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RESEARCH ARTICLE

Chlorophyll *a* fluorescence in sweet potatoes under different copper concentrations

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Abstract

Photosynthesis is one of the main plant metabolic processes affected by copper deficiency and toxicity. The objective of this study was to evaluate the effects of different copper concentrations on transient chlorophyll *a* fluorescence and modulated fluorescence in sweet potatoes. Sweet potatoes were placed in a hydroponic system and grown for six days with a complete nutrient solution. The plants were then transferred to solutions with different copper concentrations (0.041, 0.082, 0.123 and 0.164 mM) for nine days. The solutions were renewed every three days, and the plants were evaluated at the end of the experiment. Increased copper availability (0.123 and 0.164 mM) positively influenced the structure and functionality of photosystem I (PSI). The increase in Cu availability also reduced the apparent electron transport rate in the PSII [ETR(II)], and the plants treated with 0.082 and 0.123 mM Cu were able to dissipate the excess light energy in the PSII and protect its units (NPQ and qN). A joint analysis of the data shows that high copper concentrations in the nutrient solution lead to reduced photochemical activity of the PSII, increased dissipation of light energy from this same photosystem and increased PSI efficiency in sweet potatoes.

Keywords: Micronutrient, photosynthesis, copper sulphate, fluorescence transient, modulated fluorescence

1. Introduction

Heavy metals are considered environmental pollutants due to their toxicity, persistence and non-degradability in the soil, and copper is one of the main environmental contaminants (Violante *et al*., 2010; Cambrollé *et al*., 2013). This metal, in low concentrations, is an essential micronutrient for plant growth and development, and it plays a role in important biological reactions, mainly as an enzymatic cofactor present in electron carrier molecules in photosynthetic (plastocyanin) and respiratory (cytochrome oxidase) processes. However, this element is highly toxic to plants at excess concentrations (Mateos-Naranjo *et al*., 2008).

The first effect of copper toxicity in most species occurs in the roots, but high concentrations can cause copper to be translocated to the shoots and to interfere in various physiological processes (Cambrollé *et al*., 2013). The thresholds of leaf

copper concentration causing toxicity are highly variable and range between 0.02 and 0.1 mg g^{-1} dry mass (Kabata-Pendias and Pendias, 2001). High levels of copper have been related to reductions in or damages to plant growth, mineral nutrient absorption, photosynthetic activity, membrane permeability, protein synthesis, enzymatic activity and chromatin structure (Cataldo *et al*., 2011; Cambrollé *et al*., 2011; 2012).

In contaminated soils, plants behave in different manners when faced with abiotic stress, and evaluating the physiological responses of plants subjected to toxicity by heavy metals can help unveil strategies employed in the removal, accumulation and tolerance of heavy metals (Kabata-Pendias and Pendias, 2001; Cambrollé *et al*., 2013).

Photosynthesis is one of the main plant metabolic processes affected by copper toxicity, and studies that evaluate these effects have shown that this metal causes decreased photosynthesis due to altered photochemical reactions in photosystem II (PSII) or the inactivation of active reaction centres, reduced quantic yield and decreased electron transport on the acceptor side and on oxygen evolution (Perales-Vela *et al*., 2007; Xia and Tian, 2009; Cambrollé *et al*., 2012; Oukarroum *et al*., 2012). Chlorophyll *a* fluorescence-transient analysis is an efficient tool for studying physiological aspects of structure and activity, especially in the PSII (Strasser *et al*., 2004), and it has been widely used to evaluate damages to the photosynthetic system of plants by various types of stress (Maxwell and Johnson, 2000).

The analysis of modulated chlorophyll *a* fluorescence is a technique that enables the collection of qualitative and quantitative information about the organisation and functioning of the photosynthetic apparatus of plants based on the saturation pulse method (Rohácek and Barták, 1999). The principle employed by this method has been used in plant physiology studies that need to quantify the contributions of the photochemical processes and the dissipation of non-photochemical energy in the PSII (Rohácek and Barták, 1999). According to studies by Cambrollé *et al*. (2011, 2012, 2013), high copper concentrations decreased PSII yield and efficiency as a consequence of the photoinhibition induced by light stress in yellow hornpoppy (*Glaucium flavum*), sea purslane (*Halimione portulacoides*) and 'sapeira' (*Limoniastrum monopetalum*).

The sweet potato [*Ipomea batatas* (L.) Lam.] is economically important in developing nations because in addition to providing essential nutrients, it is easily cultivated, has high biomass, is resistant to drought and has a low production cost (Low *et al*., 2007). In addition to these characteristics, sweet potatoes have been used as a model plant in studies of plant nutrition and metabolism due to their ease of propagation and fast growth (Adamski *et al*., 2011; 2012).

The objective of this study was to evaluate the effect of different copper concentrations on the chlorophyll *a* fluorescence transient and modulated fluorescence in sweet potatoes.

2. Materials and Methods

2.1. Plant material and growth conditions

The sweet potato plants used in the study were obtained from apical branches that were approximately eight centimetres long and had four leaves per branch from plants rooted for five days in distilled water in a greenhouse. After rooting, the plants were placed in a continuous-flow root floating hydroponic system and were cultured using the Hoagland and Arnon (1938)

complete nutrient solution for six days. The plants were then cultured in a nutrient solution for nine days with different copper concentrations: 0.041; 0.082; 0.123 and 0.164 mM in the form of copper sulphate ($CuSO_4.5H_2O$). The concentrations were determined after preliminary experiments that showed that cultivation in concentrations lower than those cited did not cause changes in growth parameters, and the control concentration was set at 0.041 mM copper. Every three days, the solution was renewed, and the pH was adjusted to ±5.8. After the plants were exposed to the treatments for nine days, the chlorophyll *a* fluorescence transient and modulated fluorescence were evaluated.

2.2. Chlorophyll fluorescence transient

The chlorophyll *a* fluorescence transient was measured in intact, fully expanded leaves in the second node from the apex (20-25 leaves/treatment) with a Handy-Pea portable fluorometer (Hansatech, King's Lynn, Norfolk, UK), with a gain of 0.5. The leaves were first adapted to the dark for 30 minutes and then subjected to a saturating light pulse (approximately 3,000 μmol m^{-2} s^{-1}). The fluorescence intensities were measured for one second. The JIP test parameters were calculated based on the fluorescence intensities of 50 μs (minimum fluorescence – F_0), 100 μs, 300 μs, 2 ms (F_J), 30 ms (F_I) and F_M (maximum fluorescence) (Strasser *et al*., 1995).

2.3. Modulated fluorescence

Modulated chlorophyll *a* fluorescence was measured with a Dual-PAM-100 fluorometer (Heinz Walz, Effeltrich, Germany). For this purpose, unexpanded leaves were exposed for 2 minutes to an increasing photon flux density (PFD) (varying from 0 to 531 μmol photons $m^{-2}s^{-1}$). The following intensities were determined after exposure to each PFD: F_0' (initial fluorescence in the light-adapted state), F_M (maximum fluorescence in the dark-adapted state), F_M' (maximum fluorescence in the light-adapted state obtained after applying a saturating light pulse) and F_S (fluorescence intensity in equilibrium, or steady state). The following items were calculated from these parameters: effective photochemical quantum yield of the PSII [$\varphi_{PSII} = (F_0' - F_S)/F_M'$)], photochemical extinction coefficient [$qP = (F_M' - F_M)/(F_M' - F_0')$] (Genty *et al*., 1989), non-photochemical extinction coefficient [$qN = (F_M - F_M')/(F_M - F_0')$] (Genty *et al*., 1989) and non-photochemical extinction [$NPQ = (F_M - F_M')/F_M'$)] (Demmig-Adams, 1990). The apparent electron transfer rate of the PSII [ETR(II)] was calculated as 0.5 x PFD x φ_{PSII} x 0.84, where 0.5 is the proportion of energy that reaches the PSII, and PFD is the irradiance absorbed by the leaf, considering 0.84 or 84% light intensity (Baker, 2008).

3. Results

3.1.Chlorophyll a fluorescence transient: Normalisation and subtraction of transients

The transient fluorescence intensity in leaves grown under different copper concentrations was represented by typical polyphasic curves (Figure 1a-1b). The plants cultivated in higher copper concentrations (0.123 and 0.164 mM) had decreased fluorescence from the F_J (2 ms) to F_M (1 s) steps, particularly at a concentration of 0.164 mM.

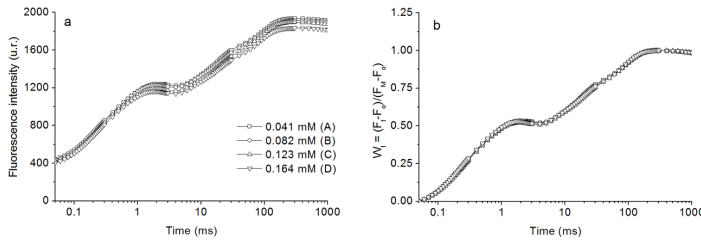

Figure 1. (a) Chlorophyll *a* fluorescence transient in dark-adapted sweet potato (*Ipomea batatas* L.) leaves cultivated under different copper concentrations (F_t); (b) Relative variable fluorescence [$W_t = (F_t - F_0)/(F_M - F_0)$].

To allow the possible identification of L-bands (approximately 150 μs), the relative variable fluorescence was normalised between the points 0 (50 μs) and K (300 μs) ($W_{OK} = [F_t - F_0]/[F_K - F_0]$) (Figure 2a) and the kinetic difference was shown through the following equation: $\Delta W_{OK} = [W_{OK(treatment)} - W_{OK(control)}]$ (Figure 2b).

The appearance of the positive L-band indicates low energetic connectivity (or grouping) between the units of the PSII (Strasser and Stirbet, 1998; Yusuf *et al*., 2010). The present study identified the positive L-band in all the treatments, although with lower amplitude in the 0.164 mM concentration (Figure 2b).

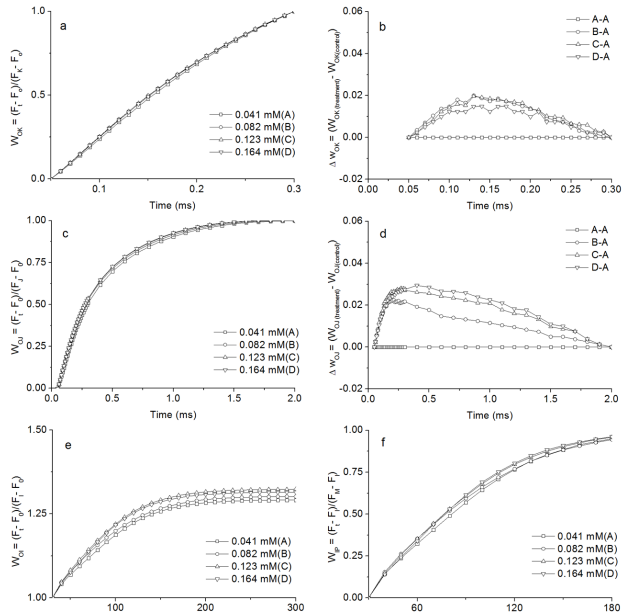

Figure 2. Chlorophyll *a* fluorescence transient in dark-adapted sweet potato (*Ipomea batatas* L.) leaves cultivated with different copper concentrations: (a) Variable fluorescence between points O and K [$W_{OK} = (F_t - F_0)/(F_K - F_0)$]; (b) Kinetic difference of W_{OK}, $\Delta W_{OK} = [W_{OK(treatment)} - W_{OK(control)}]$; (c) Variable fluorescence between points O and J [$W_{OJ} = (F_t - F_0)/(F_J - F_0)$]; (d) Kinetic difference of W_{OJ}, $\Delta W_{OJ} = [W_{OJ(treatment)} - W_{OJ(control)}]$; (e) Variable fluorescence between points O and I [$W_{OI} = (F_t - F_0)/(F_I - F_0)$] in the time interval of 30 to 330 ms; (f) Variable fluorescence between points I and P [$W_{IP} = (F_t - F_I)/(F_M - F_I)$] in the time interval of 30 to 180 ms.

The K-band (approximately 300 μs) was observed when the variable fluorescence was normalised at the steps 0 (50 μs) and J (2 ms), $W_{OJ} = [F_t - F_0]/[F_J - F_0]$) (Figure 2c) and the kinetic difference was $\Delta W_{OJ} = [W_{OJ(treatment)} – W_{OJ(control)}]$ (Figure 2d). It was thus possible to identify the positive K-band in all of the concentration treatments studied. The positive K-band indicates the inactivation of the oxygen-evolving complex (OEC) and/or increased functional size of the antenna of the PSII (Yusuf *et al*., 2010).

To evaluate the effect of variation in copper concentration on the IP phase (30 to 300 ms) of the fluorescence transient, the normalisations proposed by Yusuf *et al*. (2010), which are represented in Figure 2e-2f, were used. The relative variable fluorescence curve between steps 0 (50 μs) and I (30 ms) ($W_{OI} = [F_t - F_0]/[F_I - F_0]$) when $W_{OI} \geq 1$ (on a linear scale of 30 to 300 ms) reflects the size of the final electron acceptor pool on the acceptor side of the PSI (Figure 2e) (Yusuf *et al*., 2010). The highest concentrations (0.123 and 0.164 mM) resulted in increased acceptor abundance compared to the other concentrations. However, in the fluorescence normalisation of the IP phase ($W_{IP} = [F_t - F_I]/[F_M - F_I]$), there were no differences in the overall abundance of final electron acceptors (Figure 2f).

3.2. Chlorophyll fluorescence transient: Parameters derived from the JIP Test equations

The structural and functional parameters estimated from the OJIP transients by the JIP test are represented in the form of a radar chart (Figure 3), where all of the values were normalised for those obtained from the plants cultivated with 0.041 mM copper (control).

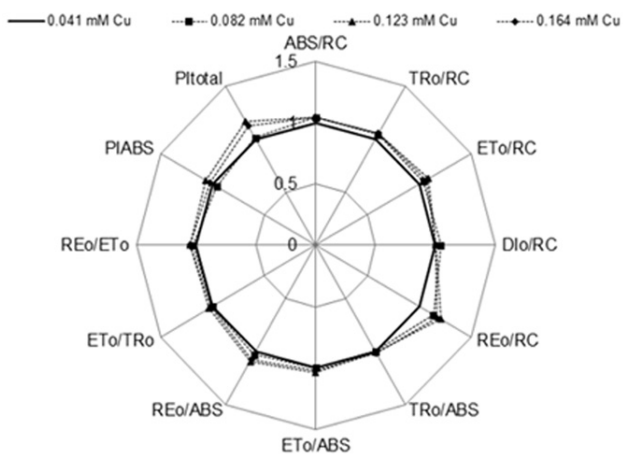

Figure 3. Chlorophyll *a* fluorescence parameters obtained using the JIP test in sweet potatoes (*Ipomea batatas* L.) cultivated with different copper concentrations: 0.041; 0.082; 0.123 and 0.164 mM.

The highest copper concentrations used (0.082; 0.123 and 0.164 mM) did not alter the energy flux absorbed (ABS/RC), captured (TR_0/RC), transported (ET_0/RC) and dissipated (DI_0/RC) by the active reaction centres of the PSII compared to the control (0.041 mM). However, there was an increase of approximately 16% in the reduction flux of the final PSI electron acceptors (RE_0/RC) per reaction centre in the plants cultivated in the higher copper concentrations (0.082; 0.123 and 0.164 mM).

The treatments with different copper concentrations did not alter the parameters that expressed the maximum quantum yield ($\varphi_{Po} = TR_0/ABS$) and the quantum yield of the electron transfer from Q_A^- to the intersystem electron acceptors ($\varphi_{Eo} = ET_0/ABS$). However, there was a slight increase in the quantum yield of the reduction of the final PSI electron acceptor per absorbed photon ($\varphi_{Ro} = RE_0/ABS$) in the plants cultivated in high copper concentrations (0.082; 0.123 and 0.164 mM), which follows the results obtained in RE_0/RC. Increased copper concentration did not affect the parameters that describe the efficiency with which an exciton captured in the reaction centre can move an electron from Q_A^- to the intersystem electron acceptor ($\psi_{Eo} = ET_0/TR_0$) and the efficiency with which an electron from the intersystem electron-carrier pool can reduce the final PSI electron acceptors or the probability of reduction of a final PSI electron acceptor ($\delta_{Ro} = RE_0/ET_0$).

The photosynthetic performance index relative to absorption (PI_{ABS}) (proposed by Strasser *et al*., 2004) was not affected by the copper concentrations. However, the total photosynthetic performance index (PI_{total}), which measures the flux of electrons to the final PSI electron acceptors (Tsimilli-Michael and Strasser, 2008), was altered by high copper concentrations. Regarding this parameter, the plants cultivated in a concentration of 0.082 mM did not differ from the control, but the PI_{total} increased 15% in the plants that had a greater copper availability (0.123 and 0.164 mM).

3.3. Modulated fluorescence

The effective photochemical quantum yield of the PSII (φ_{PSII}) and the photochemical extinction coefficient (qP) (Figure 4a-4b) decreased from 22 μmol m^{-2} s^{-1} in all of the copper treatments, and in the plants treated with 0.164 mM of copper, the values of φ_{PSII}, from this light intensity on were lower compared to the other concentrations. The parameters of the non-photochemical extinction (NPQ) (Figure 4c-4d) and the non-photochemical extinction coefficient were highly influenced by increased light intensity from 22 μmol m^{-2} s^{-1} on, and the plants cultivated in 0.082 and 0.123 mM copper had higher NPQ and qN values than the control plants. In both of the cases, the plants cultivated in the copper concentration of 0.164 mM had an intermediate response.

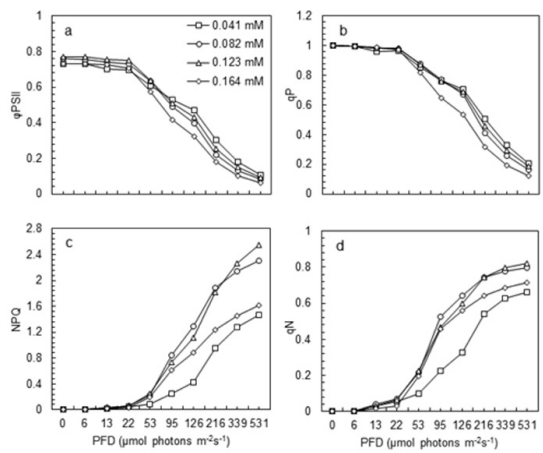

Figure 4. Light intensity response curves in sweet potatoes (*Ipomea batatas* L.) cultivated with different copper concentrations: (a) Effective photochemical quantum yield of the PSII (φ_{PSII}); (b) Photochemical extinction coefficient of variable fluorescence of photosynthesis (qP); (c) Non-photochemical extinction (NPQ); (d) Non-photochemical extinction coefficient of variable fluorescence of photosynthesis (qN). PFD = photosynthetically active photon flux density (μmol photons $m^{-2}s^{-1}$).

The copper concentrations increased the apparent electron-transfer rate in the PSII [ETR(II)] up to 53 µmol $m^{-2}s^{-1}$ light intensity. However, from this intensity on, there were different responses to the copper concentrations used (Figure 5). The control plants had the greatest ETR(II), followed by the intermediate doses (0.082 and 0.123 mM), and the lowest ETR(II) value was observed in the 0.164 mM concentration.

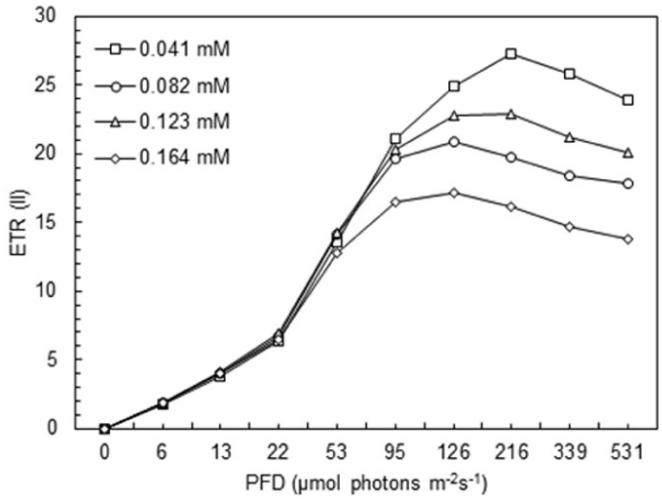

Figure 5. Variation of the electron transfer rate in the PSII, ETR (II) in sweet potatoes (*Ipomea batatas* L.) cultivated with different copper concentrations: 0.041; 0.082; 0.123 and 0.164 mM. PFD = photosynthetically active photon flux density (µmol photons m^{-2} s^{-1}).

4. Discussion

Excess copper can cause toxic effects in plants, such as inhibited growth, damage to the photosynthetic apparatus and oxidative stress (Shi-Sheng, 2007; Cambrollé *et al*., 2013). Many researchers have conducted experiments on the effects of copper on physiological and biochemical processes of plants, and many studies have focused on the photosynthetic electron transport chain in plants (Cambrollé *et al*., 2011). However, few researchers have analysed detailed photosynthetic responses to increased copper levels using chlorophyll fluorescence analysis.

The fluorescence transient of the study plants had typical OJIP polyphasic curves for all of the copper treatments, which indicates that the photosynthetic units were active even under high copper concentrations. Fluorescence decreased from step J (2 ms) to F_M (1 s) for the plants grown with the higher concentrations (0.123 and 0.163 mM) (Figure 1a). Decreased fluorescence at these levels is usually due to inhibition of electron transport on the donor side of the PSII, which is a result of the accumulation of $P680^+$ (Perales-Vela *et al.*, 2007; Oukarroum *et al*., 2012).

Analysis of the kinetic difference of the fluorescence transient curves (Figure 2b-2d) shows the appearance of the L-band and K-band. According to Strasser *et al*. (2004), the appearance of these positive bands indicates deleterious effects in plants. The positive L-bands in the sweet potato leaves cultivated under the three highest concentrations indicate that the

PSII units were less grouped, which suggests a low connectivity between the PSII antenna units (Strasser *et al*., 2004). Decreased energetic connectivity is a partial protection mechanism in that it is necessary to increase dissipation to improve utilisation of the chlorophyll excitation energy in non-photochemical processes (Redillas *et al*., 2011). Furthermore, the ability to resist via decreased connectivity could be a measure of the ability to withstand alterations in the stacking or unstacking of the thylakoid membranes (Oukarroum *et al*., 2007).

A positive K-band indicates damages in the oxygen-evolving complex (OEC) or increased functional size of the PSII antenna (Yusuf *et al*., 2010) (Figure 2d). In this study, the formation of K- and L-bands in plants cultivated in the presence of copper (0.082; 0.123 and 0.164 mM) is likely related to damages in the OEC of the PSII. This result can be explained by the accumulation of $P680^{+}$ (Figure 1a). Similar results were found for magnesium deficiency in *Citrus* species (Yang *et al*., 2012) and excess iron in sweet potatoes (Adamski *et al*., 2011), but no L- and K-bands appeared in *Alternanthera tenella* when using excess copper (Cuchiara *et al*., 2013).

Nevertheless, according to the results of chlorophyll *a* fluorescence in a light-adapted state, the increased availability of copper did not decrease the PSII yield, as indicated by the results for φ_{PSII} and qP (Figure 4a-4b). Leaves cultivated in high copper concentrations had a typical reduction in energy distribution in the PSII (φ_{PSII}) and energy dissipation in photochemical processes (qP) with increased light intensity (Figure 4a-4b), and the greatest reduction occurred at the 0.164 mM copper concentration.

Decreases in the parameters φ_{PSII} and qP are reflected in the non-photochemical extinction (NPQ) and the non-photochemical extinction coefficient (qN) because both of these factors describe dissipation of excess energy in the form of heat in the PSII antenna complexes (Rohácek and Barták, 1999). Forms of absorbed energy dissipation include i) changes in the ΔpH gradient, ii) disconnection of the mobile light-harvesting complexes, iii) transformation of violaxanthin into zeaxanthin and iv) protonation of the PsbS (integral membrane subunit of the PSII) (Rohácek and Barták, 1999; Baker, 2008). Greater values of NPQ and qN in the plants cultivated in 0.082 and 0.123 mM copper suggests greater ability to dissipate energy in the form of heat as a strategy to protect photoinhibition of the PSII compared to the control plants. The plants cultivated under 0.164 mM transferred more energy to other systems.

However, copper concentrations intensified PSI efficiency, as was observed in the chlorophyll *a* fluorescence transience data. Analysis of the IP phase (Figure 2e-2f) revealed an increased final electron-acceptor pool on the acceptor side of the PSI ($W_{OI} \geq 1$) with increased copper availability (0.123 and 0.164 mM). Higher copper concentrations positively influenced the structure and functionality of the PSI due to increased reduction flux (RE_0/RC) and yield (RE_0/ABS) of the final PSI electron acceptors (Figure 3). Furthermore, the photosynthetic performance index also increased, related as $PI_{total} = PI_{ABS} \cdot \delta_{Ro}/(1 - \delta_{Ro})$, which incorporates the maximum performance for electron transfer from water to plastoquinone (PQ) and plastocyanin (PC) by chlorophyll (PI_{ABS}) and the performance of reduction of the final PSI acceptor (Strasser *et al*., 2010).

The joint analysis of the chlorophyll *a* fluorescence in dark- and light-adapted states indicates that the copper concentrations had little effect on PSII structure and functionality because there were no marked alterations in the JIP test parameters. Under these conditions, there was only a reduction in the apparent electron-transfer rate in the PSII [ETR(II)] with increased light intensity (Figure 5). In addition, the plants treated with 0.082 and 0.123 mM copper were able to dissipate

the excess light energy in the PSII and protect its units through NPQ and qN (Munekage and Shikanai, 2005). NPN and qN activation depends on the generation of a proton gradient through the thylakoid membrane (ΔpH), which is induced by the linear and cyclic electron flow (Huang *et al*., 2012). Alternative electron transport pathways, cyclic electron transport in the PSI and the water-water cycle can regulate the induction of heat dissipation, which modifies the rate of ΔpH generation (Munekage and Shikanai, 2005). However, PSI efficiency was intensified in the plants that grew in concentrations of 0.123 and 0.164 mM copper. These results suggest that there may have been an increase in cyclic electron transport in the PSI. In this case, electrons returned from ferredoxin to plastoquinone, with ATP production and without NADPH accumulation (Munekage and Shikanai, 2005). Similar results were found in studies of water deficit (Huang *et al*., 2012), which suggests that plants cultivated in copper concentration of 0.123 mM simulated drought-related situations and developed strategies to avoid photoinhibition of the photosynthetic apparatus caused by light energy absorption.

5. Conclusions

High copper concentrations in the nutrient solution alter transient and modulated chlorophyll *a* fluorescence, reduce photochemical activity, increase the light energy dissipation of the PSII and increase PSI efficiency in sweet potato plants.

Acknowledgements

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