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Article

Chromium Differentially Affects Hydrogen Peroxide Distribution in Primary and Adventitious Roots of *Arabidopsis thaliana* L.

Aarón Giovanni Munguía-Rodríguez[1,2], José López-Bucio[1], Gerardo Rangel Sánchez[1,2], León Francisco Ruiz-Herrera[1], Yazmín Carreón-Abud[2] and Miguel Martínez-Trujillo[2,*]

[1]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B3, Ciudad Universitaria, C. P, 58030, Morelia, Michoacán, México

[2]Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo. Edificio R, Ciudad Universitaria, C. P, 58030 Morelia, Michoacán, México

[*]Corresponding Author: Miguel Martínez-Trujillo. Email: codigogenetico@gmail.com

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Abstract: The post-embryonic growth of the *Arabidopsis thaliana* root system can be modified by different types of stress, such as sublethal concentrations of metals, which may induce the production of reactive oxygen species (ROS). In this study, the effects of different concentrations of potassium chromate (K_2CrO_4) on the distribution and relative quantity of hydrogen peroxide (H_2O_2) were determined in primary and adventitious roots in *A. thaliana* HyPer line seedlings. This line has a biosensor that specifically reports H_2O_2 levels within tissues as fluorescence. Primary root growth was inhibited at 100 μM Cr (VI); in contrast, adventitious root formation was induced over the main root growth axis. These structures proliferated from 100-160 μM Cr (VI), and much higher concentrations (180-200 μM) of K_2CrO_4 were required to affect their growth. The H_2O_2 distributions were observed in the columella and lateral root cap of primary roots of plants grown in medium lacking dichromate, but following the development of toxicity symptoms, H_2O_2 changed its distribution to the meristem and differentiation zones. Conversely, adventitious roots had comparable H_2O_2 distribution patterns in untreated plants and those exposed to Cr (VI) supplementation. Thus, differential H_2O_2 distribution correlates with the resistance of adventitious roots, but not primary roots, to dichromate and underlies cell reorganization at the apex to support growth.

Keywords: Chromate; hydrogen peroxide; root growth; meristem; ion toxicity; adaptation

1 Introduction

The sessile nature of plants, coupled with changing environmental conditions, enabled regulatory mechanisms to be established during evolution, allowing plants to generate diverse adaptive responses to survive. These responses may include changes in growth and development, adjustments in metabolism, and modifications of gene expression [1]. Because the root is the organ responsible for the uptake of nutrients and water, local cues sensing may induce profound changes in root morphology [2].

In dicotyledonous plants, such as *Arabidopsis thaliana*, a primary root is developed from the embryo. Its growth depends on a group of stem cells with intense mitotic activity located at the apex that gives rise to all tissues, including the vasculature, pericycle, endodermis, cortex, epidermis, lateral root cap and the columella [3]. Gradually, lateral roots are formed, which extends soil exploration for water and nutrient acquisition and allows an improved anchorage to the substrate [4,5]. Lateral roots are derived from the pericycle, an inner cell layer surrounding the vascular tissues of the primary root, through reactivation of the cell cycle, giving rise to a primordium that emerges and grows and retains a similar organization to

the primary root [6-8]. Occasionally, adventitious roots can be formed, which are roots developed from the stem upon genetic cues or as responses to damage or hormonal or environmental stimuli [9-11].

The root system architecture (RSA) consists of the spatial arrangement of different root types and determines the volume of soil that a plant can exploit [12-14]. For example, primary root growth is inhibited and lateral root formation is stimulated in media with low phosphate concentrations, which involves a tight interrelation with iron nutrition, auxin response and mitogen-activated protein kinase signaling [15-18].

Nonessential elements, such as chromium (Cr), lead (Pb) and arsenic (As), among others, can be found in the biosphere in the form of ions that interact in many ways with biological systems. Chromium is the seventh most abundant element in the earth and is found at highly variable concentrations in the soil [19,20]. However, its utilization in the automotive industry and leather tanning has increased its concentration, and it has become an important contaminant [21,22]. The most stable and abundant forms of Cr are the trivalent Cr (III) and the hexavalent Cr (VI) forms, whereas the other valence states are unstable and short-lived in biological systems [23]. Cr (VI) can react with oxygen, forming chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions, whereas Cr (III) can form oxides or hydroxides [24]. The toxicity of Cr is mainly related to the reduction of Cr (VI) to Cr (III) in the cytoplasm, both by enzymatic and nonenzymatic reactions, leading to unstable intermediaries that produce reactive oxygen species (ROS) [25]. In addition, Cr (III) affects cell functions by binding the DNA phosphate backbone and carboxyl and sulfhydryl groups in proteins [26].

The effects of Cr (VI) in plants include the inhibition of germination, shoot and root growth restriction, and leaf chlorosis, drastically compromising plant productivity [19,22]. The maximum allowed limit of Cr (VI) in the soil for use for agricultural purposes in Mexico is 280 mg kg^{-1} [27]. To unravel the molecular mechanisms of Cr toxicity, previous reports employed the model plant *A. thaliana*, showing that phenotypic modifications depended upon metal concentration and the auxin response mediated by the *IAA14/SOLITARY ROOT* gene [28,29]. Of note, Cr (VI) affected iron (Fe), sulfur (S) and phosphorus (P) uptake, and its toxic effects could be reverted by application of sulphate or phosphate, suggesting that membrane protein transporters of these macronutrients may also internalize chromate [30].

The incomplete reduction of molecular oxygen in aerobic organisms generates highly reactive derivatives, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-), collectively known as reactive oxygen species (ROS) [31]. ROS are produced as byproducts during mitochondrial electron transport or by oxidoreductase enzymes and metal catalyzed oxidations, and ROS excess can lead to protein denaturation and lipid peroxidation. In addition, ROS can react with redox-sensitive signaling proteins under physiological conditions, acting as second messengers by mediating cellular signal transduction [31]. The mechanisms that maintain ROS at an adequate cellular level include the antioxidants ascorbic acid and glutathione as well as ROS-detoxifying enzymes, such as superoxide dismutases (SODs), which transform O_2^- to H_2O_2 following its conversion to H_2O by catalases (CATs) [32,33]. Chromium promotes the expression of genes encoding ROS-detoxifying enzymes in *A. thaliana* [28] and rice [34], opening the possibility of ROS acting as mediators of metal toxicity.

The superoxide anion is neither membrane-permeable nor long-lasting in cells, whereas H_2O_2 is considerably more stable and can cross the plasma membrane, thus functioning as a long-range cell-to-cell signal [36]. The higher stability of H_2O_2 is useful for analyzing its levels in root tissues, especially when using the *A. thaliana* HyPer transgenic line, which harbors a highly specific molecular biosensor for H_2O_2 [36,37].

To better understand the ROS mechanisms that underlie the responses of the root system to Cr (VI), H_2O_2 distribution was analyzed in *A. thaliana* primary and adventitious roots using the HyPer transgenic line. The results unravel the differential sensitivity of the primary and adventitious roots to Cr (VI) that correlates with H_2O_2 levels in growth zones.

2 Materials and Methods

2.1 Plant Material

In this study, we used plants of *A. thaliana* expressing HyPer, a biosensor for intracellular H_2O_2. Hyper consists of a *circularly* permuted YFP (cpYFP) inserted into the regulatory domain of the *Escherichia coli* H_2O_2-binding protein (OxyR). H_2O_2 shifts the excitation peak of cpYFP from 420 to 500 nm, while the emission peak remains unchanged at 516 nm. Because Hyper is reversibly modified, it is possible to register increases and decreases in H_2O_2 levels and its distribution in plant tissues [36,38].

2.2 Plant Growth Conditions

Seeds were treated superficially with 95% (v/v) ethanol for 5 min and 20% chloride solution for 7 min, washed five times with sterile distilled water and refrigerated in the dark at 4ºC for 48 h. Seeds were sown in Petri dishes with 0.2x MS medium [39], at pH 5.7, with plant agar 1% (w/v) (Phytotechnology Laboratories) and sucrose 0.6%. Media were supplemented with different K_2CrO_4 concentrations, referred to as Cr (VI). Petri plates were placed vertically to avoid root penetration into the media and to facilitate seedling analysis. Plates were incubated in a plant growth chamber (Percival AR-95 L) with a 16 h light /8 h dark photoperiod and a light intensity of 300 μmol m^{-2} s^{-1} at 23°C.

2.3 Analysis of Fluorescence

Before confocal analysis, seedlings with intact root systems were incubated in propidium iodide solution at 10 mg ml^{-1} for 3 min. A confocal microscope (Olympus FV1000) was used with a filter wheel to switch between two different excitation wavelengths, 440 nm for the H_2O_2-independent signal and 495 nm for the H_2O_2-dependent signal, and all emission spectra were registered using an emission filter at 530/20 nm; the two images were merged to produce the final image. Fluorescence was measured by calculating the green pixels in an area consisting of 100 cells above the apex using ImageJ software. Means calculated from control conditions were given a value of 1, and the means corresponding to treatments were adjusted relative to control, and thus were referred to as relative fluorescence.

2.4 Statistical Analysis

Averages and confidence intervals were calculated using the Microsoft Office Excel 2010 program. Analysis of variance (ANOVA) and Tukey significance tests were performed using the statistical software SPSS 19.0 for Windows ($p < 0.05$) [40].

3 Results

3.1 Effect of Cr (VI) on Primary Root and Adventitious Root Growth in Hyper Seedlings

To determine the effect of Cr (VI) on H_2O_2 distribution and its correlation with the growth and development of primary and adventitious roots, the *A. thaliana* HyPer line was used. The HyPer seedlings report H_2O_2 levels and distribution with higher precision than techniques based on chemical approaches. Seeds of the HyPer line were germinated under *in vitro* conditions, and plants were grown for 10 days after germination in media with different Cr (VI) concentrations. Primary root growth was stimulated by 30% in 20-40 μM Cr (VI) when compared to the control; adventitious roots were not developed in media with 20-80 μM Cr (VI) and in controls without the metal. Adventitious roots were formed in media with 100-200 μM Cr (VI); in parallel, primary root growth was inhibited by 90% at 100 μM Cr (VI). In each plant, one or two adventitious roots were developed, and when their length was determined, it was found that one was longer than the other. In 100-120 μM Cr (VI), the adventitious root growth was approximately 70% of the primary root of the control plants, whereas in 140-160 μM Cr (VI), the growth was reduced to 30%; in 180-200 μM Cr (VI), growth was almost completely inhibited Fig. 1.

Supplementation of ascorbic acid stimulated primary root growth, in contrast with the ROS-producing agent paraquat, which had an inhibitory effect Fig. 1. Thus, the root system architecture of *A. thaliana* was modified by exposition to Cr (VI), ranging from stimulation of primary root growth under

low Cr (VI) concentrations to growth inhibition upon increased Cr (VI) supplementation; concomitantly, adventitious root formation was stimulated, likely as an adaptive response.

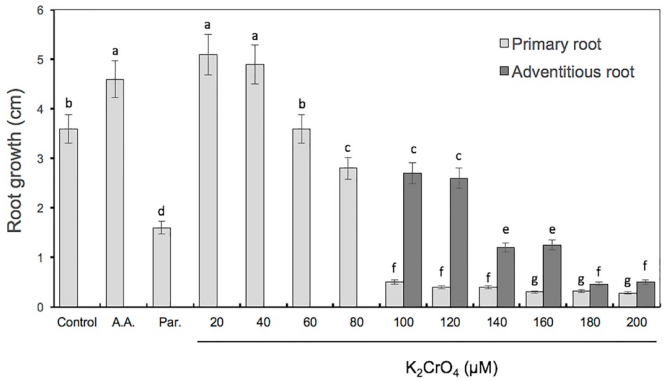

Figure 1: Effects of Cr (VI) on the growth of primary and adventitious roots of *A. thaliana*. Seeds of the *A. thaliana* HyPer line were germinated, and plants were grown for 10 days in MS media supplemented with ascorbic acid (A. A.) and paraquat (Par) treatments, included for comparison, or increasing concentrations of K_2CrO_4. The confidence interval is indicated for alpha = 0.05. The different letters indicate significant differences with the Tukey test ($p < 0.05$; n = 30). The experiment was repeated 3 times with similar results

3.2 Effect of Cr (VI) on H_2O_2 Distribution in Primary and Adventitious Roots

Paraquat and ascorbic acid increased or decreased, respectively, the fluorescence in the root, consistent with the reports that these compounds modify the amounts of ROS, including H_2O_2, thus confirming the reliability of our system using the HyPer transgenic line. In plants grown in media without Cr (VI), H_2O_2 was detected in the columella and lateral root cap cells in the primary root. This pattern was conserved in plants grown in media with 20-60 μM Cr (VI), while H_2O_2 distribution was extended to the elongation zone at 80 μM Cr (VI) Fig. 2. In 100 μM Cr (VI) or higher concentrations, the H_2O_2 distribution was extended to the elongation and differentiation zone, which correlated with the disorganization of the root apex Figs. 2 and 3.

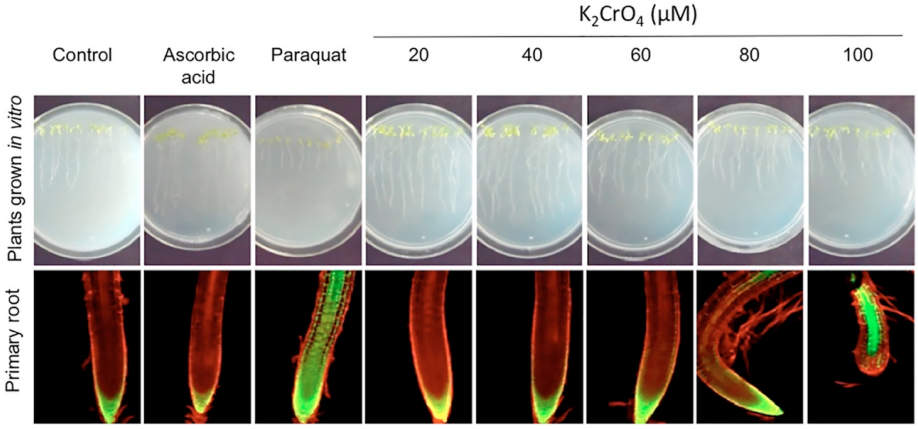

Figure 2: Effect of Cr (VI) on the H_2O_2 distribution in the primary root of *A. thaliana*. Seeds of the HyPer line of *A. thaliana* were sown and grown for 10 days in media supplemented with ascorbic acid, paraquat or increasing concentrations of K_2CrO_4. Roots were analyzed, and H_2O_2 distribution was determined as described in the Materials and Methods section. Photographs are representative of 10 plants analyzed. The experiment was repeated 3 times with similar results

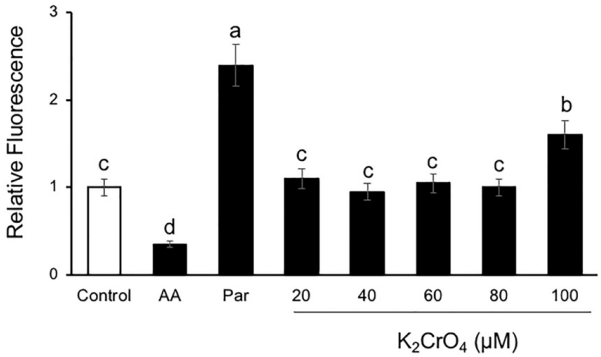

Figure 3: Effect of Cr (VI) on the hydrogen peroxide quantity in the primary root of the *A. thaliana* HyPer line. Fluorescence was measured by quantifying pixels in an area consisting of 100 cells above the apex using ImageJ software. The confidence interval is indicated for alpha = 0.05. The different letters indicate significant differences with the Tukey test ($p < 0.05$; n = 3). The experiment was repeated 3 times with similar results

The adventitious roots formed in plants grown in media supplied with 100-200 µM Cr (VI) and showed H_2O_2 distribution in columella and lateral root cap cells, comparable to the primary root of control plants (Fig. 4). However, inhibition of adventitious root growth required higher Cr (VI) supplementation (180-200 µM), and the cell organization in the apex was less affected than that in primary roots (Fig. 4). In addition, the amount of fluorescence did not decrease at higher Cr (VI) concentrations (Fig. 5). These data indicate that primary and lateral roots differ in not only their sensitivity to Cr (VI) but also H_2O_2 distribution, which coincides with changes in root tip integrity.

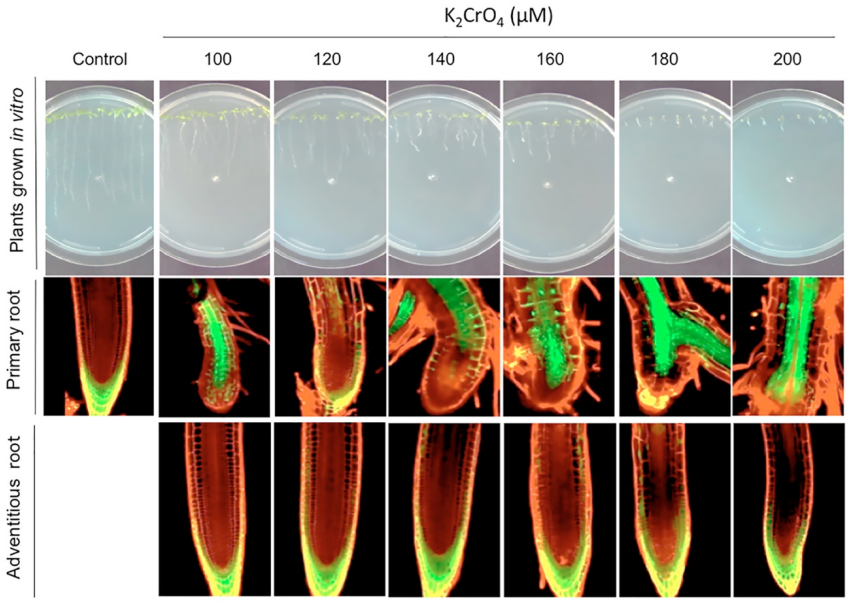

Figure 4: Effect of Cr (VI) on the H_2O_2 distribution in the primary root and adventitious roots of *A. thaliana*. Seeds of the *A. thaliana* HyPer line were sown and grown for 10 days in media with ascorbic acid, paraquat or increasing concentrations of K_2CrO_4. Adventitious roots were analyzed by confocal microscopy, and fluorescence distribution was determined as described in the Materials and Methods section. Photographs are representative of 10 plants analyzed. The experiment was repeated 3 times with similar results

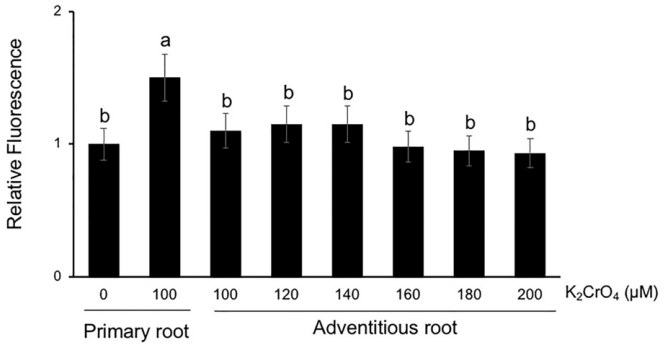

Figure 5: Effect of Cr (VI) on the relative hydrogen peroxide quantity in adventitious roots of the *A. thaliana* HyPer line. Fluorescence was measured using ImageJ software by quantifying pixels in an area consisting of 100 cells above the apex in primary and adventitious roots. The confidence interval is indicated for alpha = 0.05. Different letters indicate significant differences with the Tukey test ($p < 0.05$; n = 3). The experiment was repeated 3 times with similar results

4 Discussion

The primary root growth inhibition by Cr (VI) analyzed in this study and reported previously [41] seems to be a general response to different stressing factors, such as low nutrient availability [42] or sublethal concentrations of metals [43]. Here, we showed that Cr (VI) not only inhibited primary root growth, but one or two adventitious roots were simultaneously developed from the hypocotyl. Those roots were able to grow in media with 100-160 μM Cr (VI) and their growth was delayed at higher Cr (VI) concentrations. Accordingly, when *A. thaliana* seedlings were germinated and grown in darkness, primary root growth stopped at day six and adventitious roots were generated; this process needed sucrose or glucose as an energetic source [44]. These results suggest that in *A. thaliana*, adventitious root formation likely occurs as response to tolerate the stress caused by an effect on photosynthesis resulting from either Cr (VI) or darkness to maintain a functional root system. This hypothesis may explain why adventitious roots presented higher tolerance to stress, including maintenance of the normal cell organization at the root apex.

Primary roots displayed a higher concentration of superoxide anion and a lower H_2O_2 concentration in the meristem; the inverse distribution already occurs in the differentiation zone [45]. Furthermore, by experimentally decreasing H_2O_2 in the elongation zone, cell differentiation was delayed, which correlates with the higher H_2O_2 concentration during cell differentiation (op. cit.). This is consistent with our results showing changes in H_2O_2 distribution and quantity in the primary roots of plants grown in media supplemented with 100 μM Cr (VI) or higher concentrations; thus, the increased levels of H_2O_2 in the meristem may be related to the path to differentiation. The inhibition of primary root growth of *A. thaliana* and the decrease in meristem size could be detected following exogenous H_2O_2 application [46].

Modification in the H_2O_2 distribution by cellular uptake of Cr (VI) would be explained by the combined action of glutathione transferase and superoxide dismutase enzymes that produce H_2O_2 [47,48]. This notion is consistent with the transcriptional induction of three genes encoding peroxidases in *A. thaliana* plants exposed to Cr (VI) [28].

We previously demonstrated that supplementation of 100-200 μM Cr (VI) caused root system modifications related to the distribution of the auxin hormone and two of its polar transporters (PIN1 and PIN2). In that case, adventitious roots showed normal expression of auxin transporters, while primary roots were highly sensitive and halted their growth [29]; furthermore, when adventitious root growth was inhibited in the higher Cr (VI) concentration (200 μM), the auxin response still remained, which is comparable to the Cr (VI) effect on H_2O_2 distribution found in this study. Notably, modification of the H_2O_2 patterns occurred in primary roots but not in adventitious roots, which is reminiscent of their different sensitivity/tolerance to Cr (VI) and possibly other environmental stressors. It is necessary to speculate that either the auxin response

induced the shift from cell division to differentiation via ROS, or oxidative stress following metal exposure caused alterations in auxin response and/or transport. Tsukagoshi et al. [45] demonstrated that the induction of ROS-detoxifying enzymes in *A. thaliana* is independent of auxin and cytokinin signaling. This response implies that Cr (VI) has possibly modified the H_2O_2 distribution independently of auxin, but the disturbance in both auxin responsiveness and endogenous H_2O_2 then leads to the inhibition of primary root growth upon loss of meristem activity. Regarding the latter hypothesis, it has been reported that exogenous H_2O_2 inhibits primary root growth and decreases the expression levels of the auxin transporters PIN1 and PIN2, and, as a consequence, the normal distribution of auxin cannot be accomplished [49]; thus, the primary messenger during root sensing of Cr (VI) would be a ROS-derived signal.

5 Conclusions

Taken together, our results support the following findings: i) in root system responses to Cr (VI) exposition, changes in the H_2O_2 distribution may explain the growth inhibition of the primary root and the cell disorganization at the apex, and ii) the generated adventitious roots are more tolerant to Cr (VI), being able to maintain normal H_2O_2 homeostasis. We conclude that adventitious roots are more tolerant to Cr (VI), likely via a ROS-detoxifying mechanism, protecting macromolecules from damage and improving plant survival.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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