.2 or 1 mM of Cu, *A. filiculoides* "Ensenada" and "Xalapa" electrolyte leakage than *A. caroliniana* "CEG" (Fig. 1A, B & C). On the other hand, at 2 mM of Cu^{2+} the elec-trolyte leakage values were similar in the three *Azolla* accessions after 24 h of exposure (Fig. 1A, B & C).

Total phenolic Compounds: Chlorogenic acid equivalent total phenolic constituent of *Azolla* specimens are shown in Fig. 3. Total phenolic constituents of both species showed great differences with and without Cu^{+2} concentrations.

While 80.0 ± 0.3 µg total phenolic compounds were

Fig. 1: Percentage electrolyte leakage measured in fronds of *Azolla* three collects accessions exposed to 0.02, 0.2, 1, 2 mM Cu^{+2}, Means ± Standard error. n=3

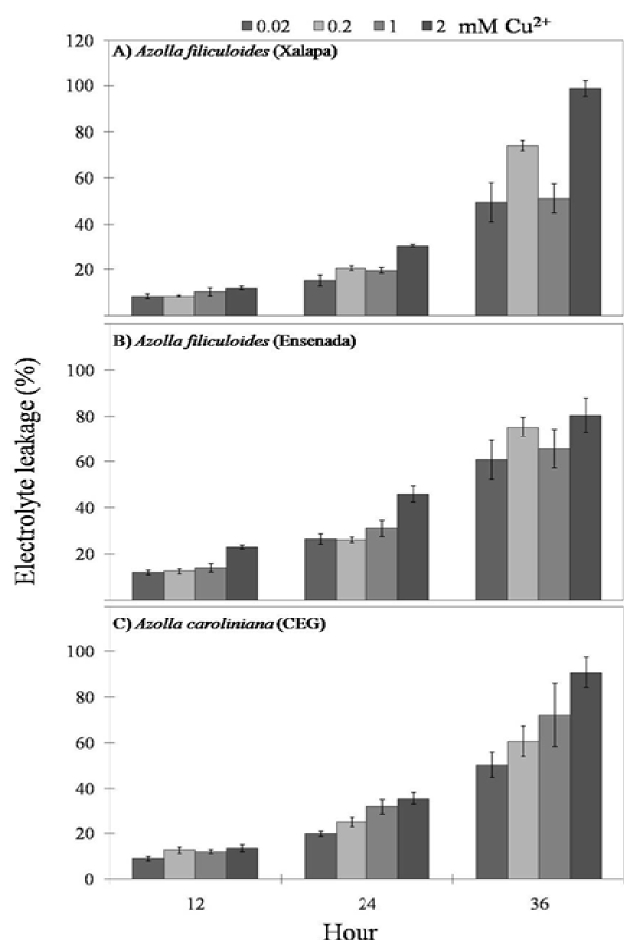


Fig. 2: Time course response of potential photochemical yields of PSII measured in fronds of *Azolla* three collects accessions exposed to 0.02, 0.2, 1, 2 mM Cu^{+2} during an exposure period of 36 h, Means ± Standard error. n=3

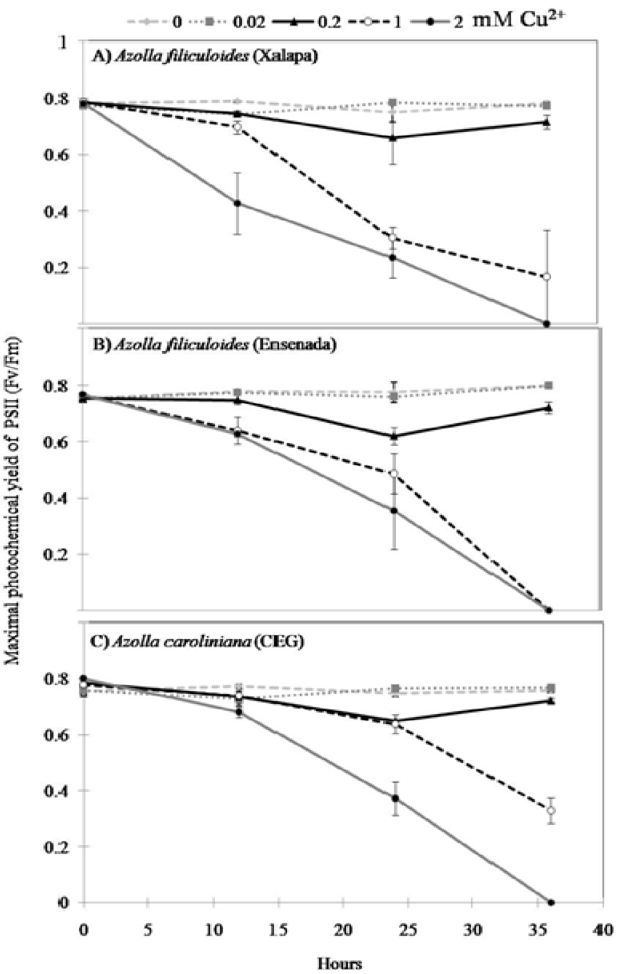
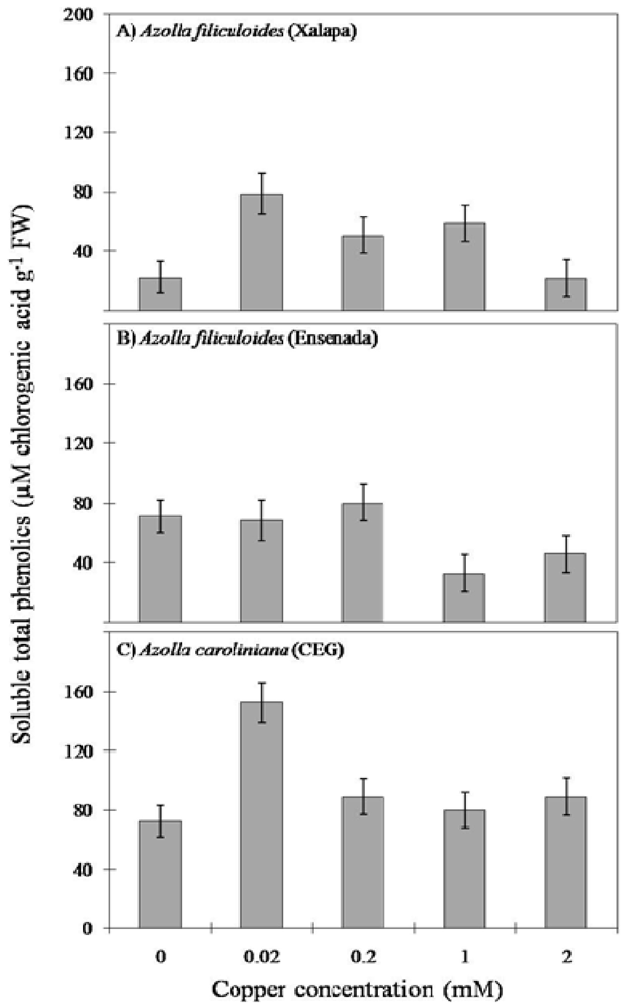


Fig. 3: Total soluble phenolics in fronds of *Azolla* three collects accessions exposed to 0.02, 0.2, 1, 2 mM Cu^{+2} during an exposure period of 36 h, Means ± Standard error. n=6



determined per gram of *Azolla* (Ensenada & CEG) only 35.0 ± 0.05 µg was determined in *Azolla* (Xalapa) after 36 h of exposure. On the other hand, 0.02 mM of Cu^{+2} the total phenolic content of *A. caroliniana* (CEG) was nearly two times higher than that of *A. filiculoides* (Xalapa & Ensenada) (Fig. 3) ($p < 0.001$). However, a non-significant decrease was not observed at 2 mM Cu^{+2} concentration for both *Azolla* species.

Chlorophyll fluorescence: The potential photochemical yield (Fv/Fm) after dark adaptation, may be used as a plant stress indicator (Björkman & Demmig, 1987). The results showed that the three *Azolla* accessions were not affected by concentrations of Cu^{2+} lower than 0.2 mM, however, the treatments with higher doses of copper during the first 12 h of exposure showed significant reductions (50-65%), when compared to their respective controls (Fig. 3).

DISCUSSION

These results suggest that Cu-induced stress causes membrane damage in *Azolla* fronds especially for the "Ensenada" accession during the first hours after exposure. In these sense, the accumulation of Cu^{2+} ions may induce the formation of reactive oxygen species (ROS) O^{-2}, H_2O_2 and HO and a subsequent decrease of antioxidants to avoid cell damages due to ROS-accumulation. Additionally, Cu^{2+} ions can interact with S and N groups in cell proteins and cause an alteration of the ionic channels of the membrane, which promotes a higher flow of ions of the leaf cells in the leaf (Bačkor *et al*., 2007). Similar results were reported by Gonzalez-Mendoza *et al*. (2009) and Tamas *et al*. (2006), where electrolyte leakage was increased by the generation of ROS in *Avicennia germinans* and *Hordeum vulgare*, respectively, when exposed to cadmium and copper.

On the other hand, our data show that phenolic compounds only increased significantly in ferns treated with 0.02 mM when compared to controls, suggesting that phenolic compounds may be as part of in the plant's detoxification mechanism. However, phenolic compounds production in response to Cu^{2+} addition was not always dose-dependent and did not increase linearly with exposure time, which is similar to that reported for other plant species (Sivaci *et al*., 2008). Finally, the reduction on Fv/Fm may be explained in part due to the negative effects of Cu^{2+} on the photochemical reactions and thus, affecting the efficiency of PSII photochemistry in which the electron transport is blocked (Tyystjärvi, 2008). Similar results were found by Küpper *et al*. (2002), where the suppressed relative yield of potential photochemical yields (Fv/Fm) by Cu^{+2}, in *Scenedesmus quadricauda*, demonstrated a rapid inactivation of PS II induced by the metal. In summary, the concerted decreases found on chlorophyll fluorescence and electrolyte leakage in plants treated with lower and higher Cu^{+2} concentrations clearly indicate that Cu^{+2} may induce important alterations on the photosynthetic apparatus of *Azolla* ferns.

CONCLUSION

Our results suggest that one of the negative effects of copper, based on chlorophyll fluorescence and electrolyte leakage tests, may be related to a rapid inactivation of PSII and membrane damage. Additionally, the chlorophyll fluorescence and electrolyte leakage tests may be used as physiological indicators' to understand in part, the main mode of action of heavy metals on the photosynthetic apparatus of *Azolla* ferns.

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