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ECOPHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS FOR ASSESSING Cr⁺⁶ STRESS CONDITIONS IN *Pterogyne nitens* Tul.: NEW AND USUAL METHODS FOR THE MANAGEMENT AND RESTORATION OF DEGRADED AREAS

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Abstract

Heavy metals, such as Cr (chromium), have received attention during recent years due to their disposal in soil and water. Brave peanut (*Pterogyne nitens* Tul.), a tropical tree, was cultivated in 500 $\mu\text{mol L}^{-1}$ Cr⁺⁶ to observe how this species responds to this stress, with a view to its possible application in the restoration of degraded areas. In the present study, we report the use of a new method, photoacoustic spectroscopy, to evaluate the emission of CO₂ and ethylene and the use of other standard techniques for assessing stress (chlorophyll *a* fluorescence, photosynthetic pigment determination, leaf relative water, specific leaf area, NO (nitric oxide) and polyamines. Photoacoustic spectroscopy appears to provide an innovative and efficient technique for detecting stress induced by heavy metals soon after their contact with plants. Polyamine content, as well as ethylene and NO, were seen to be sensitive to Cr⁺⁶; polyamines were decreased in leaves and increased in roots, ethylene was increased in the whole plant and NO was increased in the roots. Our results demonstrating alterations in ethylene in response to Cr⁺⁶ are, to date, unreported and the technique used for these measurements is novel. With regard to the polyamines, modulations in their concentrations may have protected the photosystem II, since no photosynthetic alterations were observed for pigments and chlorophyll *a* fluorescence.

Key words: arboreal tropical species, chlorophyll *a* fluorescence, nitric oxide, photoacoustic spectroscopy, polyamines

Received: September, 2011; Revised final: June, 2012; Accepted: June, 2012

1. Introduction

Heavy metal pollution is one of the most serious environmental problems in current society. Urban, agricultural and industrial activities have contributed, over the years, to the increase in levels of metals in the soil and water (Spongberg et al., 2008). Among the different metal species

encountered are chromium (Cr), lead (Pb), zinc (Zn), iron (Fe), cadmium (Cd) and copper (Cu); as such, some industrial activities have favored widespread Cr contamination in the environment.

Although Cr is an essential element for humans, it can be toxic for plant growth (Paiva et al., 2009; Panda and Choudhury, 2005) and fungi (Murugavelh and Mohanty, 2014) and some

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processes are used to remove environmental Cr (Srivastava et al., 2014). Cr phytotoxicity in the environment is controlled by a number of factors, including the element-uptake site, bioavailability, competition for binding sites and ionic speciation (Smical et al., 2008; Vernay et al., 2007). Ionic speciation or oxidation state has a direct relationship with heavy metal toxicity, where the hexavalent ionic form of Cr (Cr^{+6}) is the most toxic (Paiva et al., 2009). Cr^{+6} is capable of indirectly generating reactive nitrogen (NOS) and oxygen species (ROS), leading to oxidative stress (Shanker et al., 2005). NOS and ROS have negative actions on cell function; however some of these species can also act as signaling molecules, preparing the organism against the negative effects of the presence of metal (Kopyra and Gwózdź, 2003).

The involvement of ROS in Cr stress in plants has been well described in the literature (Prasad, 2004; Shanker et al., 2005; Vajpayee et al., 2000). The degradation of membrane lipids, due to ROS damage, causes several physiological and biochemical processes in plants, including alterations in respiration and photosynthesis. Cr^{+6} stress has also been shown to lead to alterations in chloroplast and membrane ultrastructure, gas exchange, chlorophyll *a* fluorescence parameters and pigment contents (Paiva et al., 2009; Shanker et al., 2005; Vernay et al., 2007).

NO is a small and gaseous free lipophilic radical that is an important signaling molecule with diverse physiological functions in plants (Arasimowicz and Floryszak-Wieczorek, 2007; Besson-Bard et al., 2008). Recent research indicates that NO is involved in the regulation of multiple plant responses to a variety of abiotic and biotic stress conditions (Arasimowicz-Jelonek et al., 2011; Kopyra and Gwózdź, 2004; Qiao and Fan, 2008; Xiong et al., 2010). Depending on the biological model, the presence of metals has been found to both induce (Bartha et al., 2005) and inhibit (Rodríguez-Serrano et al., 2006) NO production.

Polyamines are other molecules that also participate in responses to biotic and abiotic stress in plants. These molecules act on several regulatory processes in plants, such as the promotion of growth, cell division, DNA replication and cell differentiation (Baron and Stasolla, 2008). Diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm) are the main polyamines found in all plant cells. Excesses in heavy metal have been shown to produce changes in polyamine metabolism (Balestrasse et al., 2005; Pirintsos et al., 2005). Although these changes are controversial for different plant species, they are all related to the protective function of polyamines in plant cells at high metal concentrations (Xiong et al., 2010).

In addition to NO and polyamines, ethylene (C_2H_4) is a stress-related signaling gaseous molecule in plants. Many of the adverse responses to plants caused by heavy metals appear to be linked to ethylene, which is the simplest of the plant hormones

(Arteca and Arteca, 2007). Although heavy metals, such as Cu, Cd, Ni, Zn and others, have been shown to have an effect on ethylene production, their effects are not clear (Arteca and Arteca, 2007; Fuhrer, 1982; Pezzarossa et al., 1991). Some reports have suggested that these metals can either promote or inhibit ethylene production in several experimental systems with Cd appearing to be the most phytotoxic inorganic ion (Fuhrer, 1982; Pezzarossa et al., 1991). Gas chromatography (CG) has been the most widely-used technique for ethylene detection (Arteca and Arteca, 2007; Fuhrer, 1982; Pezzarossa et al., 1991).

Although CG is a widely-used technique, photoacoustic spectroscopy has shown advantages in terms of sensitivity and accuracy, non-invasive and real-time measurements (Corrêa et al., 2011). Photoacoustic spectroscopy is one of several photothermal (PT) techniques that are based on the conversion of optical energy absorbed into heat. The basic principle of PT techniques is the absorption of light in a sample with a subsequent change of its thermal state. The sample heating, which produces the PT signal, is correlated directly to the absorbed electromagnetic energy. If the temperature rise in the absorbing sample volume occurs faster than this volume can expand, a local pressure increase (wave) occurs. This pressure wave can be considered as a sound signal (Dumitras et al., 2010).

Despite the changes that may occur in plants, in response to heavy metals, many species show tolerance to these metals as a function of anatomical, morphological, biochemical and physiological acclimatization. The demand for arboreal species that are tolerant to heavy metals has increased due to the need for reforestation near inactive mining areas (Pulford and Watson, 2003) and/or restoration of riparian vegetation with high anthropogenic activity and therefore with a higher concentration of heavy metals (Madejón et al., 2004). *Pterogyne nitens* Tul., commonly known as brave peanut, is recommended for planting in riparian forest with periodic floodings of rapid duration, resetting and restoration of degraded areas (Santos et al., 2008). These species naturally occur in Atlantic rainforest areas, mainly in semi-deciduous broadleaf. Despite the fact that woody species are commonly used for ecological restoration in tropical environments, little is known about how these species respond to metal stress (Fuentes et al., 2007).

The presence of metal in plants generates a range of responses involving molecules and organelles in their physiological processes. Some of these responses are well-established; however some aspects of these responses, such as the main signaling molecules involved, still need further investigation. As such, using traditional methods of research on stress, including the evaluation of chlorophyll *a* fluorescence, and innovative physical methods, such as photoacoustic spectroscopy to quantify ethylene and carbon dioxide (CO_2), we have evaluated the following: 1) Whether impairment of the photosynthetic process of *P. nitens* occurs when it is

exposed to Cr⁺⁶; 2) Whether physical methodologies, such as photoacoustic spectroscopy, could be valuable techniques that are as good as the well-established chlorophyll *a* fluorescence assay to detect physiological changes caused by heavy metal stress, and 3) Whether polyamines and NO modulate the response to heavy metal stress in this arboreal specie.

2. Material and methods

2.1. Plant growth and treatments

Pterogyne nitens Tul. (Fabaceae, Caesalpinioideae) were cultivated under two different conditions, according to analyzes to be used.

1) Seedlings were cultivated in Plantmax® substrate under a photosynthetic photon flux density (PPFD) of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and at room temperature (about 25 °C) for 2 months years old. Seedlings were submitted to 500 $\mu\text{mol L}^{-1}$ Cr⁺⁶ (K₂Cr₂O₇) for 7 days. After this period, chlorophyll *a* fluorescence, photosynthetic pigments, leaf relative water, specific leaf area, ethylene, CO₂ and NO contents were analyzed. NO analyses were carried out at 2 h and 7 days as NO is involved in primary response (short term) stress (Besson-Bard et al., 2009).

2) Seeds were germinated in agar in the presence (500 mol L⁻¹) or absence of Cr⁺⁶. After 7 days, three samples from roots and leaves from each treatment were collected in order to determine the free polyamine contents.

2.2. Chlorophyll *a* fluorescence measurements

These measurements were carried out between 11:00 a.m. and 12:00 a.m., using a pulse amplitude modulation fluorimeter (FMS2, Hansatech Instruments Ltd., Norfolk, UK). Three leaves from each plant (of a total of three plants) were kept in the dark for 30 min and then exposed to a weak, modulated light beam (approximately 6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 660 nm), followed by exposure to 0.8 s of high intensity (10.000 $\mu\text{molm}^{-2}\text{s}^{-1}$) actinic white light beam, as adapted from Genty et al. (1989) and van Kooten and Snel (1990).

The maximum quantum yield of PSII (F_v/F_m), the variable chlorophyll fluorescence ratio (F_v/F_0) and the extinction coefficients: qP (photochemical quenching) and NPQ (non-photochemical quenching) were measured. Values are presented as means of three repetitions for each treatment.

2.3. Photosynthetic pigment contents

Three leaf discs of 1.1 cm in diameter were taken from each plant, sliced and placed in plastic tubes containing 5mL of dimethyl sulfoxide (DMSO) in the dark. The plastic tubes were then placed in a thermostatic water bath (CIENITEC-246) at 70 °C for 2 h.

The photosynthetic pigments were analyzed with a spectrophotometer at wavelengths of 480, 649 and 665 nm and quantified using the equations described by Wellburn (1994) for carotenoids, chlorophyll *a* and chlorophyll *b*. All these procedures were carried out in a low-light environment. Values were expressed in nmol cm^{-2} .

2.4. Leaf relative water content

In order to determine the relative water content (RWC) of the leaves, three leaf discs of 1.1 cm in diameter were taken from each plant. The leaf discs were weighed to obtain the fresh mass (FM) and turgid mass (TM), leaves were infiltrated with distilled water using a vacuum system.

Subsequently, the water surfaces of the discs were gently wiped with tissue paper and dried at 60 °C, for 48 h and the dry mass (DM) was obtained. The leaf relative water content (RWC) was calculated by the following (Eq. 1):

$$RWC(\%) = \frac{(FM - DM)}{(TM - DM)} \cdot 100 \quad (1)$$

2.5. Specific leaf area

The specific leaf areas (SLA) were given by the leaf areas from the disc and the total dry masses of the disc leaf, expressed in $\text{cm}^2 \text{g}^{-1}$, according to the (Eq. 2):

$$SLA = \frac{A_{\text{leaf}}}{m_{\text{leaf}}} \quad (2)$$

where, A_{leaf} is the disc leaf area and m_{leaf} is the DM of the disc leaf.

2.6. Ethylene and CO₂ measurements: photoacoustic spectroscopy technique

In order to monitor ethylene, a laser-driven photoacoustic system, based on infrared absorption was used. A commercial infrared detector (URAS14, ABB), connected in series to the photoacoustic spectrometer, was used to monitor plant respiration (CO₂ emission) (Corrêa et al., 2011). For this, each plant was enclosed in the analyzer's chambers. Chemical filters were used to eliminate the contributions from CO₂ and H₂O present in the carrier gas.

To avoid interferences due to water vapor and hydrocarbons, the gaseous sample was passed through a "cold trap" prior to entering the photoacoustic cell. To calibrate the photoacoustic detector, a standard mixture of 1 ppmv of ethylene in nitrogen was used. Ethylene released from the plants was then determined using a continuous flow rate of 2.0 L h⁻¹, controlled by electronic flow controllers (model 5850S, Brooks Instrument). Ethylene and CO₂ concentrations were calculated, taking into

account the values of the flow and the plant shoot masses, obtained by FM.

2.7. NO determination

Intracellular NO levels were analyzed by fluorescence microscopy using fluorescent probes, according to Tun et al. (2006) with modifications. After 2 h and 7 days of incubation, root tip segments (2 cm) from both treatments were obtained (10 root tip/treatment, in triplicate) and incubated in 5 mM Hepes-Tris HCl buffer pH 7,5 containing 5 $\mu\text{mol L}^{-1}$ DAF-FM-DA (4,5-diaminofluorescein diacetate; Calbiochem), a cell permeable fluorescent dye to analyze endogenous NO.

Samples were incubated in the dark, at 25 °C, on a rotatory shaker (50 rpm) for 2 h. Subsequently, samples were washed twice with Hepes-Tris HCl buffer prior to observation, and plates were prepared and observed under a fluorescence microscope Axioskop 2 (Carl Zeiss, Germany). The filter was set for DAF-FM-DA excitation at 515 nm and emission at 525 nm. Digital photos were taken with exactly the same settings of a digital camera, and were not further processed. All experiments were repeated at least twice, with similar results, and the photos presented are representative of a single experiment.

2.8. Polyamine determination

Polyamines were determined according to Silveira et al. (2004). The samples were ground in 1.6 ml of 5% (v/v) perchloric acid and, after 1 h, the samples were centrifuged for 20 min at 2.0×10^4 g at 4°C. Free polyamines were determined directly from the supernatant, being derivated by dansyl chloride and identified by HPLC (Shimadzu Shinpack CLC ODS), using a 5 μm reverse-phase column. The gradient was developed by mixing increasing proportions of absolute acetonitrile with 10% acetonitrile in water (pH 3.5).

The gradient of absolute acetonitrile was programmed to 65% over the first 10 min, from 65% to 100% for between 10 min and 13 min, and 100% between 13 min and 21 min, at 1 mL min^{-1} flow and 40°C. The polyamine concentration was determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparison with Put, Spd and Spm standards.

2.9. Statistical analysis

The results of chlorophyll *a* fluorescence variables, photosynthetic pigments, relative water content, specific leaf area and polyamines were analyzed by one-way analyses of variance (ANOVA), and the means were compared using Tukey's test ($P < 0.05$).

Data were presented as means \pm standard deviation. The correlation between extinction NPQ

coefficients and carotenoids were evaluated by Pearson's correlation coefficient (R).

3. Results and discussion

None of the values for photosynthesis parameters were statistically different at $P < 0.05$ (Table 1). Mean values of F_v/F_m and F_v/F_0 rates for the controls and for the plants treated with Cr^{+6} were 0.814 and 4.57 and 0.845 and 4.89, respectively. A very low correlation between this pigment and NPQ was obtained ($R=0.118$) (data not shown). Photosynthetic studies that employ chlorophyll *a* fluorescence (measuring parameters such as F_v/F_m and F_v/F_0 ratios) are an important tool to evaluate disturbances in PSII caused by heavy metals in plants (Maxwell and Johnson, 2000). Bolhar-Nordechamp et al. (1989) suggested that values of F_v/F_m between 0.75 and 0.85 indicate that the photosynthetic apparatus operates in environmental conditions favorable to the healthy development of the plant. In the present study, the values of F_v/F_m remained in the range proposed by these authors, suggesting the absence of stress in the photochemical phase. According to Roháček (2002), the F_v/F_0 ratio in healthy plants may vary from 4 to 6, representing changes between the photochemical quantum yield and non-photochemical processes that occur in PSII. Although the F_v/F_0 ratio is more sensitive for detecting stress (Oliveira et al., 2009; Paiva et al., 2009), the values found in this study demonstrate an absence of photochemical stress (according to F_v/F_m) and reflect the integrity of the PSII. A significant decrease in F_v/F_m ratio was observed for *Lolium perenne* L. plants in 250 $\mu\text{mol L}^{-1}$ Cr^{+6} after 45 days. The decrease was even higher when the plants were exposed to 500 $\mu\text{mol L}^{-1}$ Cr^{+6} (Vernay et al., 2007). Significant decreases in F_v/F_m , F_v/F_0 and qP parameters for water hyacinth (*Eichhornia crassipes*), a metal hyperaccumulator aquatic species, were observed when the plants were exposed to Cr^{+6} . Even for a hyperaccumulator species, the treatment with the higher concentration of Cr^{+6} (10 mmol L^{-1}) was lethal (Paiva et al., 2009).

The above-mentioned studies show that these ratios were efficient for assessing stress caused by Cr^{+6} . However, it is important to emphasize the difference between the availability of metallic chemical species for aquatic and terrestrial plants. Once in aqueous solution, the metal ion has more mobility and availability for absorption (Antoniadis and Alloway, 2002).

In soils, in addition to the presence of heavy metal ions in the mineral structure, these ions may be adsorbed to solid surfaces, in interstitial solution, in their complexed form or associated with colloids. Such factors limit the mobility and, therefore, their availability for absorption (Remon et al., 2005). Another important consideration regarding Cr pollution is the fact that this element presents various chemical species.

Chromium ⁺⁶ is reported as the most harmful to plants due to its high oxidizing power (Paiva et al., 2009; Shanker et al., 2005). Despite this feature, Cr⁺⁶ toxicity strongly depends on the concentrations that are available for absorption, the plant species and the development stages of the plant (Remon et al., 2005).

In the present study, the pigment levels did not change under stress (Table 1). However, several studies have demonstrated changes in photosynthetic pigment contents for plants exposed to metal (Paiva et al., 2009; Pandey and Tripathi, 2011). This could occur due to the substitution of Mg⁺² ion in the chlorophyll molecule by heavy metal ions (Küpper et al., 1998) or by an increase in carotenoid production, aiding thermal energy dissipation from photosynthetic impairments (Demmig-Adams, 1990). In the present study, the unchanged values of the pigments, especially carotenoids and NPQ, also suggest that the plants of this study did not dissipate energy to prevent photo-oxidative damage, perhaps because the PSII was protected by binding with polyamines, as will be discussed.

Table 1. Chlorophyll *a* fluorescence, photosynthetic pigment contents (nmol cm⁻²), relative water content (RWC, %) and specific leaf area (cm² g⁻¹) in brave peanut seedlings exposed to 500 μmol L⁻¹ Cr⁺⁶ during 7 days. Values followed by different letters differ significantly (*P* < 0.05) for the same parameter. Mean ± standard deviation, *n* = 5. Chl = Chlorophyll

Parameter analyzed	Cr ⁺⁶ (μmol L ⁻¹)	
	Control	500
Fv/Fm	0.814 ± 0.006 A	0.845 ± 0.012 A
Fv/F ₀	4.57 ± 0.028 A	4.89 ± 0.565 A
Qp	0.898 ± 0.021 A	0.891 ± 0.033 A
NPQ	0.149 ± 0.013 A	0.150 ± 0.014 A
Chl <i>a</i>	45.486 ± 0.713 A	45.660 ± 2.366 A
Chl <i>b</i>	13.903 ± 0.042 A	13.013 ± 3.862 A
Chl <i>a</i> / Chl <i>b</i>	3.271 ± 0.041 A	3.747 ± 0.901 A
Carotenoids	13.758 ± 1.426 A	13.906 ± 2.105 A
Total Chl/Carotenoids	4.337 ± 0.395 A	4.358 ± 1.077 A
RWC	25.214 ± 1.4695 A	24.952 ± 0.3787 A
SLA	447.94 ± 14.361 A	434.31 ± 13.344 A

Relative water content (RWC) and specific leaf area (SLA) obtained from the plants incubated with Cr⁺⁶ did not show any variation in the control plants (Table 1). The effects of heavy metal on anatomical and physiological processes that influence the plant's water regulation have been well documented. In general, these studies suggest a reduction for RCW and photosynthetic pigments in the presence of Cr, as described for pea (*Pisum sativum* L.) (Tiwari et al., 2009), Chinese fern (*Pteris vittata* L.) (Su et al., 2005) and cauliflower (*Brassica oleracea* L.) (Chatterjee and Chatterjee, 2000). For other metals such as Cu, Co, Ni and Cd, the responses seem to be more heterogeneous, with an increase (Chatterjee and Chatterjee, 2000), a decrease

(Llamas et al., 2008) and no changes (Lin and Kao, 2007) in RCW.

It has been hypothesized that some plants have a mechanism for phytotoxicity for excess heavy metals, based on the disturbance of cell membrane function, where the membrane potential increases as a function of heavy metal absorption. This may cause a decrease in intracellular K⁺ ions, which, in turn, may decrease the water content in plants (Llamas et al., 2008). Although further data regarding cell membranes were not collected in the present study, the maintenance of values for RWC suggests that the Cr⁺⁶ treatment was not sufficient to cause changes in membrane potential, as reflected by this parameter. Like RCW, SLA and leaf thickness have important functions in plants, since the amount of light absorbed by the leaf and the routes of CO₂ diffusion are directly dependent on these parameters (Syvertsen et al., 1995). Chromium(VI) treatment, described in the present study, did not alter the SLA. The interaction between SLA and heavy metals did not show a direct relationship in Syncamore Maple (*Acer pseudoplatanus* L.) (André et al., 2006). These authors found that the SLA remained unchanged after 120 days in the presence of Cu (640 mg kg⁻¹ soil), Zn (3000 mg kg⁻¹ soil), Cd (10 mg kg⁻¹ soil) and Pb (90 mg kg⁻¹ soil).

This result was obtained for tree species, as used in the present study, suggesting a certain tolerance to higher concentrations of metals, in comparison with annual plants (for example the species of agronomic and environmental interest that are the focus of most of studies with heavy metals) (Cardoso et al., 2002; Llamas et al., 2008; Tiwari et al., 2009; Vitória et al., 2010). Great tolerance in tree species has also been observed in salt stress (Passos et al., 2005).

Photoacoustic spectroscopy permitted the detection of an increase in ethylene emission from the plants following exposure to Cr⁺⁶ (Fig. 1A), while changes in the plant respiration rates, i.e. the CO₂ emission (Fig. 1B) after 7 days of treatment with Cr⁺⁶ did not show statistical differences. Another parameter widely used to assess the extent of stress in plants is the ethylene emission rate (Arteca and Arteca, 2007; Fuher, 1982; Poschenrieder et al., 1993).

Plants can regulate ethylene production by genetic means, according to development stage, organ and/or a variety of environmental factors such as temperature, light, oxygen, nutrition and heavy metals (Arteca and Arteca, 2007; Poschenrieder et al., 1993). Arteca and Arteca (2007) suggested that changes in ethylene concentration vary depending on the metal. The authors observed that, in *Arabidopsis thaliana* Thal., Cu⁺² and Cd⁺² provoke positive effects, while Ni⁺² and Zn⁺² do not affect ethylene concentrations. Cd⁺² and Cu⁺² also promoted an increase in the ethylene production in wheat leaves (*Triticum aestivum* L.). On the other hand, for sunflower leaves (*Helianthus annuus* L.), only Cu⁺² increased ethylene production (Groppa et al., 2003).

Despite the fact that changes in ethylene concentration are considered to be an indication of stress, specific data regarding Cr stress are still needed.

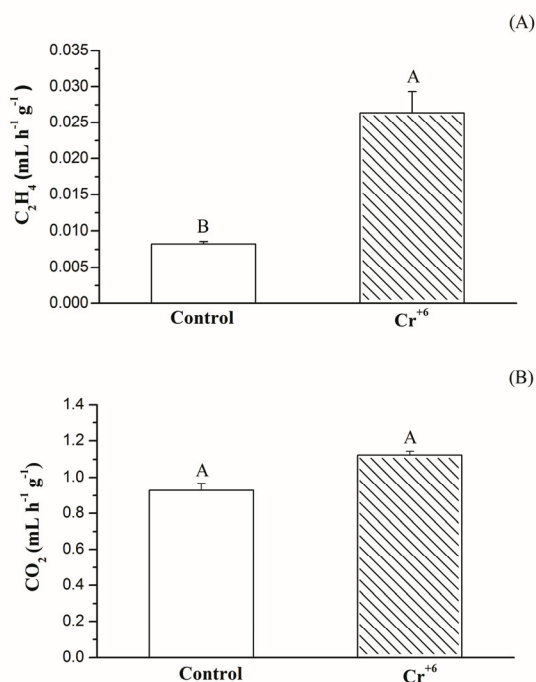


Fig. 1. Photoacoustic spectroscopy measurements of (A) Ethylene (C_2H_4 , $\mu L h^{-1} g^{-1}$) and (B) Carbon dioxide (CO_2 , $mL h^{-1} g^{-1}$) in brave peanut seedlings exposed to $500 \mu mol L^{-1} Cr^{+6}$ for 7 days. Values followed by different letters differ significantly ($P < 0.05$) for the same parameter (mean \pm standard deviation, $n = 3$)

A single study that relates ethylene and Cr^{+6} was developed with bean seedlings (*Phaseolus vulgaris* L.) (Poschenrieder et al., 1993) and presented conflicting results to ours. These authors used GC and demonstrated an inhibition of ethylene emission in the presence of Cr^{+6} . This contradiction may have occurred as a function of the differences in Cr^{+6} concentration used by Poschenrieder et al. (1993) ($2 mmol L^{-1}$ and $10, 20$ and $40 \mu mol L^{-1} Cr^{+6}$) and in the present study ($500 \mu mol L^{-1}$) and/or the differences in plant species.

The lack of work in this area reinforces the need for more data to demonstrate the association between ethylene and Cr. This would help to elucidate the role of metals in this important physiological change, since ethylene emission may lead to consequences ranging from the early senescence of the organ where it is produced (Taiz and Zeiger, 2010) to the premature ripening of fruits (da Silva et al., 2005). With regard to the method used for ethylene detection in the present study, it is important to emphasize the use of photoacoustic spectroscopy as a powerful tool in the study of plants exposed to heavy metals; this method that has not been, to our knowledge, previously employed in this manner. The literature shows that GC is the technique most commonly used for this purpose (Arteca and Artica, 2007; Pezzarossa et al., 1991).

The detection of low ethylene concentrations, by means of photoacoustic spectroscopy, is an advantage of this method of detection of gases. The sensitivity and selectivity of the photoacoustic method are due primarily to the use of laser as a radiation/excitation source. This device can be chosen according to the molecule to be analyzed, i.e. according to the wavelength at which the molecule absorbs the radiation, discriminating or minimizing other chemical species that could interfere in the detection. In addition, photoacoustic spectroscopy provides a great advantage for non-destructive and real-time measurements, compared to GC, since for GC, samples must be collected at intervals of time and then be evaluated.

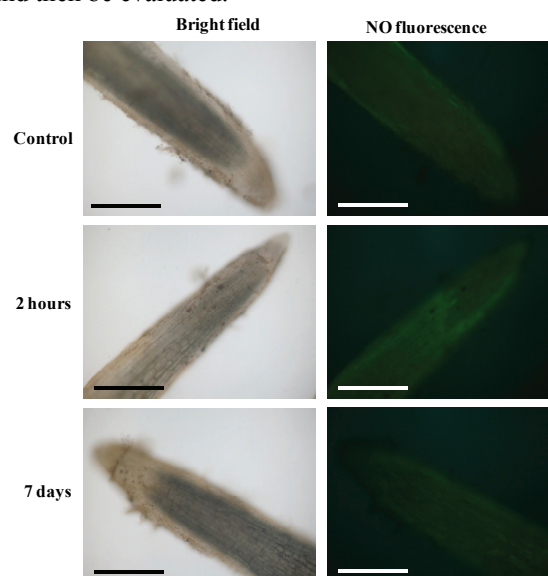


Fig. 2. Fluorescence microscopy of intracellular NO (nitric oxide) in brave peanut seedlings treated with $500 \mu mol L^{-1} Cr^{+6}$ for 2 h and 7 days. Root samples were incubated with cell permeable DAF-FM-DA (4,5-diaminofluorescein diacetate) and analyzed using fluorescence microscopy. Scale bars = 300 μm

The concentrations of polyamines (Put, Spd and Spm) were higher in leaves than in roots (Fig. 3). In leaves, Spd was the most abundant polyamine in both treatments. Although the pattern of polyamine production was similar between the control plants and the plants treated with Cr^{+6} , lower concentrations were found in Cr^{+6} plants. On the other hand, in the roots, an increase in polyamine levels was observed in the presence of Cr^{+6} . This increase was greatest for Spd, followed by Spm and Put.

Analyzing the different free polyamines, Put presented a significant decrease in the leaves, while it was increased in roots of the plants exposed to Cr^{+6} . The concentration of Spd showed no variation in the leaves; however, in the roots, an increase of about 6 times was observed in the presence of Cr^{+6} . For Spm, an inverse behavior was observed, i.e. it decreased in leaves and increased in roots when compared to the control samples.

Studies have suggested the interaction of polyamines with thylacoid proteins during stress,

imparting a greater tolerance in the plant (Hamdani et al., 2011). According to these authors, high concentrations of polyamines in the PSII caused significant loss of PSII activity, while lower concentrations, especially of Spm, stabilized the PSII conformation and improved the photosynthetic functioning under stress. Under stressful conditions, changes occur in the binding pattern of Put and Spm to the Light-harvesting Complex II (LHCII), adjusting its size. A decrease in the Put/Spm ratio would increase the size of the LHCII. This could lead to an increase in energy dissipation, reducing the photochemical activity (Hamdani et al., 2011).

In the present study, we observed reductions in Put and Spm concentrations in the presence of Cr⁺⁶, suggesting the stability observed for PSII. However, the Put/Spm ratio did not change, possibly explaining the lack of increase in energy dissipation. Polyamines have been reported to have other functions such as the electron transport maintenance, energy transfer between PSI and PSII (mainly due to Spm) and maintaining chlorophyll content under stress conditions (Humdani et al., 2011).

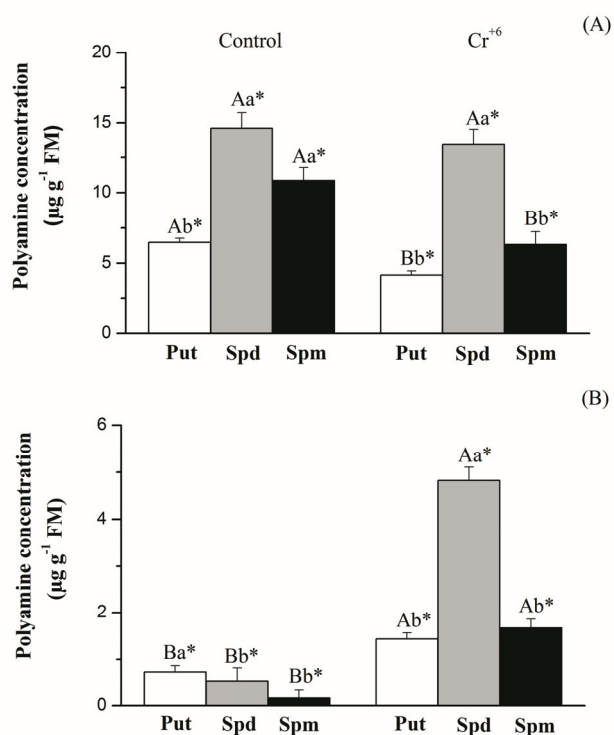


Fig. 3. Free polyamine concentrations (µg g⁻¹ FM) in (A) Leaf and (B) Roots of brave peanut seedlings exposed to 500 µmol L⁻¹ Cr⁺⁶ for 7 days. Upper case letters compare PA concentrations between control and Cr⁺⁶ treatments.

Lower case letters compare PA concentrations in the control plants and in the Cr⁺⁶ treated plants. Asterisks (*) compare PA (□ Put – Putrescine, ■ Spd – Spermidine and ■ Spm – Spermine) concentrations between leaves and roots. (*P* < 0.05) for the same parameter (*n* = 3). FM = fresh mass

NO fluorescence emission from roots exposed to Cr⁺⁶ for 2 h was more intense when compared with the control, and compared to plants exposed to Cr⁺⁶

for 7 days (Fig. 2). In plants, stress can trigger a signaling cascade, inducing the production of signaling molecules, such as NO, which leads to changes in concentration or modulation of secondary messengers, consequently activating defense responses (Xiong et al., 2010). The greater NO fluorescence after 2 h of treatment with Cr⁺⁶ may be associated with the rapid synthesis of this compound, resulting in the activation of protective responses. This together with its rapid diffusion and reactivity characterizes the NO as a secondary messenger to the initial responses in the protection against stress (Wendehenne et al., 2004).

NO has an antioxidant capacity, as this molecule may sequester ROS and stimulate the antioxidant system, alleviating the oxidative stress caused by heavy metals (Xiong et al., 2010). Additionally, in studies on salt stress, treatments with an NO donor led to plant growth, with the maintenance of PSII activity and increased the activity of antioxidant enzymes, resulting in stress tolerance (Uchida et al., 2002).

Although the antioxidant activity was not evaluated in this study, it may be suggested that the increase in NO at the beginning of the treatment (2 h) may have induced an increase in antioxidant activity and protection of PSII, alleviating the effects of Cr⁺⁶. Increases in NO production under stress conditions induced by heavy metals have been described for several species, especially for the annual species of agronomic interest (tree species) (Bartha et al., 2005). As such, the results obtained for *P. nitens* are new and reveal the role of NO under heavy metal stress in long-cycle (tree) species, suggesting that the defense mechanism involving NO is maintained by a wide range of species, independently of their life form.

4. Conclusions

The presence of Cr⁺⁶ in *P. nitens* did not affect the photosynthetic process and other parameters associated with it, e.g. pigments. This may be due to the modulations in polyamine and NO concentrations.

The photoacoustic spectroscopy was effective for detecting physiological changes from exposure to Cr⁺⁶. This technique opens new possibilities for analyzes conducted in association with the usual methods for evaluating stress.

Physiological adjustments verified as a result of Cr⁺⁶ exposure and the maintenance of the photosynthetic processes suggest that *P. nitens* is a good choice for reforestation of contaminated areas and restoration of riparian areas in anthropized rivers.

Acknowledgments

The authors would like to thank Dr. Debora Guerra Barroso (Laboratório de Fitotecnia, UENF, Brazil) for providing the brave peanut seeds used in our experiments. The authors are indebted to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Rio de Janeiro (FAPERJ), and Coordenação de

Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

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