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Polyamine metabolism in sunflower plants under long-term cadmium or copper stress

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Summary. The effect of different doses of cadmium and copper was studied in relation to growth and polyamine (Pas) metabolism in shoots of sunflower plants. Cadmium accumulated to higher levels than copper and shoot length was reduced by 0.5 and 1 mM Cd, but only by 1 mM Cu. At 1 mM of Cd or Cu, Put content increased 270% and 160% with Cd^{2+} and Cu^{2+}, respectively. Spermidine (Spd) was modified only by 1 mM Cd, while spermine (Spm) declined after seeds germinated, increasing thereafter but only with 1 mM Cd or Cu (273% over the controls for Cd and 230% for Cu at day 16). Both ADC and ODC activities were increased by 1 mM Cd, whereas 1 mM Cu enhanced ADC activity, but reduced ODC activity at every concentration used. The role of Pas as markers of Cd or Cu toxicity is discussed.

Keywords: Polyamines – Cadmium – Copper – Sunflower

Abbreviations: ADC, arginine decarboxylase; DAO, diamine oxidase; ODC, ornithine decarboxylase; Pas, polyamines; Pro, proline; Put, putrescine; Spd, spermidine; Spm, spermine

Introduction

The naturally occurring polyamines spermidine (Spd) and spermine (Spm), and the diamine putrescine (Put) are basic, aliphatic nitrogenous molecules, widely distributed in plants, animals and microorganisms, implicated in a wide range of regulatory processes such as promotion of growth, cell division, DNA replication and cell differentiation (Slocum et al., 1984; Evans and Malmberg, 1989; Tiburcio et al., 1997; Bouchereau et al., 1999). Polyamine involvement in defense mechanisms during biotic and abiotic stresses (such as mineral nutrient deficiency, osmotic, salt, heat, chilling, heavy metals, oxidative stress, and pathogen infection) has also been demonstrated (Bouchereau et al., 1999; Mackintosh et al., 2001; Perez-Amador et al., 2002; Groppa et al., 2001, 2003; Walters, 2003).

In plants, Put is synthesized from arginine or ornithine by arginine decarboxylase (ADC, EC 4.1.1.19) and ornithine decarboxylase (ODC, EC 4.1.1.17), respectively. The following addition of two aminopropyl groups to Put (catalyzed by Spd and Spm synthases) leads to the synthesis of Spd and Spm. Polyamines content is also regulated by their degradation through the action of amine oxidases, which include the copper-containing amine oxidases (CuAO; EC 1.4.3.6), oxidizing the diamines Put and cadaverine at the primary amino groups, and the flavin-containing polyamine oxidases (PAO, EC 1.5.3.3), which oxidize Spd and Spm at their secondary amino groups (Federico and Angelini, 1991). The enzymes involved in polyamine catabolism and the products deriving from their action contribute to important physiological processes (Martin-Tanguy, 1997; Sebela et al., 2001). The production of H_2O_2 through polyamine oxidation has been correlated with the oxidative burst, cell death, lignification, and suberization processes occurring during development and defense responses (Allan and Fluhr, 1997; Møller and McPherson, 1998; Rea et al., 1998, 2002; Cona et al., 2003; Walters, 2003).

In the last years, heavy metal pollution has become a matter of study due to the increase of industrial and urban activities that contribute to soil contamination. Cadmium is not essential for plant growth and it is phytotoxic to living cells, even at very low concentrations. This metal is naturally present in soils but its content has dramatically increased due to several human activities that release it into the environment (Sanità di Toppi and Gabrielli, 1999). It is recognized as an extremely significant pollutant due to its high toxicity and large solubility in water (Pinto et al., 2004). Since Cd is fairly immobile, its accumulation in soils can become strongly phytotoxic and it can accumulate in

diverse plant tissues, mainly in roots (Vitória et al., 2001). In plants, cadmium produces chlorosis, oxidative damage (Stroinski and Kozlowska, 1997; Fornazier et al., 2002), growth inhibition and even plant death (Somashekaraiah et al., 1992; Boussama et al., 1999). This metal produces changes in membrane lipids (Ouariti et al., 1997) and affects enzyme activities (Van Assche and Clijsters, 1990). Copper is widely distributed in nature. It is an essential element for normal plant growth since it is constituent of many enzymes and proteins involved in different metabolic processes. However, it is toxic for plants at high concentrations, since it can interfere with numerous physiological processes (Fernandes and Henriques, 1991). Due to the fact that copper is widely used in industrial and agronomic activities, copper soil contamination has become an important environmental problem. Copper toxicity is mediated by the formation of free radicals (Luna et al., 1994) and by the catalysis of the Haber-Weiss reaction (Halliwell and Guteridge, 1984). These radicals are highly toxic and can oxidize biological macromolecules such as lipids, thus disturbing membrane permeability (Weckx and Clijsters, 1996).

The aim of this work was to evaluate polyamine metabolism in sunflower plants grown under cadmium or copper stress during 16 days for further establishing a relationship between metals toxicity and the Pas pattern of accumulation.

Materials and methods

Chemicals

Put, Spm, Spd, 1,6 hexanediamine were from Sigma Chemical Company (Saint Louis, Mo). L-[1-^{14}C]-arginine (specific activity 320 mCi/mmol), L-[1-^{14}C]-ornithine (specific activity 50–60 mCi/mmol), 1,4-[^{14}C]-putrescine (specific activity 110 mCi/mmol), and protosol were from New England Nuclear. All other chemicals were of analytical grade.

Plant material and treatments

Sunflower (*Helianthus annuus* L.) seeds were surface sterilized with NaClO (8% active Cl_2) at 50% for 10 min and then thoroughly rinsed with distilled water. They were germinated in plastic pots filled with vermiculite and irrigated during 16 days with the following solutions:

- Control plants, with Hoagland (Hoagland and Arnon, 1957) solution.
- Cd-treated plants, with Hoagland solution containing 0.1, 0.5 or 1 mM $CdCl_2$.
- Cu-treated plants, with Hoagland solution containing 0.1, 0.5 or 1 mM $CuCl_2$.

Plants were grown with a 16/8 h day/night photoperiod at 26/20 °C, under fluorescent white light (a photon flux density of 175 µmol m^{-2} s^{-1}), in a controlled environmental growth chamber. For all determinations plants were harvested at 0, 3, 7, 10 and 16 days of growth and cotyledons and/or leaves (shoots) were used for determinations.

Metal content

Shoots and roots of sunflower plants were thoroughly rinsed to eliminate the metal that could be superficially adsorbed. Plant material was dried at 80 °C during 48 h, weighed and ground to a fine powder. Cadmium and copper determinations were made on HNO_3:$HClO_4$ (3:1 v/v) digests by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300).

Growth analysis

Shoot length and relative water content (RWC)

A sample of 15 to 20 plants per treatment was used to evaluate shoot length, at the times described previously. Relative water content (RWC), expressed as a percentage, was determined in sunflower plants according to the formula:

$$RWC(\%) = (FW - DW)^{*}100/DW$$

where FW and DW means fresh weight and dry weight, respectively. FW was measured just after harvesting the plants and DW was measured after drying the plants at 80 °C for 48 h.

Chlorophyll content

Chlorophyll was extracted by boiling 300 mg fresh weight of leaves in 5 ml of 96% ethanol. The chlorophyll content was measured spectrophotometrically at 654 nm, as described by Wintermans and De Mots (1965).

Proline determination

Proline was determined using ninhydrin according to Bates et al. (1973). Extracts were made by homogenizing 300 mg of plant material with 3% (p/v) 5-sulphosalicylic acid and centrifuged at 5000 *g*. A sample of 500 µl of the supernatant was used for the assay.

Analysis of polyamines

Plant tissues (300 mg FW) were homogenized with 5% (v/v) perchloric acid, kept 30 min on ice and centrifuged at 3000 *g* for 10 min. The supernatants were derivatized using the dansylation method described by Smith and Meeuse (1966) and 1,6-hexanediamine was used as an internal standard. Standards of Put, Spd and Spm were dansylated simultaneously. The dansylated derivatives were extracted with 1 ml ethylacetate. Polyamines were separated and identified by TLC, performed on high resolution silica gel plates (JT Baker, silica gel plates IB 2-F) using n-hexane:ethyl acetate (1:1) solvent system. Dansylated polyamines were identified by comparing the Rf values of dansylated standards. Silica plates were observed under UV light and bands corresponding to the polyamines in the samples and standards were scraped off the plates and eluted with 1 ml ethylacetate. Their fluorescence was measured at 365 nm excitation and 510 nm emission, in an spectrofluorometer (Aminco Bowman).

Determination of ADC, ODC and DAO activities

All assays were performed on fresh extracts according to the method described by Flores and Galston (1984). Samples (300 mg FW) were homogenized in a chilled mortar with 2 ml of 50 mM phosphate buffer (pH 7.8) containing 0.5 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF and 1 mM pyridoxal phosphate. They were centrifuged at 25,000 *g* for 20 min and the supernatants were immediately used for enzyme assays. The incubation mixture for arginine decarboxylase consisted of 100 µl of the crude extract, 70 µl of the extraction buffer, 10 mM pyridoxal phosphate, 25 mM ditiothreitol, and the substrate [1-^{14}C] arginine (320 mCi/mmol, New England Nuclear) diluted with cold arginine to give a final concentration of 20 mM, in a final reaction volume of 200 µl. Ornithine decarboxylase was assayed in a similar way using [1-^{14}C] ornithine (54.3 mCi/mmol, New England Nuclear), diluted with cold ornithine to give a final concentration

of 20 mM. Reaction mixtures were incubated for 60 min at 37 °C under continuous shaking. The reaction was stopped adding 100 µl of TCA 20% (w/v) and the incubation continued for 45 min. For blanks, TCA was added at zero time. The $^{14}CO_2$ released in the reaction was trapped in Whatmann filter papers moistened with Protosol (New England Nuclear) and placed above the reaction mixture in glass tubes similar to the plastic wells of Kontes. When the reaction was finished, the filter papers were put in a scintillation solution in glass vials and the radioactivity was measured in a Beckmann LS 1801 scintillator counter. Specific activities of the enzymes were expressed as nmol $^{14}CO_2\,g^{-1}$ FW h^{-1}. Diamino oxidase (DAO) activity was assayed using 200 µl of crude extract, 50 µl of 100 mM Tris–HCl buffer, and 40 µl of [1-^{14}C] Put (110 mCi/mmol), New England Nuclear) diluted with cold putrescine to a final concentration of 4 mM, in a final reaction volume of 290 µl. The reaction mixture was incubated for 60 min at 37 °C under continuous shaking. The ^{14}C-labelled pyrroline produced was extracted with 3 ml toluene from the reaction mixture after addition of 200 µl of $NaCO_3$ to bring the pH to 8.0. For blanks, TCA was added at zero time. One milliliter of toluene extract was placed in a scintillation solution in glass vials and the radioactivity was measured in a Beckmann LS 1801 scintillator counter (Martin-Tanguy, 1997).

Protein determination

Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

Statistics

Values in the text and tables indicate mean values ± S.E. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Cadmium and copper content of shoots and roots

In order to evaluate the proportion of the metals that were translocated from the roots to the shoots, Cd and Cu were measured in both parts of the plants. Both metals entered the roots and shoots of sunflower plants increasingly along the 16 days of treatment, according to the age of the plant and the metal used, and proportionally to the metal concentration in the growth solution (Table 1).The concentration in roots was about 18 to 12 times higher than that in shoots for 1 mM Cd and Cu respectively, with a huge translocation of both metals to the shoots. Cadmium accumulated to higher levels than copper after the 7th day of exposure (Table 1). However, at the highest concentration used (1 mM), Cd reached the maximum level in the plant shoots (451.5 mg kg^{-1} DW) on the tenth day of treatment, while copper continued increasing in root and shoot tissues until 16th day, at the three concentrations used.

Shoot length

When sunflower seeds were germinated and grown for 16 days with cadmium and copper, an evident inhibition of growth was observed as the metals increased in the nutrient solution. However, no difference in the radicle emergency was observed among treatments, all cadmium and copper concentrations used rendered germinating seeds and all plants survived the exposure time. Despite both metals negatively affected shoot length (Fig. 1A and B) in a dose-dependent manner, the magnitude of the growth reduction was different according to the metal. While shoot length was reduced by 0.5 and 1 mM Cd (80% and 90% respect to the controls, respectively), copper reduced shoot length only at the highest concentration used (Fig. 1A and B). Although 0.1 mM cadmium or copper did not alter shoot growth (Fig. 1A and B), plants treated

Table 1. Cadmium and copper content in shoots and roots of sunflower plants along 16 d of treatment. Metal concentration is expressed as mg kg^{-1} DW. Samples were extracted as described in Materials and methods

	Days of treatment	Metal accumulation							
		mg Cd kg^{-1} DW				mg Cu kg^{-1} DW			
		C	Cd 0.1 mM	Cd 0.5 mM	Cd 1 mM	C	Cu 0.1 mM	Cu 0.5 mM	Cu 1 mM
Shoot	0	3.5	3.5	3.5	3.5	15.6	15.6	15.6	15.6
	3	3.0	10.2	33.4	56.5	28.9	30.2	32.5	35.6
	7	3.0	68.2	142.3	232.1	31.6	35.4	76.5	126.2
	10	2.9	167.9	286.9	451.5	29.3	42.1	93.4	238.3
	16	3.9	259.1	312.2	414.5	30.3	52.2	135.3	324.5
Root	0	3.5	3.5	3.5	3.5	15.6	15.6	15.6	15.6
	3	3.0	120.7	386.9	853.0	16.2	30.2	102.3	456.6
	7	7.3	254.7	898.3	1999.1	21.2	64.3	374.0	896.3
	10	9.8	471.4	2153.5	4108.9	19.9	115.4	845.5	1634.7
	16	12.4	891.0	3568.1	7723.0	19.1	322.2	1235.3	3964.4

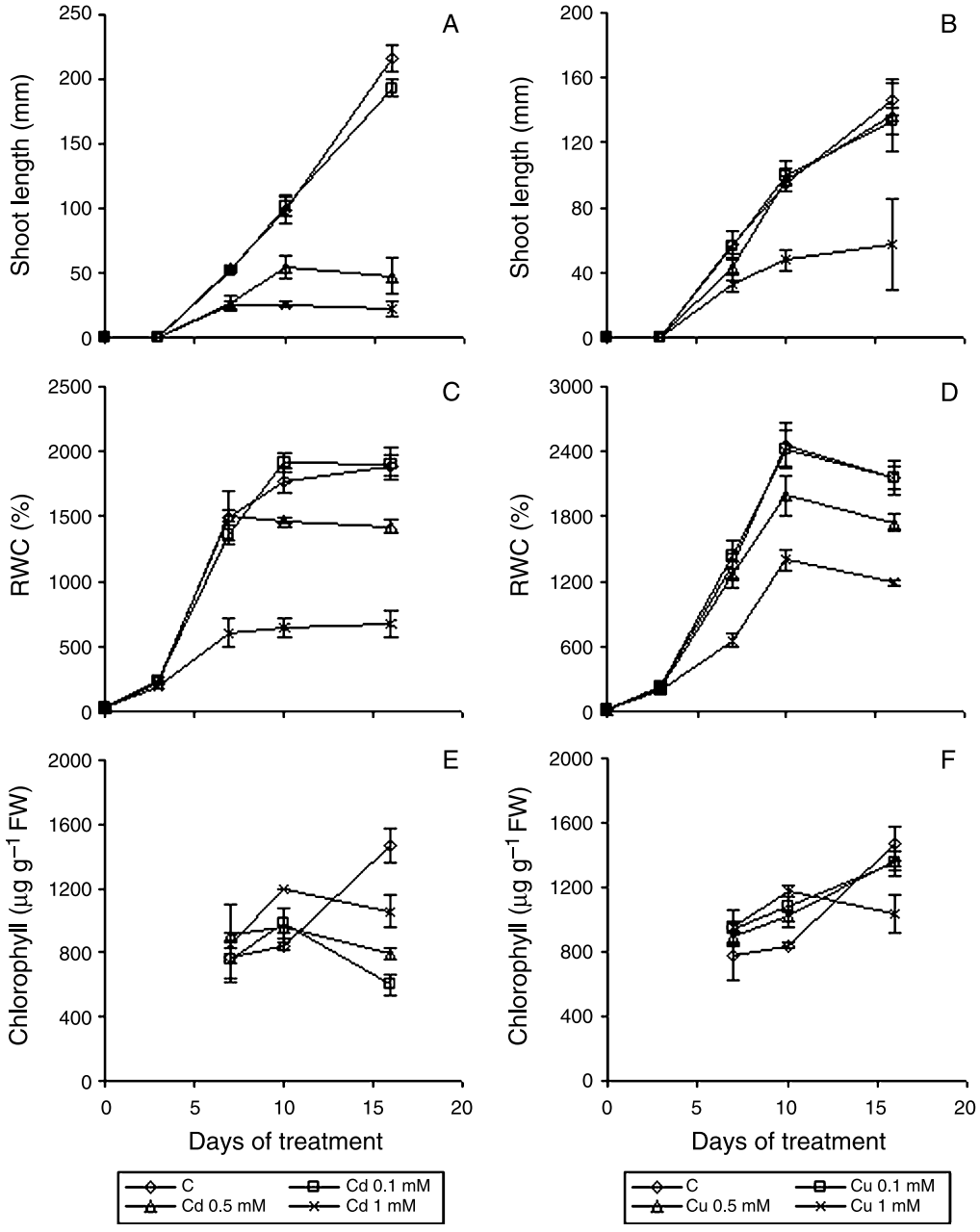

Fig. 1. Shoot length variation (**A**, **B**), RWC (**C**, **D**) and chlorophyll content (**E**, **F**) measured in sunflower plants treated with 0.1, 0.5 or 1 mM of $CdCl_2$ (left panel) or 0.1, 0.5 or 1 mM of $CuCl_2$ (right panel). Plants were treated for 16 days in a control acclimated room at $24 \pm 2\,^{\circ}C$ and samples were taken at 0, 3, 7, 10 and 16 d. Values are the means ± S.E. of three different experiments with three replicated measurements for chlorophyll determination. For shoot length and RWC determination, 20 plants were measured each time

with 0.1 mM $CdCl_2$ exhibited shorter roots and a greater number of lateral roots, compared with controls (data not shown).

Relative water content

Relative water content (RWC) was measured in order to evaluate cadmium and copper effect on the water status of sunflower plants along 16 days of treatment. RWC did not differ between controls and 0.1 mM Cd or Cu all along the experiment, and decreased accordingly to the metal with 0.5 and 1 mM Cd or Cu from the 7th day of treatment. Cadmium or copper, at a concentration of 0.5 mM, reduced RWC around 20% and 25%, respectively, from the tenth day of treatment (Fig. 1C and D). However, the last day of the experiment, the RWC of the plants treated with 1 mM Cd or Cu was 35% and 55% of the control levels, respectively (Fig. 1C and D).

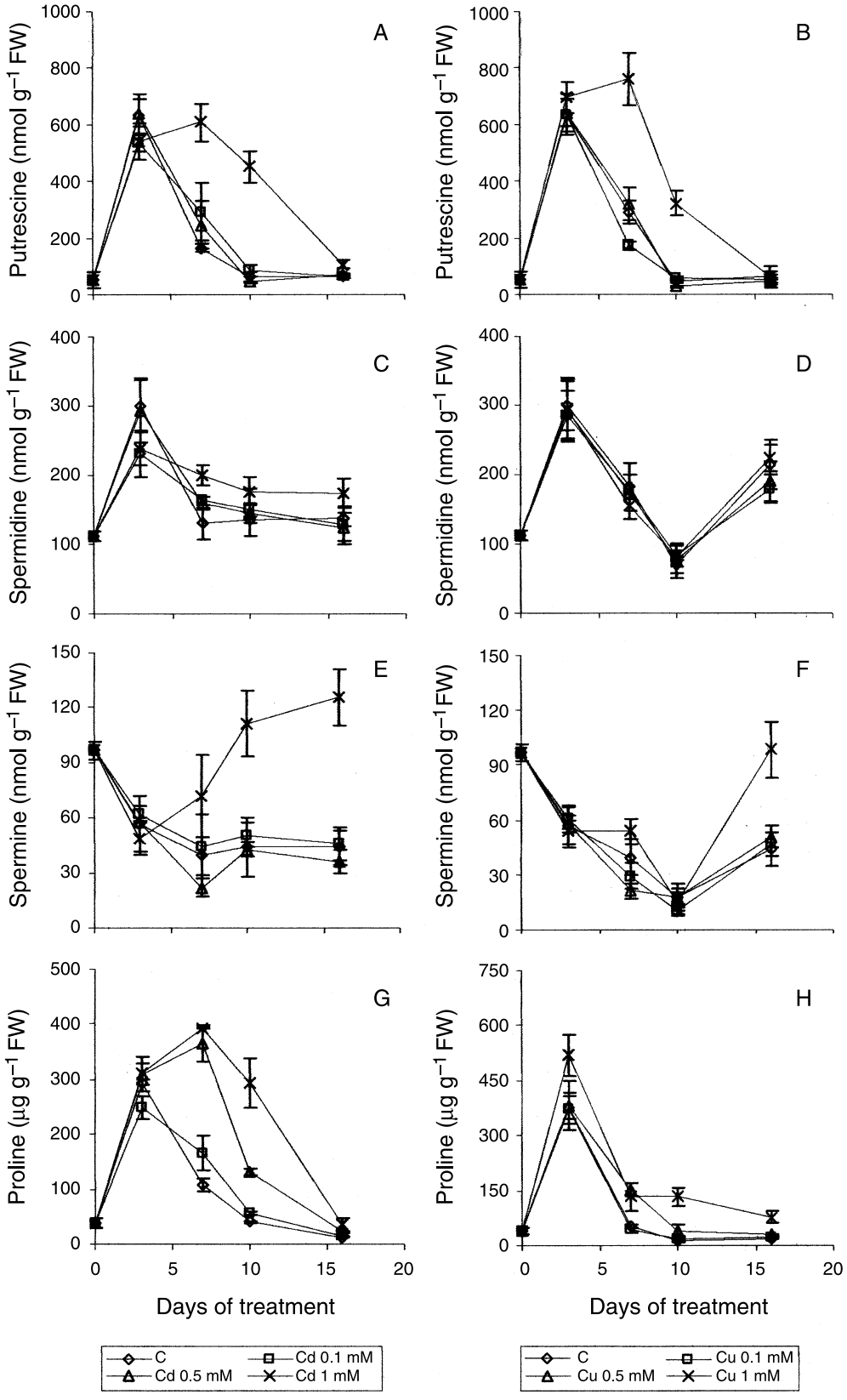

Fig. 2. Changes in the amount of Put (**A**, **B**), Spd (**C**, **D**), Spm (**E**, **F**) and Pro (**G**, **H**), in shoots of sunflower plants treated with 0.1, 0.5 or 1 mM of $CdCl_2$ (left panel) or 0.1, 0.5 or 1 mM of $CuCl_2$ (right panel). Plants were treated for 16 days in a control acclimated room at 24 ± 2 °C and samples were taken at 0, 3, 7, 10 and 16 d. Tissues were extracted as detailed in Materials and methods. Values are the means ± S.E. of three different experiments with three replicated measurements

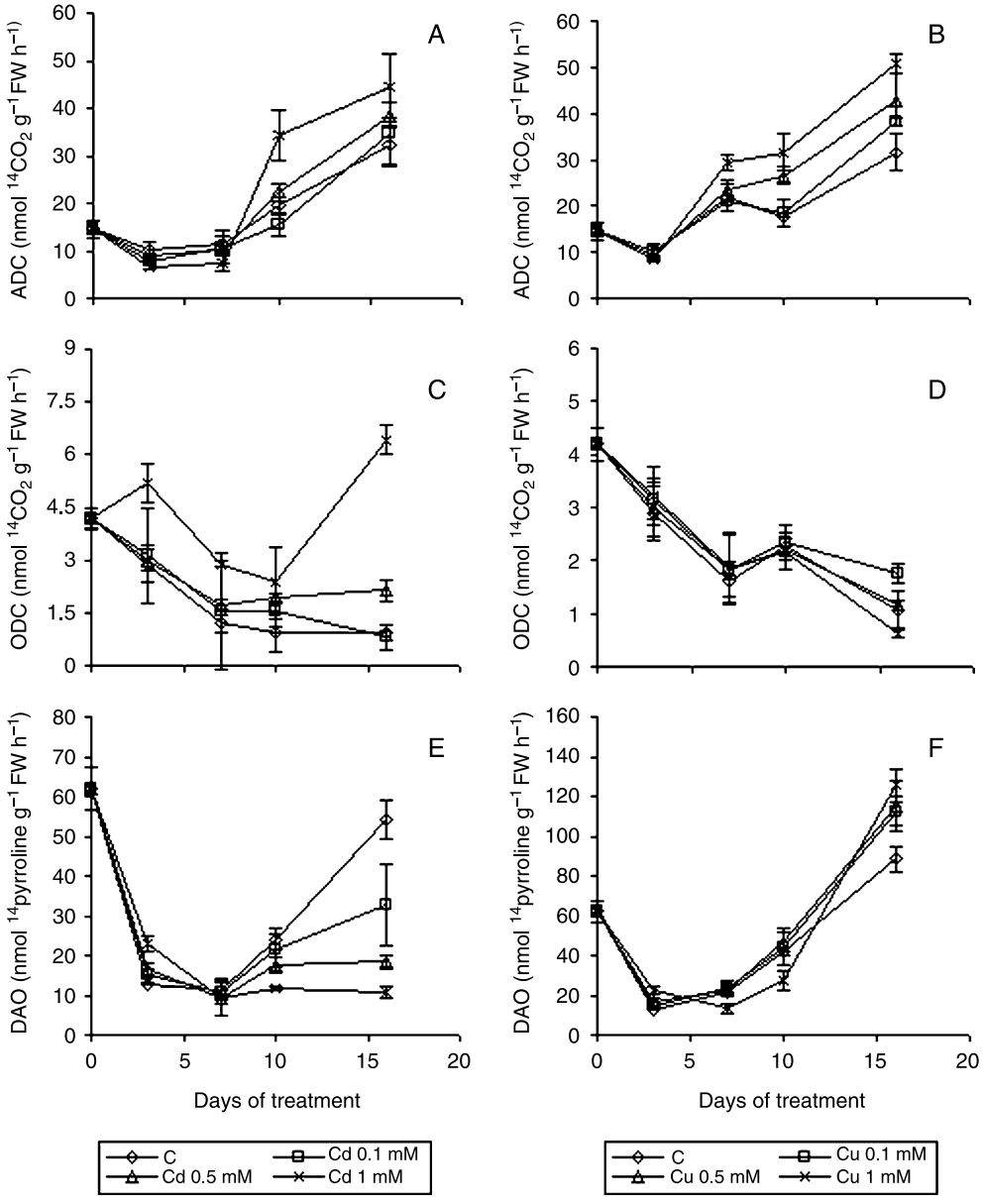

Fig. 3. Time course effect of 0.1, 0.5 and 1 mM of $CdCl_2$ (left panel) or $CuCl_2$ (right panel), on arginine decarboxylase (ADC) (**A**, **B**), ornithine decarboylase (ODC) (**C**, **D**) and diamine oxidase (DAO) (**E**, **F**) activities in shoots of sunflower plants. Plants were treated for 16 days in a control acclimated room at 24 ± 2 °C and samples were taken at 0, 3, 7, 10 and 16 d. Tissues were extracted as detailed in Materials and methods. Values are the means ± S.E. of three different experiments with three replicated measurements

Chlorophyll content

Chlorophyll content was measured as another marker of metal toxicity. Significant differences among treatments were observed from the 10th day of treatment (Fig. 1E and F). Surprisingly, chlorophyll content was mainly affected by the lower Cd concentration (0.1 mM), which produced by day 16, a decrease of 60% on the pigment concentration compared to the control, while 0.5 and 1 mM Cd reduced its content by 50% and 30%, respectively. Furthermore, the effect of copper was less evident than that of cadmium, considering that chlorophyll content dropped the last day an average of 10 to 30%, with the three Cu concentrations tested (Fig. 1E and F).

Polyamine levels

The three main Pas most commonly found in plants were measured in shoots of sunflower plants. On the third day of growth, Put content of control plants was 12-fold higher

than the level found in the seeds. Thereafter, Put content decreased until reaching the seed levels on the sixteenth day (Fig. 2A and B). Cadmium and copper also enhanced Put content after the onset of germination, independently of the concentration used and, except for 1 mM of the metals, all treatments showed the same pattern of variation than that of control plants. At the highest concentration of Cd or Cu, this polyamine remained higher than controls until the last day of the experiment, reaching a peak of increase of 270% and 160% with Cd^{2+} and Cu^{2+}, respectively, by day 7. On the 16th day, Put returned to control levels (Fig. 2A and B). Spermidine content showed a very similar pattern of variation that Put, increasing after germination and decreasing thereafter, but its content was modified respect to the controls only by 1 mM Cd, which increased this polyamine an average of 33% between days 7 and 16 of the metal exposure (Fig. 2C). Copper did not modify Spd levels all throughout the experiment, independently of the concentration used (Fig. 2D). Contrary to the pattern of variation observed with Put and Spd, Spm content declined after seeds germinated (Fig. 2E and F), increasing thereafter but only with 1 mM Cd or Cu from the third day of exposure (273% over the controls for Cd and 230% for Cu at day 16) (Fig. 2E and F).

Proline content

Both cadmium and copper modified proline content in sunflower plants (Fig. 2G and H). The pattern of proline variation resembled that of Put along the treatments either with Cd or Cu, but 0.5 mM Cd also increased the osmolite content between days 3 and 10, and the rise in Pro was less pronounced with 1 mM Cu. Cadmium produced a significant increase in proline content between days 3 and 10 at every concentration used. The most pronounced differences were observed by day 7 under 1 mM Cd, where Pro increased 3.7-fold over the controls (Fig. 2G). However, on the sixteenth day of metal exposure, Pro content returned to control levels. Cu-treated plants (0.5 and 1 mM of the metal) also increased shoot proline content from the 3rd day of treatment, but only the higher concentration of the metal maintained proline level over the controls until the last day of exposure (Fig. 2H).

ADC and ODC activities

In order to evaluate the ways of Put synthesis in sunflower plants under Cd or Cu stress, ADC and ODC activities were measured. Both Put biosynthetic enzyme activities were increased by 1 mM Cd (Fig. 3A and C). While ADC activity increased 75% and 29% on the tenth and the sixteenth day of treatment, respectively (Fig. 3A), ODC began to increase on day 3 (80% over the controls), reaching an activity 6-fold higher than controls on day 16 (Fig. 3C). Copper enhanced ADC activity from 30% to 65% with 1 mM of the metal, according to the day of exposure (Fig. 3B), while 0.5 mM and 0.1 mM of the metal increased the enzyme activity from the seventh and tenth day of treatment, respectively, but the increase was slighter (Fig. 3B). Contrary to the results observed for ADC, ODC activity was negatively affected by copper at every concentration used. A decrease of 40% respect to the controls was observed on the sixteenth day of treatment when 1 mM Cu was used (Fig. 3D).

DAO activity

Diamine oxidase is the enzyme responsible of putrescine catabolism, therefore it is also involved in the regulation of Put level. This enzyme was differently affected according to the metal. Cadmium inhibited DAO activity from the 7th day of treatment and the inhibition was related to the concentration used. The last day, the enzyme activity decreased below controls to 34%, 62% and 80% with 0.1, 0.5 and 1 mM Cd, respectively (Fig. 3E). On the other hand, plants treated with 0.1, 0.5 and 1 mM Cu increased DAO activity around 27%, 30% and 42% over the controls, by day 16 of treatment, respectively (Fig. 3F).

Discussion

In the present work we analyzed the effect of cadmium and copper on polyamine metabolism along 16 days of exposure, in order to evaluate if these aliphatic nitrogenous compounds could be used as biochemical markers of Cd or Cu stress response. First of all, we decided to estimate the level of the metals both in roots and shoots just to compare the magnitude of the accumulation and the proportion of the metal that reached the shoots. It has been well documented that Cd accumulated preferentially in roots of many species (Vitória et al., 2001; Wu and Zhang, 2002; Wu et al., 2004; Drazic and Mihailovic, 2005). In accordance with these reports, sunflower roots accumulated significantly great amount of metals than shoots (18/1 and 12/1 for 1 mM of Cd and Cu, though this relationship decrease to 3/1 and 6/1 for 0.1 mM Cd or Cu, respectively). The magnitude of Cd accumulation in sunflower roots and shoots was significantly higher than

that of Cu. However, a different pattern of accumulation was observed between both metals. Whereas the exposure to 0.5 and 1 mM Cd resulted in a maximum raise of the metal by day 10, the exposure to Cu resulted in a continuous increase in cellular Cu at every concentration used within the experimental period of 16 days.

Heavy metals may disturb the hormonal balance and further decrease growth via cell expansion (Poschenrieder and Barceló, 1999). One hundred micromolar of Cd or Cu did not affect shoot growth. However, 0.1 mM Cd resulted in a significant accumulation of the metal by day 16 (65 times more than the controls), contrary to that observed for Cu, which accumulation by the end of the exposure period was less than twice of the controls. Lower Cd concentrations than we used in this work (40–50 μM) resulted in the inhibition of shoot growth of poplar (Schützendübel et al., 2002) and pea plants (Dixit et al., 2001; Sandalio et al., 2001). For some radish varieties, 0.5, 1 and 5 mM $CdCl_2$ proved to be lethal after 4 days of Cd treatment (Vitória et al., 2001). On the other hand, *Arabidopsis thaliana* plants treated with 0.5 mM Cd accumulated 3 times less Cd in shoots than sunflower, in a similar experimental design (Cho and Seo, 2004). Sunflower has been postulated as a good candidate for soil reclamation in heavy metal-polluted sites. A prerequisite for Cd tolerance is the ability of roots to sustain growth and maintain cellular homeostasis in heavy metal-polluted soil. Our results demonstrated that Cu was much less accumulated than Cd in sunflower shoots, and only 1 mM of the metal reduced shoot length. On the other hand, 0.5 and 1 mM Cd significantly decreased shoot length, but when plants were exposed to low Cd levels (0.1 mM), they were capable of a relevant Cd accumulation in shoots without reducing growth. It is well known that Cd inhibits photosynthesis and reduces chlorophyll content. Sunflower plants treated with 1 mM Cd showed reduced growth, but were capable of preserving the pigment level over the controls up to day 10, when chlorophyll started to decrease, but still maintaining a higher level than that observed with 0.1 or 0.5 mM Cd. Moreover, the water status of the plants was not modified by 0.1 mM of the metals, suggesting that there was no threat to sunflower growth up to this metal concentration. Much lower Cd levels proved to be lethal for other species (Sandalio et al., 2001; Vitória et al., 2001).

Polyamines accumulate in plants during cell division and differentiation. Therefore, several types of stress including osmotic stress, UV irradiation, chilling, heavy metals and pathogen attack have been documented that altered polyamine metabolism in several plant species (Santa-Cruz et al., 1997; Balestrasse et al., 2003; Groppa et al., 2003; Walters, 2003). However, their role in stress responses is still elusive and even contradictory. For example, though the fact that Put accumulation under several stress conditions might be indicating that this aliphatic diamine acts as a compatible solute, an excessive content over the normal level is usually toxic to plants. Proline has also been reported as an efficient osmolite with antioxidant properties, as well as the higher polyamines Spd and Spm (Lovaas, 1997; Hare et al., 1998). In sunflower plants, the increase in Put, but also in Pro, took place almost exclusively (except for Pro under 0.5 mM Cd) with 1 mM of Cd or Cu. This enhancement of Put and Pro did not seem to be indicating a plant response that occurred to maintain the water status or shoot growth, since these parameters were significantly reduced below controls early after treatments begun, independently of Put or Pro levels. The peak in Put content by the 7^{th} day with 1 mM Cd or Cu was coincident with the start of decrease in the RWC and growth of the shoots, and the high proline content in the case of Cd. In our work, Put or Pro elevations were part of a physiological response of sunflower plants to the imposition of an intense metal stress and might be indicative of toxicity symptoms rather than to a protection mechanism in a long term exposure. It could also be suggested that Pro is involved in detoxification of heavy metals by its direct function or by way of biosynthesis of chelating peptides (Wu et al., 2004). Putrescine also increased greatly in nodules of Cd-treated soybean plants but in this work the metal was used at a considerable lower concentration (50 μM) (Balestrasse et al., 2003). Groppa et al. (2001, 2003), using sunflower leaf discs, have reported that Cd or Cu at 0.5 mM reduced Put and Spd content, without affecting Spm, while in wheat leaf discs, Cd and Cu, used at the same concentration, increased Put level to a great extent, decreased Spm content and did not modify Spd.

Both with 1 mM Cd or Cu, Spm greatly increased, earlier and more significantly with Cd, but in both cases, the point of maximum Spm level was coincident with the lowest Put level, suggesting that the increase in the tetramine synthesis occurred at expense of Put. Regarding Put biosynthetic enzymes, the decay in Put content occurred, even though the activity of ADC and ODC for Cd, and ADC for Cu remained elevated by day 16. It is commonly believed that there is a strong homeostatic regulation of the polyamine biosynthetic pathway in plants as well as animals, and this is achieved by feedback regulation of the three decarboxylases, i.e. ADC, ODC, and SAMDC (for reviews, see Kumar et al., 1997; Walden et al., 1997).

Data presented here showed neither a feedback inhibition of ADC or ODC activity by Put nor an inhibition of Put synthesis due to ADC or ODC diminished activity towards the end of the exposure time.

The enhanced levels of Put observed until day 10 in sunflower shoots could be attributed to ADC and ODC for Cd-treated plants, and almost totally to ADC in the case of Cu. It was noteworthy that Pro levels remained over the controls in plants under 1 mM Cu exposure until the last day, and this pathway could be consuming ornithine for Pro synthesis, in detriment of its use for Put synthesis. ODC seemed to be the main enzyme responsible for Put biosynthesis in wheat leaf discs treated with Cu (Groppa et al., 2003), while both ADC and ODC accounted for Put synthesis when Cd was used.

On the other hand, it has been well documented in the literature that the cellular contents of Spd and Spm in plants are much more tightly regulated than those of Put (Minocha and Minocha, 1995; Andersen et al., 1998). Several reports have been published where transgenic expression of *samdc* cDNA has been used to modulate Spd and Spm levels (Noh and Minocha, 1994; Pedros et al., 1999). In most cases, where Put is significantly reduced, there was only a small increase in cellular Spd and Spm. However, in sunflower plants under a severe Cd or Cu stress, the drop in Put levels as stress progressed reflected an increased utilization for Spm formation.

In plants, the relationship between increased Put production and its catabolism is not clear. The amount of free Put present in a plant tissue is the net result of its biosynthesis, conjugation with phenolic acids, transport to other tissues, conversion to Spd/Spm and its degradation during a given period of time. The first step in Put breakdown is catalyzed by DAO. In sunflower Cd-treated plants, the increase in Put from day 3 was accompanied by an unmodified DAO activity, but the abrupt decline in 1 mM Cd-treated plants was clearly not a consequence of a higher DAO activity. The enzyme activity increased significantly in control and 0.1 mM Cd-treated plants from day 7, though Put decay started before (after day 3), in a close relationship with the beginning in Spm elevation. However, under 1 mM Cu stress, the drop in Put content observed by day 16 was coincident with an enhancement in DAO activity observed from day 10. In poplar transgenic cells, Put degradation increased, and this increase occurred without induction of the catabolic enzyme DAO (Bhatnagar et al., 2002). In nodules and roots of soybean plants, elevated Put levels under 0.5 mM Cd were accompanied by a decreased or unmodified DAO activity (Balestrasse et al., 2005). In sunflower leaf discs treated with 0.5 mM Cd or Cu, DAO activity diminished or did no vary, respectively, while both metals used at 0.5 mM inhibited DAO activity in wheat leaf discs (Groppa et al., 2003). Another reason for high Put and unchanged Spd levels is that Spd could also be rapidly degraded to Put via an interconversion pathway well known in animals and recently characterized in higher plants (De Agazio et al., 1995).

On the other hand, the effects of metal ions on the enzyme activities related to PA synthesis and degradation have received little attention in plants. The diamine putrescine can be converted into Spd and Spm through the consecutive activity of two distinct aminopropyltransferases, spermidine synthase and spermine synthase. Both enzymes use decarboxylated S-adenosyl-L-methionine as an aminopropyl donor (Pegg et al., 1998; Alabadi and Carbonell, 1999). Yoon et al. (2000) have reported that metal ions such as Cu^{2+} activated Spd synthase of soybean at concentrations ranging from 10 to 100 μM. Taking into account the fact that Spd is a substrate for Spm synthesis, the regulation of Spd synthesis by Cu could be another fact affecting Spm content through the addition of aminopropyl groups to Spd.

Polyamines, mostly the tetramine Spm, are postulated as membrane stabilizers and protectors of the photosynthesizing apparatus (Besford et al., 1993; Groppa et al., 2003). The elevated levels of Spm from the 7^{th} day of exposure with 1 mM Cd could be a beneficial trait against chlorophyll degradation in sunflower plants under Cd or Cu stress. However, it has been reported that Spm is not essential for plant survival (Imai et al., 2004) and so its accumulation under stress might not represent a strong metal tolerance trait. Previous reports of our group (Groppa et al., 2001, 2003; Balestrasse et al., 2003, 2005) show clear evidence that polyamine metabolism can be severely disturbed in plants under metal stress, and not only the metal class and concentration has to be taken into account at the time the results are analyzed, but also the plant species, the developmental stage and the experimental model used.

The data presented here let us to suggest that Put and Spm could be considered as useful early (when the metals inside the tissues had not still reach the maximum), and late markers of Cd and Cu stress in plants, respectively, though neither of them seemed to be biologically active in sunflower response to metal stress. Furthermore, sunflower plants have proved to tolerate Cd or Cu levels that would be normally lethal for other plant species (0.1 mM for Cd or 0.1 and 0.5 mM for Cu), maintaining growth at normal rates.

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