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Hormone-induced protection of sunflower photosynthetic apparatus against copper toxicity

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Abstract

The effects of excess Cu as affected by the application of exogenous hormones (gibberellic acid - GA_3 and indole-3-acetic acid - IAA) with respect to sunflower (*Helianthus annuus* L.) growth, physiology, and metabolism were studied. Application of 100 µM IAA lessened the toxic effects of 80 µM Cu in roots indicating greater root length and root hair formation, while addition of 100 µM GA_3 ameliorated the toxic effect mainly to the shoot. The content of photosynthetic pigments significantly declined under Cu stress, whereas application of hormones led to a substantial preservation of chlorophylls and carotenoids. Under Cu stress, the rate constant of energy trapping by photosystem 2 (PS2) reaction centres (RCs) was reduced as a result of physical dissociation of the light-harvesting complex (LHC) from PS2 core, while application of IAA and especially GA_3 resulted in stability of the LHC of PS2 RCs. The drop in net photosynthetic (P_N) and transpiration (E) rates with preserved or slightly reduced variable to maximum fluorescence ratio (F_v/F_m) in the presence of 80 µM Cu could be explained by a possible inhibition of the enzymatic processes in the Calvin cycle. Application of 100 µM IAA and 100 µM GA_3 lessened Cu effects mainly on P_N. Water use efficiency was also improved under hormone exposure.

Additional key words: auxin, carotenoids, chlorophylls, CO_2 assimilation, gibberellin, *Helianthus annuus* L., photosynthetic quantum yield, photosystem 2, transpiration.

Introduction

Among pollutants of agricultural soils, Cu has become increasingly hazardous due to its involvement in fungicides, fertilizers, pesticides, *etc*. In spite of its physiological importance, an increase in Cu contents threatens plant health because it interacts with several enzymes, and disturbs membrane permeability and electron transport in chloroplasts (Van Assche and Clijsters 1990, Ouzounidou *et al.* 1997). Its toxicity to plants is well documented (Baker 1990, Moustakas *et al.* 1997, Ouzounidou *et al.* 1998).

A large number of metabolic changes occur during plant growth, differentiation, and development. Various plant regulators such as cytokinins, gibberellins, auxins, abscisic acid, and ethylene affect these processes (Davies 1995). In general, cytokinins and in some systems gibberellins or auxins delay the loss of chlorophyll (Chl), whereas ethylene and abscisic acid enhance their loss (Jordi *et al.* 1995). Growth regulators influence mineral uptake as well. However, little information is available on the effects of additional gibberellic acid (GA_3) and indole-3-acetic acid (IAA) on plants grown in excess of toxic metals in the soil (Tromp 1989, Panou-Philotheou *et al.* 2002). In parallel, many environmental factors can influence the optimum conditions for plant growth and cell metabolism.

Chl fluorescence is often used to study several aspects of photosynthesis because it reflects changes in the thylakoid membrane organisation and functioning. At the same time, Chl fluorescence shows complex phenomena so that the interpretation of these data is not easy (Ouzounidou *et al.* 1997). Variations in leaf photosynthetic (P_N) and transpiration (E) rates in response to

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Abbreviations: Car - carotenoids; Chl - chlorophyll; E - transpiration rate; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; LHC - light-harvest complex; P_N - net photosynthetic rate; PS 2 - photosystem 2; RC - reaction centre; WUE - water use efficiency.

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changes in environmental conditions such as temperature, irradiance, relative humidity, and concentration of toxic metals are commonly observed in different plant species and genotypes under natural conditions. Variation also exists under greenhouse conditions but to a lesser extent (Gimenez *et al.* 1992, Issa *et al.* 2001). The Chl fluorescence and gas exchange measurements compare well with other methods of quality assessment (Pospíšilová *et al.* 1998, Ouzounidou and Constantinidou 1999).

We investigated the effects of two chemically pure plant growth regulators (GA_3 and IAA) on the loss of photosynthetic pigments and plant senescence of sunflower caused by toxic concentration of Cu. In addition, we studied the ameliorative role of additional hormones to photosynthetic function of sunflower as a result of Cu-stress by analyzing Chl fluorescence parameters and CO_2 assimilation.

Materials and methods

Plants and treatments: Ten-day-old seedlings of sunflower (*Helianthus annuus* L. cv. Aida) were randomly mounted on nylon-mesh floats (each one supporting 10 plants per pot) in aerated Hoagland nutrient solution, containing 1.6 mM N, 0.6 mM K, 0.4 mM Ca, 0.2 mM P, 0.1 mM S, 0.1 mM Mg, 50 μM Cl, 25 μM B, 20 μM Fe, 2 μM Zn, 2 μM Mn, 0.5 μM Cu and 0.5 μM Mo (Ouzounidou *et al.* 1998). The Hoagland solution contained 80 μM Cu ($CuSO_4 . 5 H_2O$) or 80 μM Cu plus 100 μM IAA or 80 μM Cu plus 100 μM GA_3. GA_3 and IAA were obtained from *Sigma*, the purity of both hormones was higher than 95 %.

The four nutrient solutions (control, Cu+IAA, Cu+GA_3, and Cu) were adjusted to pH 5.5 ± 0.2, with KOH. Plants were subjected to Cu stress for 8 d in a phytotron programmed for 6-h photoperiod with photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} at plant level, at a temperature of 22 °C and relative humidity of 70 %. At the end of the treatment the plants were harvested and root and shoot elongation were determined. The same leaves were used for Chl fluorescence measurements, gas exchange, and pigments determination.

Chlorophylls *a* and *b* and total carotenoids of the fully expanded leaf were measured in the same 100 % acetone extract by spectrophotometer (*LKB Ultraspec II*) according to Lichtenthaler (1987).

The fast Chl fluorescence induction kinetics were measured on pre-darkened leaves (30 min) at room temperature in order to relax all energy-dependent fluorescence quenching coefficients. A Plant Efficiency Analyzer (*PEA*, *Hansatech*, King's Lynn, UK) monitored them with 600 W m^{-2} of red (630 nm) radiation on the upper surface of the leaf (for detail see Ouzounidou *et al.* 2003). From each fluorescence transient of the type OJIP, different values were selected in order to determine any structural and functional changes of the photosynthetic apparatus under Cu, Cu+IAA, or Cu+GA_3 treatments. Following parameters were measured: F_0 - initial fluorescence intensity when all RCs are open, F_m - maximum fluorescence when all RCs are closed, F_v - variable fluorescence ($F_v = F_m - F_0$), t_{max} - time to reach the maximum fluorescence, Area - summation of all values ($F_m - F_t$) for t = 0 to t = t_{max}, k_N (= $1/F_m$) - expression proportional to the sum of all rate constants of non-photochemical deexcitation events, k_P ($=1/F_0 - 1/F_m$) - expression proportional to the rate constant of primary photochemistry (Moustakas *et al.* 1998).

Gas-exchange measurements were made on the fully expanded leaf with a portable photosynthetic apparatus *Li-6200* (*LiCor*, Lincoln, USA). The analyzer was calibrated with two standard CO_2/air mixtures. Measurements were made at 25 °C under a photon flux density of about 750 μmol m^{-2} s^{-1} (for details see Ouzounidou *et al.* 1998). P_N, and E were calculated from gas exchange measurements according to Caemmerer and Farquhar (1981). Water use efficiency (WUE) is given as P_N/E.

Results

Plant growth: Root elongation was more sensitive to Cu exposure than shoot elongation. On exposure to 80 μM Cu, root length was significantly inhibited by 81 % of the control, while the respective shoot inhibition was only 44 % of the control ($P < 0.05$). Application of 100 μM IAA induced less growth loss (64 and 28 % for root and shoot length, respectively (Fig. 1*A*, $P < 0.05$). On the contrary, supply of 100 μM GA_3 induced less Cu toxicity amelioration of root elongation compared with the 80 μM Cu exposure, whereas shoot elongation decreased only by 16 % of the control (Fig. 1*A*). The addition of metal and hormones was associated with distinct morphological changes: 80 μM Cu caused blackening of root and no root hair formation, 80 μM Cu + 100 μM IAA induced root hair formation, and 80 μM Cu + 100 μM GA_3 limited root hair formation.

Pigments contents: The highest pigment contents were recorded in control leaves with absolute values 1.050 and 0.830 mg kg^{-1} (FM) for Chls and Cars, respectively. Cars of sunflower leaves were more tolerant to Cu stress than Chl (*a*+*b*), showing a decrease of 30 and 38 % of the control, respectively ($P < 0.05$, Fig. 1*B*). Addition of hormones caused a significant preservation of the pigments. On exposure to Cu+GA_3 Chl content declined by 13 % ($P > 0.05$), while the Car content by 22 % ($P<0.05$) of the control. The reduction in Car/Chl ratio was maximum under Cu stress, by 26 % ($P < 0.05$) of the control, and minimum under Cu+GA_3 treatment by 18 % ($P > 0.05$, Fig. 1*B*).

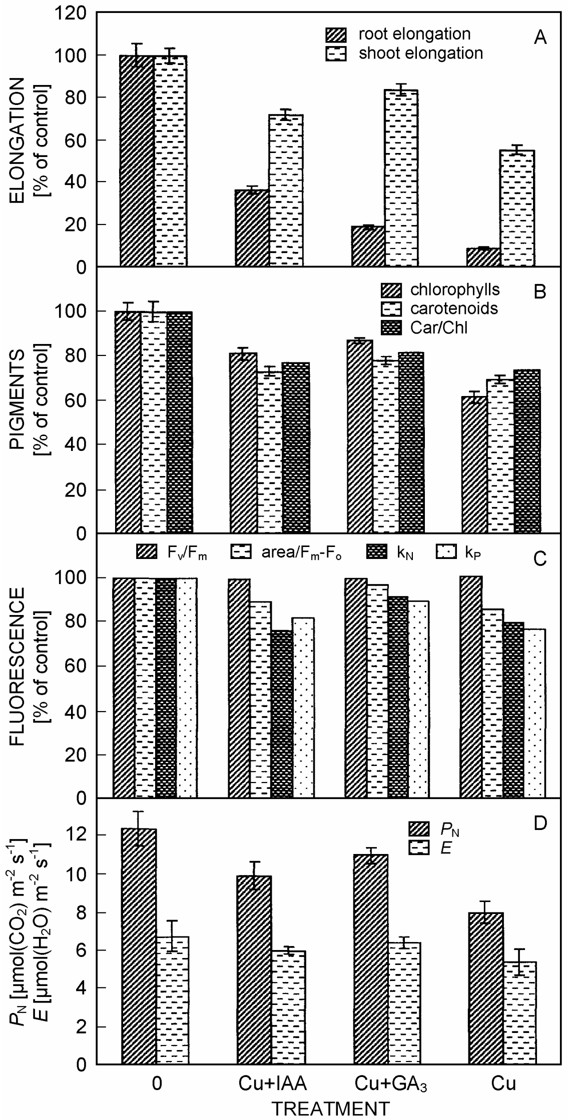

Fig. 1. Changes of root and shoot elongation (*A*; $n = 15$), photosynthetic pigment contents (*B*; $n = 5$), some chlorophyll fluorescence parameters (*C*; $n = 5$), and net photosynthetic rate, P_N and transpiration rate, E (*D*; $n = 5$) of *Helianthus annuus* L. cv. Aida fully expanded leaves, as a function of 8 d exposure to 80 µM Cu, 80 µM Cu+100 µM IAA and 80 µM Cu +100 µM GA_3. *Vertical bars* represent the standard errors.

Chl fluorescence: F_0, F_m and F_v as well as the time to reach the maximal fluorescence (t_{max}) were significantly higher under Cu and Cu+IAA treatments compared to the control ($P < 0.05$, Table 1). A slight increase of the above parameters under Cu+GA_3 was also detected.

Table 1. Changes in chlorophyll fluorescence indices of sunflower fully expanded leaves as determined from fluorescence induction curves under control, Cu, and Cu + hormone treatments. Means ± SE of five separate measurements (* - differences from control significant at $P < 0.05$).

Parameters	Control	Cu+IAA	Cu+GA_3	Cu
F_0	0.387±0.06	0.479±0.08*	0.429±0.03	0.495±0.05*
F_m	2.615±0.08	3.437±0.10*	2.857±0.12	3.242±0.09*
F_v	2.228	2.958*	2.428	2.747*
t_{max}	0.054	0.081*	0.073*	0.082*

After the 30-min dark adaptation, control leaves showed typical Chl fluorescence induction curves, variable fluorescence accounting for 85 % of the total fluorescence ($F_v/F_m = 0.85$). These values indicate a good photosystem 2 (PS2) photochemical efficiency. The F_v/F_m was not influenced by the exposure to Cu stress and hormone treatments (Fig. 1*C*). This parameter is considered a reliable indicator of PS 2 photochemical trapping efficiency in the dark adapted state (Gonzalez-Moro *et al.* 1997). However, Ouzounidou *et al.* (2003) reported that F_v/F_0 is a much better indicator of change in the rates of photosynthetic quantum conversion than F_v/F_m. F_v/F_0 was more sensitive to Cu stress showing a slight decline by 4 % of the control. F_m/F_0 reflecting the structural integrity of the reaction centres (RCs) of PS2, had also a similar pattern as the above parameters under the treatments.

Area/(F_m - F_0) ratio, related with the total number of electrons that have gone through the RCs of PS 2, diminished considerably becoming 86 % of the control value under 80 µM Cu. Application of 100 µM IAA and 100 µM GA_3 resulted in lower diminution of this ratio (Fig. 1*C*).

The rate constant corresponding to the photochemical and non-photochemical events k_P and k_N decreased significantly by 23 and 20 % of the control under Cu stress, respectively ($P < 0.05$, Fig. 1*C*). Addition of exogenous IAA and GA_3 resulted to a lesser suppression of both indices (Fig. 1*C*).

Leaf gas-exchange responses to treatments: Upon Cu exposure P_N and E significantly decreased over eight days, by 35 and 20 % of the control, respectively

(P < 0.05, Fig. 1*D*). Plants treated with additional IAA and GA_3 performed higher P_N as compared to Cu stress; these plants, however, displayed a reduction of 20 and 12 % (P < 0.05) of the control, respectively. A slight and not significant decline in E was detected under the two hormone treatments (11 and 5 % of the control, respectively, Fig. 1*D*). WUE of Cu stressed leaves was suppressed considerably by 19 % of the control, whereas the supply of exogenous IAA and GA_3 resulted in preservation of this index.

Discussion

Growth performance of the plants under excess Cu was seriously affected: mainly root elongation ceased within the first days. Shoot elongation of sunflower was more tolerant to Cu stress, because plant shoot accumulates less Cu than the roots (Baker 1990, Ouzounidou *et al.* 1998, Wojcik and Tukiendorf 2003). Application of IAA lessened toxic effects in roots indicating greater root length and root hair formation, while addition of GA_3 ameliorated the toxic effect of Cu mainly to the shoot. Our results are in accordance with the findings of Davies (1995) that auxin stimulates root initiation on stem cuttings and branch root development. It also stimulates shoot growth. Application of GA_3 resulted in the increase of height in Cu-treated oregano plants (Panou-Philitheou *et al.* 2002) while it enhanced stem growth by stimulating both cell division and cell elongation in dwarf peas (Reid 1988, Daykin *et al.* 1997). Moreover, according to Bosabalidis and Exarchou (1995) and Panou-Philitheou *et al.* (2002) in Cu-cultivated plants an excess of endogenous GA_3 and NAA was found. Thus, the reduced height of those plants compared to the controls was not due to the lack of hormones, but to the toxicity that Cu induces to certain physiological processes (Ouzounidou 1996).

The content of photosynthetic pigments significantly declined under Cu stress, whereas application of hormones led to a substantial preservation of Chl and Car. IAA and even more GA_3 delayed Chl loss more than that of Car. The involvement of IAA in Chl preservation was reported by Dai and Paull (1991) and Van Doorn *et al.* (1992). In addition, according to Jordi *et al.* (1995) *Alstroemeria* growers use GA_3 in a post-harvest treatment to prevent leaf yellowing. However, GA_3 shows a concentration depent effect on the retention of Chl in the concentration range 10^{-8} to 10^{-5} M. In general, GA_3 caused retention of Chl and delay of leaf senescence, but it is unclear whether GA_3 acted indirectly on Chl loss by affecting the endogenous cytokinin concentration in the leaves (Jordi *et al.* 1994).

The observed reduction in contents of the photosynthetic pigments induced by excess Cu is considered to be an indirect effect of Cu on PS 2 activity (Ouzounidou *et al.* 1998). Indeed, Cu-induced acceleration of the degradation rate of cell components, such as pigments, indicates decreased leaf density and simultaneous inhibition of the restoration of products of the less efficient photosynthetic apparatus (Maksymiec and Baszynski 1996). Actually, by the OJIP-test we detected the vitality of sunflower plants under Cu-stress and addition of exogenous hormones. We interpret the significant increase of F_0 under Cu stress as a reduction of the rate constant of energy trapping by PS 2 RCs and this could be the result of a physical dissociation of LHC from PS 2 core, as it has been observed under several stresses (Armond *et al.* 1980, Havaux 1993, Ouzounidou and Constantinidou 1999, Ouzounidou *et al.* 2003). The lower increase of the initial fluorescence yield observed under Cu+GA_3 reflects the most ameliorative role of GA_3 at the membrane level compared to IAA. In addition, Cu-stressed plants had a significant by higher F_m value which correlated well with the increased t_{max}, whereas application of GA_3 protected the excitation energy transfer between LHC and PS 2 RCs.

Because there is no apparent change in F_v/F_m under Cu exposure, the slower t_{max} might be due to a decrease in LHC size of the PS 2 RCs (Ouzounidou and Constantinidou 1999). Compared to the controls, the functional Chl antenna size of the photosynthetic apparatus was smaller in Cu-treated sunflower plants, as indicated by the increased t_{max}. Application of exogenous GA_3 resulted in a substantial stability of the LHC of PS 2 RCs in sunflower plants. Copper ions seem to affect t_{max} in a different way depending on the supplied concentration and plant species, since in spinach plants a reduction of this index was observed (Ouzounidou *et al.* 1998). In parallel, on exposure to Cu, the total number of electrons through the RCs declined which is judged from decreased Area/(F_m - F_0) ratio, while application of hormones lessened this disturbance. Thus, the tested growth regulators and especially GA_3 might trigger some protective mechanisms on photosynthetic apparatus, resulting to the stability of PS 2.

The drop in P_N and E with preserved or slightly reduced quantum yield of photochemistry in the presence of toxic amounts of Cu could be explained by a possible inhibition of the enzymatic processes in the Calvin cycle of sunflower plants. Several heavy metals have been suspected of inducing inhibition of photosynthesis through reduced carbon metabolism (Van Assche and Clijsters 1990, Maksymiec *et al.* 1994, Baron *et al.* 1995, Ouzounidou and Constantinidou 1999). In addition, the impact of Cu on sunflower leaf WUE indicates the reduced control over water loss (Lawlor and Mitchell 1991, Moustakas *et al.* 1997). Mineral elements can affect E by their effect on leaf development, stomatal opening, and energy metabolism. Application of IAA and GA_3 lessened Cu effects, mainly in P_N of sunflower; WUE was also improved under hormone exposure.

Applied IAA increased opening of adaxial stomata, but there was very little effect on abaxial stomata under favourable conditions (Pemadasa 1982, Davies 1995). However, when opening of the abaxial stomata is restricted by a reduced supply of K ions in the medium, IAA causes enhanced opening. Restriction of aperture can happen under Cu stress, since the excess of Cu reduces significantly the uptake of K ions (Ouzounidou *et al.* 1998). Moreover, IAA enhances photosynthetic CO_2 uptake, probably by increasing the coupling between electron transport and phosphorylation (Tamas *et al.* 1974).

GA_3 should also be considered as possible promoter of photosynthesis (Arteca and Dong 1981). Generally, there are experimental data indicating that hormones may act at a number of steps involved in the assimilation and transport of photoassimilates to developing organs. Plant hormones might also function by enhancing sink potential *via* increasing cell number and/or regulating cell differentiation such as plastid biogenesis and DNA amplification, or by modifying the duration or rate of dry mass accumulation of a developing reproductive organ (Davies 1995). In conclusion, our results suggest that GA_3 and IAA may alleviate metabolic and physiological disturbances of Cu-stressed sunflower plants.

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