# Copper Stress on Photosynthesis of Black Mangle (*Avicennia germinans*)

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## ABSTRACT

The effects of copper toxicity on the photosynthetic activities of *Avicennia germinans* was investigated using two $CuSO_4$ concentrations (0.062 and 0.33 M) added in Hoagland's solution in an aerated hydroponic system. Photosynthesis and chlorophyll fluorescence were measured after 30 h of copper stress. Results obtained in this study show that increasing levels of $Cu^{+2}$ of 0.062 and 0.33 M $Cu^{+2}$ resulted in a general reduction of the stomatal conductance (28 and 18%, respectively) and 100% of inhibition of net photosynthesis. Additionally, at these concentrations of $Cu^{+2}$, reductions of chlorophyll fluorescence parameters were also observed. These changes suggested that the photosynthetic apparatus of *Avicennia germinans* was the primary target of the $Cu^{+2}$ action. It is concluded that $Cu^{+2}$ ions causes a drastic decline in photosynthetic gas exchange and Chlorophyll fluorescence parameters in *A. germinans* leaves.

**Key words**: *Avicennia germinans*, chlorophyll fluorescence, copper, photosystem II, photosynthesis.

## INTRODUCTION

Mangrove ecosystems are among the most important features of the coastal environment in many tropical and subtropical areas. Many mangrove ecosystems are located close to urban development areas, which may be impacted by effluents from industrial sources and urban runoff that often contains toxic concentrations of heavy metals (Cuong et al. 2005, Defew et al. 2005). Despite this, mangroves possess a great tolerance to relatively high levels of heavy metal pollution (Peters et al. 1997, De Lacerda 1998). *Avicennia* species in particular, are considered to be especially robust to heavy metals

and accumulate metals in greater quantities than other mangrove species, before any visible signs of toxicity are evident (MacFarlane et al. 2003). Copper ($Cu^{+2}$) is essential plant micronutrients, and often occurs in high concentrations in mangrove forest due to their prevalence in sediments, up to 900 mg/kg (Chen et al. 2007). The uptake of $Cu^{+2}$ in excess to nutritional requirements by mangroves may initiate a disruption of many physiological functions that can cause damage at the cellular level (MacFarlane and Burchett 2001). Most of the in vitro studies using isolated chloroplasts or excised leaves have reported a direct effect of copper on the photosynthetic electron transport chain (Mallick and Mohn 2003). These disturbances are correlated

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with lipid peroxidation of thylakoid membranes or with the alteration of lipid protein interactions in the chloroplast membrane, affecting the light reaction processes, especially those associated with PSII (Perales-Vela et al. 2007). In vivo, the first contact between copper and the plant is not directly at the chloroplast level and therefore, the mechanism of photosynthesis inhibition probably differs from that observed *in vitro*.

In this context, the objective of this work is to research if plants were able to tolerate higher concentrations of metal. We investigated the effect of an additional supply of copper on the photosynthetic activity of *Avicenia germinans*. The net photosynthetic rate and the chlorophyll a fluorescence were measured to determine the physiological modifications induced by copper excess.

## MATERIALS AND METHODS

### Field Collection and Germination of Plant Material

*Avicennia germinans* (L.) Stearn., black mangle, seeds were obtained from Chabihau Bay, (21° 20' 38" N and 89° 05' 08" W) Yucatan, Mexico. The seeds of black mangle were germinated between moistened filter paper at 25°C for 3 d. The disinfection process was achieved by sequential steps starting with 1% NaOCl (Clorox) for 5 min, followed by a rinse with deionized sterile water. The propagules were then peeled with a sterile scalpel and the pericarp discarded. The naked propagules were further disinfected with 0.1% NaOCl and finally rinsed with deionized sterile water six times. One hundred fifty propagules were then planted in 13.5 cm-diameter plastic pots (one plant per pot) filled with sterilized soil and grown at 28–35°C /24–26°C (day/night temperature regime) under a 12-h light-dark photoperiods and 60% relative air humidity in a greenhouse.

### Copper Exposure

Twenty individuals, three-months-old *Avicennia germinans* seedlings were randomly allocated (n=4) and transferred to different flasks containing Hoagland's solution supplemented with different concentrations (0.062 and 0.33 M) of $CuSO_4.5H_2O$.

Control plants were transferred to plastic containers with 500 mL Hoagland's solution without $CuSO_4.5H_2O$. Treated and control plants were exposed during a period of 30 h under hydroponics conditions and these exposures were performed in quadruplicates. Treated started at 9 am and the measures taken at 16 h were made during the night period.

### Gas Exchange

The rate of net photosynthesis ($P_n$), and stomata conductance ($g_s$) of young fully expanded leaves were measured at 4, 8, 16, 24 and 30 h after exposure to treatments with copper using a portable infrared gas analyzer (LI-Cor Model 6200, Lincoln, NE, USA). All the photosynthetic measurements were standardised at 23°C, 400 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation and each treatment was measured four times. Treatments started at 9 am and the measurements taken at 16 h were made during the night period.

### Fluorescence Measurements

The determination of chlorophyll fluorescence was carried out using a portable fluorometer (Plant Efficiency Analyzer-MK2–9600–Hansatech, Norfolk, UK) on completely expanded leaves of appropriate phytosanitary condition. The data were recorded from 10 ms up to 1 s with a data acquisition of every 10 ms for the first 300 ms, then every 100 ms up to 3 ms and later every 1 ms. The signal resolution was 12 bits (0–4,000). For each treatment, the chlorophyll (Chl) a fluorescence transients of 4 individual leaves were measured. Leaves were maintained in darkness for 5 min before taking the data on chlorophyll fluorescence. The maximal intensity of the light source, providing an irradiance saturating pulse of 3,000 $mmol\ photons.m^{-2}.s^{-1}$ was

used. Different chlorophyll fluorescence parameters like the $F_v/F_m$, $F_v/F_0$ and $F_0/F_v$ ratios were calculated from measurement of $F_v$, $F_m$ and $F_0$ using the software Biolyzer 2.5 (Maldonado-Rodriguez 2002). The ratio of variable fluorescence to maximal fluorescence ($F_v/F_m$) is an indicator of the efficiency of the photosynthetic apparatus, while the ratio of variable fluorescence to unquenchable portion of fluorescence ($F_v/F_0$) is an indicator of the size and the number of active photosynthetic reaction centers and ($F_0/F_v$) represent the efficiency of the water-splitting apparatus (Kriedemann et al. 1985).

STATISTICS ANALYSIS

All data presented are the mean values. Statistical analysis was carried out by one-way ANOVA for repeated measures followed by posthoc analysis by the Fisher test of least significant difference with significance set at $P \leq 0.05$.

**RESULTS**

PHOTOSYNTHESIS PARAMETERS

The increase of $Cu^{+2}$ dose decreases all leaf gas exchange values, having the strongest negative effect on stomatal conductance ($g_s$), and net photosynthesis ($P_n$) (Fig. 1a, b). The values of stomatal conductance were lower in treated plants compared to control plants (Fig 1a). The reduction of stomatal conductance was similar in plants treated with 0.332 and 0.062 M $Cu^{+2}$ (83 and 72 %, respectively). The net photosynthesis was significantly (100% of inhibition) and most similarly affected by both $Cu^{+2}$ concentrations (Fig 1b).
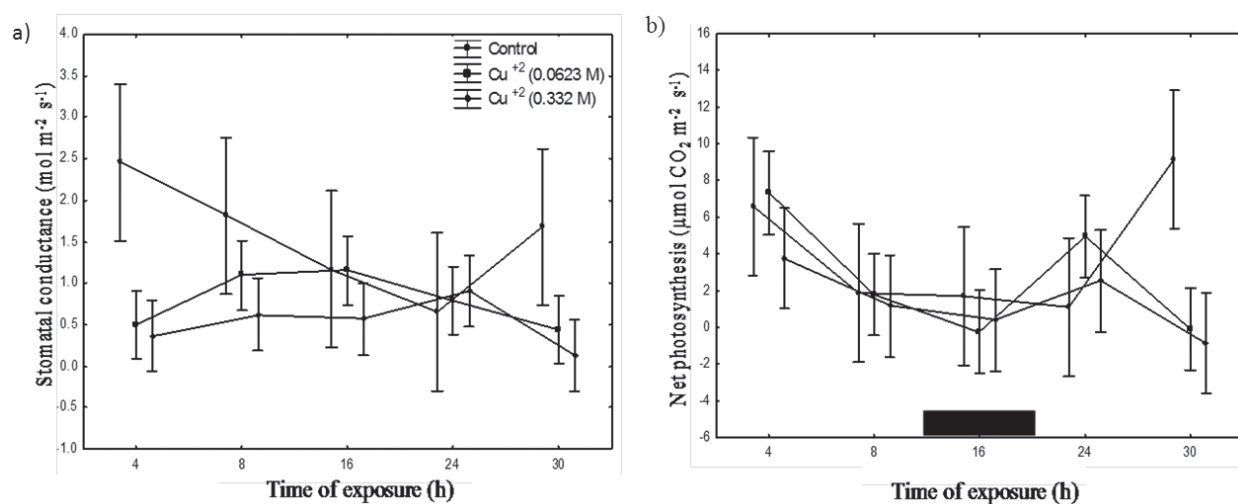


**Figure 1 -** Time course response of stomatal conductance **(a)** and net photosynthesis **(b)**., from untreated plants or plants treated with 0.062 M and 0.33 M $Cu^{+2}$ during an exposure period of 30 h. The black bar indicates the dark hours. Each point is the mean from 4 replications.

CHLOROPHYLL FLUORESCENCE PARAMETERS

The measurement of fluorescence parameters in control plants showed an dropped from 4 to 8 h followed by a progressive rise from 8 to 24 h. towards the end of the dark hours to finally dropped again the following day to values similar found the previous day (Fig 2a).

On the contrary $Cu^{+2}$-treated plants with 0.332 M also showed dial fluctuations during the first 24 h, but after 30 h of exposure to the metal, significantly higher $F_0/F_{vi}$ values (around 138 %) were recorded in treated plants (Fig 2a). In contrast, the strongest augment of $F_0/F_{vi}$ caused by higt Cu-concentration was not observed in the plants treated with 0.062 M of $Cu^{+2}$.

The $F_v/F_m$ and $F_v/F_0$ values were followed during the course of exposure $Cu^{+2}$ concentrations (Fig. 2b and 2c). During the experiment, the plants exposed to 0.062 and 0.332 M $Cu^{+2}$, no difference was found in these parameters during a large part of the experiment. Effectively, only at 30 h (end of the experiment) could a slightly difference be observed between the treatments. In the case of $F_v/F_m$ and $F_v/F_0$ values for control plants, they rise slightly from 4 to 8 h followed by a progressive drop from 8 to 24 h, towards the end of the dark hours, to finally rise again the following day to values similar found the previous day (Fig 2b and 2c).

## DISCUSSION

Numerous studies on the physiological responses to excess amounts of heavy metal ions indicate that mangrove have developed various mechanisms to cope with this environmental threat. Some of them mechanisms appear to involve the presence of exclusion and sequestering processes that can moderate the metal uptake by roots and induce a substantial metal accumulation on the root tissues (De Lacerda 1998).

In this sense, Gonzalez-Mendoza and Zapata-Perez (2008) suggest that the radical architecture present in *Avicennia germinans* plants might alter the uptake of Cd by an integrated network of multiple response processes such as production of organic acids,
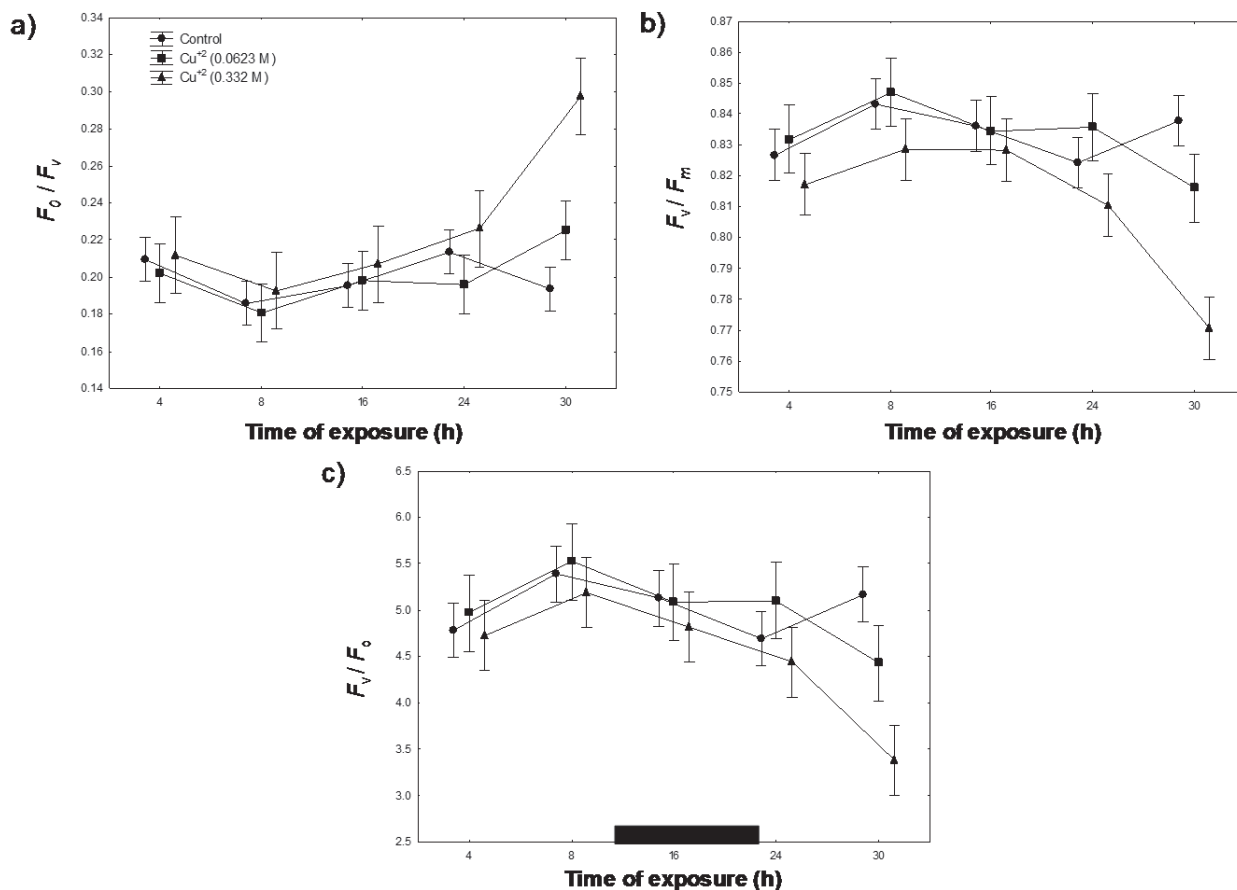


**Figure 2 -** Time course response of various fluorescent parameters for untreated A germinans plants or plants after 30 h exposure to 0.062 and 0.33 M $Cu^{+2}$ **(a)** water –splitting apparatus of PSII, $F_0/F_v$, **(b)** the maximal photochemical yield of PSII in dark-adapted leaves $F_v/F_m$; and **(c)** the ratio of variable fluorescence to unquenchable portion of fluorescence ($F_v/F_0$). The black bar indicates the dark hours. Each point is the mean from 4 replications.

antioxidative response, cell-wall lignifications and suberization. On the other hand, Gonzalez-Mendoza et al. (2007) showed that minimal concentrations of cadmium in foliar tissues affect several targets of photosystem II. More specifically the main targets of cadmium can be listed as a decrease in the number of active reaction centers and damage to the activity of the water-splitting complex. On the other hand, numerous metals are essential for living organisms at very low concentrations, but at high concentrations most of them are toxic and have a direct and adverse influence on various physiological and biochemical processes. In this sense the copper is an essential metal that participates in growth, metabolism and enzyme activities (Yruela 2005). The copper can be accumulated in the leaf tissue in high concentrations of numerous mangrove species in the field including *Kandelia* spp., *Rhizophora* spp. and *Avicennia* spp., without apparent impact on plant health (Peters et al. 1997, Macfarlane et al. 2003). Even though, it is known that higher amounts of $Cu^{+2}$ end up in the leaves to be accumulated (Defew et al. 2005), the physiological effects of copper stress in plants of mangrove like *Avicennia germinans* are basically not known. Therefore, investigations on the mechanism of copper action on the photosynthetic apparatus are of special interest. In this way, we provide important evidence that aerial tissues of *A. germinans* are affected by low and high $Cu^{+2}$ concentrations. Our data showed that the highest sensitivity to $Cu^{+2}$ was exhibited by stomatal conductance ($g_s$), and net photosynthesis ($P_n$). Our study showed that at the experimental design used chlorophyll fluorescence are less sensitive parameters of $Cu^{+2}$ toxicity.

The inactivation of net photosynthesis could be explained by a possible inhibition of the enzymatic processes in the Calvin cycle of *Avicennia* plants. Specifically, because the $Cu^{+2}$ could induced a reduction in the synthesis or activity of Calvin cycle enzymes reducing the demand for $CO_2$, resulting in reductions in stomatal conductance in this plants.

Additionally, the $Cu^{+2}$ ions may also exert its toxicity in subcellular organelles, interfering with mitochondrial electron transport, respiration, ATP production and photosynthesis in the chloroplasts and may eventually cause plant death (Yruela 2005). Similar results were found by Ouzounidou and Ilias (2005) where *Helianthus annuus* plants exposed to $Cu^{+2}$ for short time showed a decrease in the rate of net photosynthesis due to stomata closure, Rubisco inactivation and chlorophyll degradation.

Chlorophyll fluorescence induction parameters have been shown to detect even subtle biochemical damage within photosynthetic reaction centers in a wide variety of both terrestrial and marine plants. The present investigation shows that a significant decline in the $F_v/F_0$ ratio indicating an apparent change in the rate of electron transport from PS II to the primary electron acceptors in *A. germinans* plants exposed to both $Cu^{+2}$ concentrations. The changes in the $F_v/F_0$ ratio can be due to the modifications in the unquenchable fluorescence ($F_0$) that affected the energy transfer from the antenna complex to the reaction centres (DeEll et al. 1999). Additionally, the decline in the $F_v/F_0$ ratio is indicative of either a decline in the rate of photochemistry as the primary electron acceptor pool ($Q_n$) became increasingly oxidized, or a reduction of the pool size of the primary electron acceptors associated with PS II activity (Krause and Weiss 1991). Another possible explanation for the changes in the $F_v/F_0$ ratio of the copper-treated plants is the substitution of the central atom of chlorophyll, magnesium by $Cu^{+2}$. This substitution prevents photosynthetic light-harvesting in the affected chlorophyll molecules, resulting in a disruption of photosynthetic reactions (Küpper et al. 2002). Similar results were found by Ouzounidou and Ilias (2005) where the values $F_v/F_o$ were clearly decreased with lower and higher $Cu^{+2}$ concentrations in *Helianthus annuus*, and they demonstrated a rapid inactivation of PS II induced by the metal.

In conclusion, excess $Cu^{+2}$ produces a variety of toxic effects on photosynthesis. Based on both high sensitivity of leaf gas exchange parameters (stomatal

conductance and net photosynthesis) than chlorophyll fluorescence parameters to excess $Cu^{2+}$ and fastness of their measurement, we propose these parameters as suitable parameters for plant test systems, which can be utilized for successful screening for higher $Cu^{2+}$ tolerance among mangrove plants.

## ACKNOWLEDGMENTS

## RESUMO

Os efeitos da toxicidade do cobre nas atividades de fotossíntese de *Avicennia germinans* foram investigados utilizando duas concentrações de $CuSO_4$ (0,062 e 0,33 M) adicionadas à solução nutritiva em um sistema de hidroponia aerada. A fotossíntese e fluorescência da clorofila foram medidas após 30 h de exposição ao cobre. Os resultados obtidos neste estudo mostram que o aumento dos níveis de $Cu^{+2}$ de 0,062 e 0,33 M resultou numa redução geral da condutância estomática (28 e 18%, respectivamente) e 100% de inibição da fotossíntese. Além disso, nestas concentrações de $Cu^{+2}$, reduções de parâmetros de fluorescência também foram observadas. Estas alterações sugerem que o aparelho fotossintético de Avicennia germinans foi o alvo principal da ação do $Cu^{+2}$. Conclui-se que os íons $Cu^{+2}$ provocam uma drástica queda nas trocas gasosas fotossintéticas e parâmetros de fluorescência da clorofila nas folhas de *A. germinans*.

**Palavras-chave**: *Avicennia germinans*, clorofila fluorescente, cobre, fotosistema II, fotossíntese.

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