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Research article

Chromium (VI) induces toxicity at different photosynthetic levels in pea

Eleazar Rodriguez [a], Conceição Santos [a,*], Raquel Azevedo [a], José Moutinho-Pereira [b], Carlos Correia [b], Maria Celeste Dias [a]

[a] CESAM & Department of Biology, Laboratory of Biotechnology and Cytometry, University of Aveiro, 3810 Aveiro, Portugal
[b] CITAB & Department of Biology and Environment, University of Trás-os-Montes e Alto Douro, Apartado 1013, 5001-801 Vila Real, Portugal

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ABSTRACT

In order to comprehensively characterize the effects of Cr (VI) on the photosynthetic performance of *Pisum sativum*, plants irrigated with Cr solutions (ranging from 20 to 2000 mg l^{-1}) were evaluated using the following classical endpoints: gas exchange parameters, chlorophyll *a* (Chl *a*) fluorescence, leaf pigments, Rubisco activity, soluble sugars and starch content. Flow cytometry (FCM) was applied in an innovative approach to evaluate the morphological and fluorescence emission status of chloroplasts from plants exposed to Cr (VI). The parameters related to gas exchange, net CO_2 assimilation rate (*A*) and Rubisco activity were severally affected by Cr exposure, in some cases even at the lowest dosage used. While all biomarkers used to measure Chl *a* fluorescence indicated a decrease in fluorescence at the maximum dosage, pigment contents significantly increased in response to Cr (VI). The morphology of chloroplasts also was altered by Cr (VI) exposure, as a volume decrease was observed. Soluble sugars and starch showed an overall tendency to increase in Cr (VI) exposed plants, but sucrose and glucose decreased highly when exposed to 2000 mg l^{-1}. In conclusion, our results indicate that Cr (VI) affects photosynthesis at several levels, but the most Cr (VI)-sensitive endpoints were chloroplast morphology and biochemical processes; only at higher dosages the photochemical efficiency is compromised.

1. Introduction

Chromium (Cr) is one of the most abundant elements in earth crust [1] and together with its release to the environment by several industries (metallurgical, leather, and chemical) it is an important metal pollutant [2,3]. Shanker et al. [4] stated that unlike other heavy metals such as cadmium [5], Cr had not received the same level of attention from plant scientists. Since then, the overall scenario regarding Cr phytotoxicity has changed very little; few reports have addressed the deleterious effects of this metal in plants and most of what is known comes from other organisms like animals and algae [e.g Ref. [6–8]].

Chromium phytotoxic potential depends on its speciation which is responsible for its mobilization, subsequent uptake and resultant toxicity [4]. Lopez-Luna et al. [9] demonstrated, for three species of cereals, that Cr (VI) presented higher mobility, caused more toxic effects at lower levels than Cr (III) and Cr of tannery sludge. These facts are justified by the fact that Cr (VI) has higher solubility in water and enters cell membranes more easily than other Cr valences [10]. Once inside the cell, Cr (VI) is known to generate reactive oxygen species [11] and its intracellular reduction to Cr (III) allows direct interaction with DNA [12], which might in turn cause DNA degradation and cell cycle arrest [13], among others.

The little information available indicates that Cr may affect plant performance: in *Pisum sativum* seedlings it was demonstrated that Cr exposure reduced photosynthetic rate, and the authors hypothesized that Cr could act as an acceptor of electrons during photophosphorylation, inhibiting the process [14]. Later, Vernay et al. [15] verified that in *Lolium perenne*, Cr (VI) exposure affected the photochemistry of PSII and decreased CO_2 assimilation. More recently, Lopez-Luna et al. [9] showed that in sorghum, wheat and oat plants significant translocation of Cr (VI) occurred to aerial parts, supporting the possibility of interactions between this chromium valence and the photosynthetic machinery/process. In algae, Juarez et al. [16] observed changes in chloroplast matrix and morphology together with a complete pheophytinization of both Chl *a* and Chl *b* when exposed to Cr (VI).

Even with this increase in studies regarding Cr toxicity in the photosynthetic apparatus, there is still much to be done in order to fully understand the extent of effects of this heavy metal in photosynthesis, and on nutrients involved in this metabolism.

The aim of the present research is to analyze the effects of Cr (VI) in several aspects of photosynthesis, using pea as model species. To

* Corresponding author. Campus Universitário de Santiago, University of Aveiro, 3810-193 Aveiro, Portugal. Tel.: +351 234 370 766; fax: +351 234 426 408.
E-mail address: csantos@ua.pt (C. Santos).

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this end, plants were exposed to several Cr (VI) concentrations ranging from the maximum admitted level in some European Union countries for agricultural purpose waters (20 mg l^{-1} for total Cr) to values observed in waters from chrome tanning processes (2000 mg l^{-1} of Cr (VI)) [17]. The following biomarkers were used to assess the Cr (VI) toxicity in photosynthesis: gas exchange parameters, photosynthesis-related nutrients unbalances, Chl *a* fluorescence, leaf pigments, Rubisco activity, soluble sugars and starch contents. Also an emerging technique, using plant functional flow cytometry that combines morphological and fluorescence emission status of isolated chloroplast was used. With the data presented here we give a better insight on the toxicity of Cr (VI) in plants.

2. Material and methods

2.1. Plant culture conditions and exposure to chromium

Pea seeds (*P. sativum* L., cv Corne de Bélier, IPSO BP 301, 26401 Crest, France) were hydrated for 48 h and then sowed in pots containing a peat:perlite mixture 4:1 (in a proportion currently used in commercial greenhouse production of crops). Plants were grown during 28 days at 24 °C ± 1 °C, under light intensity of 200 μmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h (light/dark). The Cr was added as $K_2Cr_2O_7$ to the nutrient solution (1:10 Hoagland's solution) to give concentrations of 0; 20; 200; 1000 and 2000 mg l^{-1} of Cr. Plants were watered (at least 25 per condition) with 100 ml of the nutrient solution twice a week. Exposure lasted the aforementioned period of 28 days, after which analyses were performed.

2.2. Chromium and nutrient analysis

Contents of total Cr and of some nutrients essential to photosynthesis (Mg, Mn, Fe, S, P) were determined in dried leaves and roots of all conditions. Prior to drying (at 60 °C), roots were washed with a solution of $CaSO_4$ to remove Cr adsorbed to the root surface. Dried tissues were treated according to Azevedo et al. [18]; in brief, dried tissues were incinerated at 530 °C during 14 h. The ashes were digested with HCl solutions and heating and then filtered. Ultra pure water was added until final volume of 14 ml and samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin Yvon, JY70Plus, Longjumeau Cedex, France).

2.3. Pigments quantification

Leaf disks (0.5 cm^2) were ground in a mortar at 4 °C with 2 ml cold acetone/Tris 50 mM pH 7.8 buffer solution (80:20, v:v) and centrifuged at 2800× *g* during 5 min. The supernatant was diluted to a final volume of 3 ml with additional acetone/Tris buffer. The absorbance at 470, 537, 647 and 663 nm were determined with a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S). The contents of Chl *a*, Chl *b* and carotenoids were calculated using the formulae of Sims and Gamon [19]. According to Lichtenthaler [20], the molecular weights used to convert gram units to mole units were as follows: Chl $a = 893.5$ g mol^{-1}, Chl $b = 907.5$ g mol^{-1} and carotenoids = 550 g mol^{-1}. At least 5 samples (from 5 different individuals) were analyzed per condition.

2.4. Chlorophyll a fluorescence and gas exchange

Chlorophyll *a* fluorescence parameters were measured in the adaxial side of the leaf with a pulse-amplitude-modulated fluorimeter (FMS 2, Hansatech Instruments, Norfolk, England). Maximum quantum efficiency of photosystem II (PSII) was calculated as $F_v/F_m = (F_m - F_0)/F_m$ by measuring the fluorescence signal from a dark-adapted leaf when all reaction centers are open using a low intensity pulsed measuring light source (F_0) and during a pulse saturating light (0.7 s pulse of 15,000 μmol photons $m^{-2}s^{-1}$ of white light) when all reactions centers are closed (F_m). Leaves were dark-adapted for 30 min using dark-adapting leaf-clips for these measurements. Following F_v/F_m estimation, after a 20-s exposure to actinic light (1500 μmol $m^{-2}s^{-1}$), light-adapted steady-state fluorescence yield (F_s) was averaged over 2.5 s, followed by exposure to saturating light (15,000 μmol $m^{-2}s^{-1}$) for 0.7 s to establish F'_m. From these measurements, quantum effective efficiency of PSII was calculated as $\Phi_{PSII} = \Delta F/F'_m = (F'_m - F_s)/F'_m$ [21].

Leaf gas exchange measurements were performed using a portable IRGA (LC*pro*+, ADC, Hoddesdon, United Kingdom), operating in the open mode under growth chamber conditions. Net CO_2 assimilation rate (A), stomatal conductance (g_s), transpiration rate (E) and the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) were estimated from gas exchange measurements using the equations developed by Von Caemmerer and Farquhar [22]. The A/E ratio was used as water use efficiency (WUE).

Gas-exchange and chlorophyll fluorescence measurements were always performed between 13:00 and 14:00 h (8 h after the beginning of the light period).

2.5. Rubisco activity

Leaf discs of 0.5 cm^2 diameter were homogenized at 4 °C in a mortal with 1 ml extraction buffer according to Dias and Brüggemann [23]. The extraction buffer consisted of 50 mM Tris/HCl, pH 7.9, 8 mM $MgCl_2$, 5 mM Na-pyruvate, 1 mM EDTA, 2 mM K_2HPO_4, 20 mM dithiothreitol, and 0.3% (m/v) bovine serum albumin. The homogenate was centrifuged in Eppendorf cups at 9000 × *g*. Immediately after extraction, Ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) activity was assayed as described by Lilley and Walker [24]. This assay followed NADPH oxidation measured spectrophotometrically at 340 nm. Total activity was achieved after incubation in 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 20 min.

2.6. Soluble sugars and starch

Soluble sugars were extracted from frozen leaf discs (0.5 cm^2) with 2 ml 80% (v/v) of ethanol at 80 °C over 20 min [25]. Glucose, fructose, sucrose were quantified using a spectrometric enzyme-coupled assay [26] and starch was quantified [27] in a Thermo Fisher Scientific spectrophotometer (Genesys 10-uv S).

2.7. Chloroplast morphology and fluorescence emission by flow cytometry

For flow cytometry (FCM) analysis, chloroplast extraction was performed using the protocol developed by Rodriguez et al. [28]. In brief, leaves were harvested as needed and macerated with a mortar and pistil, in HS buffer (HEPES and sorbitol). The suspension was filtered through two 50 μm nylon meshes, and centrifuged for 2 min at 4000× *g*. Supernatant was placed in 2 ml of a 35% Percoll® solution, and centrifuged for 8 min at 1400× *g*. Pellet was re-suspended and centrifuged twice in HS buffer at 4000× *g* for 2 min. Chloroplasts were then analyzed in a Coulter Epics XL Flow cytometer. The chloroplast forward light scattered (FS), corresponding to the volume of each particle, and the autofluorescence emitted (FL) were analyzed. From the FL histogram of control plants and as established by Rodriguez et al. [28], chloroplasts were classified into two population, broken/damaged chloroplast (population A, with low fluorescence) and intact chloroplasts (population B, with high fluorescence). After this, the voltage of the cytometer and the regions characterizing these populations were