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RESEARCH ARTICLE

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Commercial growth regulator has adverse effect over soybean seedlings under different cadmium levels

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Abstract

Aim of study: Soils contaminated by heavy metals, such as cadmium, may reduce plant development. Exogenous application of plant growth regulators (PGR), are used for optimizing the crops production in stressful environments. The aim of this study was to evaluate the effects of Cd concentrations on the development of soybean seedlings under exogenous application of a commercial PGR.

Area of study: Presidente Prudente, São Paulo, Brazil.

Material and methods: Soybean seeds were pre-treated in distilled water (control treatment) and in solution with plant growth regulator (PGR treatment) and then germinated with distillated water. The germinated seeds were transferred to different levels of Cd (0, 100, 500 and 900 mg L^{-1}).

Main results: Cd exposure at increasing concentrations, decreased root development, (area, length and volume of roots) and activity of enzymatic antioxidants (SOD, CAT and APX) and enhanced MDA. These responses were accentuated by the PGR exposition. The root morphology and activity of antioxidant enzymes presented "hormesis" responses until 500 mg L^{-1} of Cd, and the proline content may have played a fundamental role in the maintenance of metabolic activities and biomass.

Research highlights: The results indicate that the use of PGR intensified the toxicity responses caused by exposure to increased Cd level. In addition, stress indicators such as MDA content and antioxidant activity in different organs (root and shoot) of soybean seedlings, responded differently according with the use of PGR under exposure of Cd.

Additional key words: antioxidant activity; heavy metals; morphology; synthetic hormones; *Glycine max.*

Abbreviations used: ABA (abscisic acid); APX (ascorbic peroxidase); CAT (catalase); GA (gibberellic acid); IBA (indolebutyric acid); MDA (malondialdehyde); PGR (plant growth regulator); ROS (reactive oxygen species); SOD (superoxide dismutase); TBA (thiobarbituric acid).

Authors' contributions: Conceived, designed and performed the experiment: GSF and HRS. Analysis of the experiments and interpretation of data: IBR and ALM. Coordinated the research, analyzed the data and wrote the paper: SCB.

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Introduction

Heavy metals such as cadmium (Cd) are considered environmental pollutants that threaten all forms of organisms (Sytar *et al*., 2013; Yan *et al*., 2016) and are available naturally in the environment or through anthropic sources (Sabiha-Javied *et al*., 2008; Wuana & Okieimen, 2011). Many industrial activities are recognized as important sources of Cd contamination in agricultural soils (Bermudez *et al*., 2010; Salazar *et al*., 2012). Cultivated areas contaminated by Cd directly impacts the growth and seeds quality of large crops (Vollmann *et al*., 2015). This happens because heavy metals are persistent in the environment and may be

transported through soils (Zhou *et al*., 2013). Plants accumulating heavy metals from soils in which they grow may either store these metals in root or translocate them to the biomass aboveground, entering in the food chain (Zhou *et al*., 2013; Vollmann *et al*., 2015).

In plants, the presence of Cd influences several physiological and biochemical processes. Excessive Cd levels results in an overproduction of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($O_2^{\bullet}$). The overproduction of ROS can lead to lipid peroxidation, macromolecule deterioration, ions leakage, membrane degradation, and DNA chain cleavage (Trivedi & Ansari, 2015).

ROS in excessive concentrations are considered dangerous molecules for plant metabolism. However, it has been shown that ROS can also play an important role in plant defence against pathogens and the signalling of molecules, which contribute to the control of plant development and the perception of the external environment (Smirnoff, 2005). A complex enzymatic network of antioxidant molecules controls the concentration of ROS and the repair of oxidative damages. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase and glutathione reductase play an important role in reducing the production of ROS and protecting the cell from damage (Prasad & Strzalka, 2002; Smirnoff, 2005; Emamverdian *et al*., 2015).

Studies have shown that ROS production is directly related to the action of plant growth regulators (PGR), such as the hormone gibberellin, auxin and cytokinin (Xu *et al*., 2006; Krishnamurthy & Rathinasabapathi, 2013; Yan *et al*., 2016). PGR are well recognized for regulating processes related to growth and development, and their levels are rapidly altered by environmental fluctuation. In addition, the action of growth regulators has been questioned of being the connecting link regulating the level of ROS and its role in damage or in triggering signalling pathways in plants under stress (Krishnamurthy & Rathinasabapathi, 2013). The production of ROS under stress conditions may influence biosynthesis, transport, metabolism and signalling of auxin (Tognetti *et al*., 2012). Abscisic acid (ABA) may increase the activation of SOD and CAT enzymes (Jiang & Zhang, 2001; Agarwal *et al*., 2005) and inhibit the transport and translocation of Cd (Hsu & Kao, 2003). Further, ABA may act synergistically with cytokinin on the physiological regulation in plants under stress caused by Cd (Ghanem *et al*., 2008).

Soybean (*Glycine max* (L.) Merrill) is one of the most important protein crops in the world for the production of oil and food. The high demand of soybeans has resulted in this crop being cultivated in the vicinity of routes with heavy vehicular traffic and areas close to human activity such as industrial centres (Rodriguez *et al*., 2011). The soybeans cultivation in polluted areas and in soils contaminated with heavy metals can result in plants with alterations in growth, development and yield (Hasan *et al*., 2009; Piršelová *et al*., 2011), which can impact grain yield and quality and consequently, human health (Alam *et al*., 2003).

The exogenous application of natural and synthetic regulators is known for contributing to the development of plants under a variety of abiotic stresses (Alonso-Ramirez *et al*., 2009; Farooq *et al*., 2017). New alternatives which seek to increase crop tolerance to stresses caused by the presence of heavy metals need to be studied, in order to find workable solutions for productivity in contaminated soils. Therefore, the aim of this study was to evaluate the effects of different concentrations of Cd on the growth and physiology of soybean seedlings, and to verify the potential attenuating effect of oxidative stress by the exogenous application of PGR. It was hypothesized that seedling growth and antioxidant activity stressed by high concentrations of Cd are attenuated by the application of PGR.

Material and methods

Seeds of soybean cultivar 'RK 7214 IPRO' were sterilized in 2% sodium hypochlorite solution for 5 min and then washed in distilled water 5 times. After that, the seeds were soaked for 12 h at room temperature, in distilled water (control treatment) and in solution with plant growth regulator (PGR treatment) in dose recommended by the manufacturer (5 mL L^{-1}). The pre-treatments followed the method of Liu TT *et al*. (2011) and Liu L *et al*. (2018). The PGR used in this study was a commercial product composed of three plant regulators in the following concentration: 0.005% of indolebutyric acid (IBA, auxin analogue), 0.009% kinetin (cytokinin) and 0.005% gibberellic acid (GA, gibberellin). After 12 h, the seeds were germinated in two layers of rolled filter paper moistened with distillated water and placed in transparent plastic bags. The plastic bags were conditioned in chamber type Mangelsdorf with temperature controlled at 25 °C in the dark, and left to grow until the radicle reached 3-8 mm in length (Piršelová *et al*., 2011). Uniformly germinated seeds were selected from control and PGR treatments, collected and transferred to in acrylic boxes (Gerbox®) with fresh Germitest® paper moistened with four concentrations of $CdCl_2.H_2O$ (0, 100, 500 and 900 mg L^{-1}). These con-

centrations were previously defined based on dose-response curve plotted from pilot experiments, conducted prior to the study, using a wide range of Cd levels (5 mg L^{-1} to 1 g L^{-1}) (Neumann *et al*., 1998; Kuriakose & Prasad, 2008). The seedlings were grown for seven days in chamber type Mangelsdorf with temperature controlled at 25 °C in the day/night, photoperiod of 12/12 h and white light of 670 lux. After that the growth and biochemical parameters of the seedlings were analysed.

The experimental design was completely randomized composed of 4 doses of Cd (0, 100, 500 and 900 mg L^{-1}) and two treatments, one with distilled water (control) and another with application of PGR.

Seedling growth

To obtain the length, area and volume of the roots, the seedlings were stained in methylene blue (1% solution) for 15 sec and then photographed with a digital camera fixed at a distance of 30 cm. To improve the image quality and to eliminate the effect of shading, the roots were positioned on a Negatoscope (Ultra Slim LED, Biotron). The images were analysed by Safira® software (Embrapa, Stonway). The seedlings were stored in labelled paper bags and placed in a ventilating oven at 60 °C until constant dry masses could be observed.

Biochemical analysis

The seedlings had their roots excised, the shoots and roots were immediately immersed in liquid nitrogen for rapid freezing and stored in falcon tubes. Frozen tissue of shoot and root was sampled and its extract for the enzymatic analyses were obtained in 5 mL of 0.1 M potassium phosphate buffer (pH 7.8).

The activity of the enzyme SOD was determined by the addition of supernatant extract to sodium phosphate buffer (pH 7.8) containing methionine, nitro blue tetrazolium (NBT), EDTA and riboflavin. The analyses were performed after the samples react in a chamber at 25°C under 15W fluorescent light for 5 min. The reaction was measured spectrophotometrically at 560 nm of absorbance (Giannopolitis & Ries, 1977).

The activity of CAT was determined by measuring the decrease of H_2O_2 in absorbance at 240 nm (Peixoto *et al*., 1999). The plant extract was added to potassium phosphate buffer (pH 7.0) mixed with H_2O_2. The absorption variation was calculated after 60 sec, the activity of the enzyme was calculated using a molar extinction coefficient $\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$. The specific activity of CAT took into account the concentration of soluble protein in the test.

The activity of APX was determined from the dilution of plant extract added to potassium phosphate buffer (pH 6.8), containing pyrogallol and H_2O_2. After 1 min the reaction was stopped with H_2SO_4 and the absorbance reading was recorded at 420 nm ($\varepsilon = 2.47\ mM\ cm^{-1}$) (Peixoto *et al*., 1999).

Lipid peroxidation was performed by quantification of the malondialdehyde content (MDA) reagent with thiobarbituric acid (TBA). Plants extract were homogenized in polyvinylpyrrolidone (PVPP) and trichloroacetic acid (TCA). The supernatant was added to TBA and then heated in a water bath at 95 °C for 20 min, and the reaction was stopped in ice bath. Absorbance of the extract was measured by spectrophotometry at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The absorbance coefficient used to determine the concentration of MDA was 1.55×10^{-5} (Gomes-Junior *et al*., 2006).

The proline content was extracted in sulphosalicylic acid, and its concentration was estimated by reacting the extract with a known quantity of supernatant with ninhydrin reagent and measuring its absorbance at 520 nm, according to the methodology described by Bates *et al*. (1973).

Statistical analysis

The reported values were averages of five replicates, with each replicate comprising of 20 seedlings. The data were analysed by two-way ANOVA with treatments (control and PGR) and Cd levels as two factors. Treatment means were compared using Tukey test at a significance level of p=0.05.

A multivariate analysis was performed via principal component analysis (PCA) to verify the grouping of the different plant responses to the treatments studied, taking into account the entire set of parameters measured.

Results

The results indicated that the dry mass of the seedlings, both shoot and roots, were not significantly changed by different Cd levels and PGR treatment (Fig. 1). Considering the root morphology, the seedlings showed a high sensitivity to the Cd concentrations and PGR treatment. In the control treatment, the length, volume and area of the roots, when the seedlings were under the dose of 100 mg L^{-1}, tended to increase compared to 0 mg L^{-1}. However, at dose of 900 mg L^{-1},

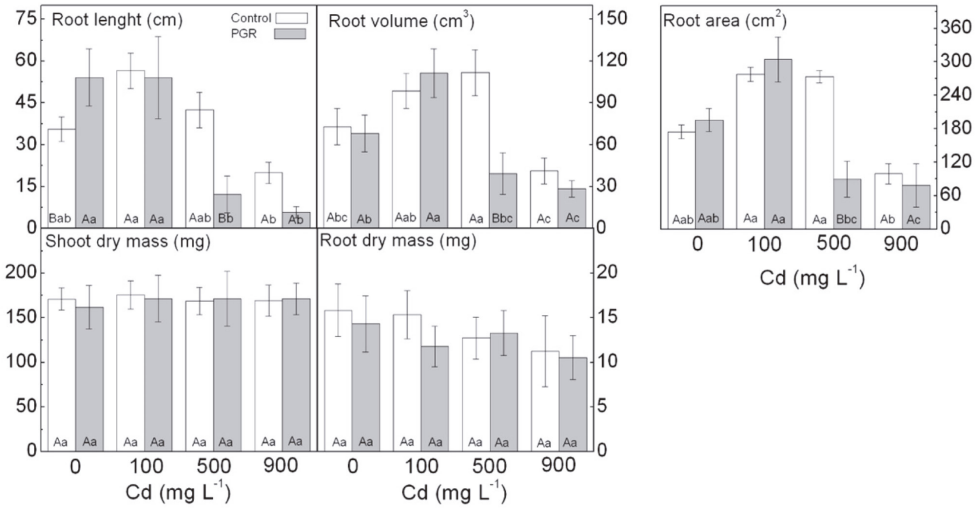

Figure 1. Root length, volume and area, shoot dry mass and root dry mass (mean ± SD) of seedlings exposed to control and plant growth regulator (PGR) treatment and four Cd concentrations (0, 100, 500 and 900 mg L^{-1}). Different capital and lowercase letters indicates $p \leq 0.05$ between control and PGR treatment and the Cd concentrations, respectively.

these same parameters were significantly reduced ($p<0.05$) when compared to the dose of 100 mg L^{-1} (61%, 58% and 64% reduction, respectively, for root length, volume and area). Under the PGR treatment, the length, volume and area of roots decreased at dose of 900 mg L^{-1} compared to the dose of 100 mg L^{-1} (64%, 67% and 74% of reduction, respectively).

Without the application of Cd (dose 0 mg L^{-1}) the root length increased 54% in PGR, compared to the control (Fig. 1) while the roots volume and area were not affected by the same treatment ($p>0.05$). However, under the Cd dose of 500 mg L^{-1} the values of length, volume and root area decreased significantly ($p<0.05$) in the presence of the bioregulator compared to the control treatment (71%, 64% and 67% reduction, respectively).

The lipid peroxidation of the seedlings expressed by the MDA content under control treatment was increased in the shoot at the dose of 500 mg L^{-1} compared to the dose 0 mg L^{-1}, the roots however, showed no significant changes of MDA content to increase of Cd treatment ($p>0.05$). On the other hand, in the PGR treatment, the MDA in the shoot increased progressively as the level of Cd enhanced, while the roots, increased significantly ($p<0.05$) in the doses of 100 mg L^{-1} and 500 mg L^{-1} compared to the dose 0 mg L^{-1}.

Regarding the response of seedlings under control and PGR treatment, the MDA content in the shoot increased by PGR treatment when compared to the control, especially in the Cd doses of 100 and 900 mg L^{-1} (Fig. 2). The MDA in the roots was increased by PGR when compared to the control treatment, but this increase was only significant at doses 100 and 500 mg L^{-1} ($p<0.05$).

The proline content was increased as the level of Cd enhanced ($p<0.05$) in both treatments (control and PGR) (Fig. 2). Regardless of the Cd doses tested, the proline was significantly higher under PGR treatment compared to control ($p<0.05$).

It was observed that the increase in the levels of Cd under the control treatment did not influence the SOD enzyme activity in the shoot. However, under the PGR application, the enzyme activity was reduced concomitantly to the increase of Cd level. In the roots under control treatment, the activity of SOD was significantly reduced as the Cd doses increased, whereas under PGR, the activity of this enzyme did not show significant changes as the level of Cd increased ($p>0.05$).

Compared to the control treatment, the PGR did not influence significantly the activity of SOD in the shoot of the seedlings ($p>0.05$) in all concentrations of Cd tested (Fig. 3). On the other hand, the SOD activity in the roots was reduced by the PGR in 2.8 times in the dose 100 mg L^{-1} of Cd, whereas in the dose of 900 mg L^{-1} it increased 2.2 times when compared to the control treatment.

Compared to the control treatment, the application of PGR significantly reduced ($p<0.05$) the activity of CAT in the roots of seedlings under the doses 0 and 100 mg L^{-1} of Cd, and in the shoot of the seedlings under the 0 mg L^{-1} dose (Fig. 3). On the other hand, at the dose of 100 mg L^{-1} the PGR increased the CAT activity in the shoot (62% higher compared to the control treatment) whereas, in the dose 500 mg L^{-1} the enzyme activity decreased 83% in this same treatment.

Under the control treatment, the dose of 900 mg L^{-1} reduced by 39% the CAT activity in the shoot compared

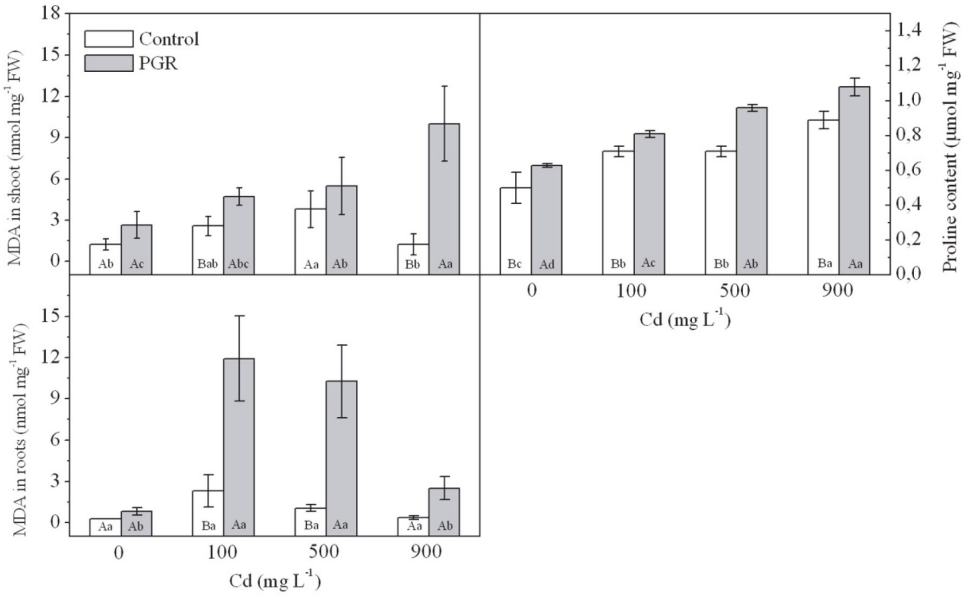

Figure 2. Effects (mean ± SD) of control and plant growth regulator (PGR) treatments on malondialdehyde (MDA) in shoot, MDA in root and proline content of seedlings exposed to 0, 100, 500 and 900 mg L^{-1} of Cd. Different capital and lowercase letters indicates $p \leq 0.05$ between control and PGR treatment and the Cd concentrations, respectively.

to the dose of 0 mg L^{-1}, while the CAT in the roots was reduced by 85% in the dose of 500 mg L^{-1} compared to the 0 mg L^{-1} dose (Fig. 3). In the PGR treatment, the activity of this enzyme was reduced by the dose of 500 mg L^{-1} in the shoot in relation to 0 mg L^{-1}, while in the roots, the CAT activity was reduced in doses ≥100 mg L^{-1}.

The activity of APX in the shoot was reduced by PGR, compared to the control treatment in all levels of Cd tested (Fig. 3). The roots showed a reduction of approximately 90% in the activity of APX under PGR when compared to the control at dose 0 mg L^{-1}.

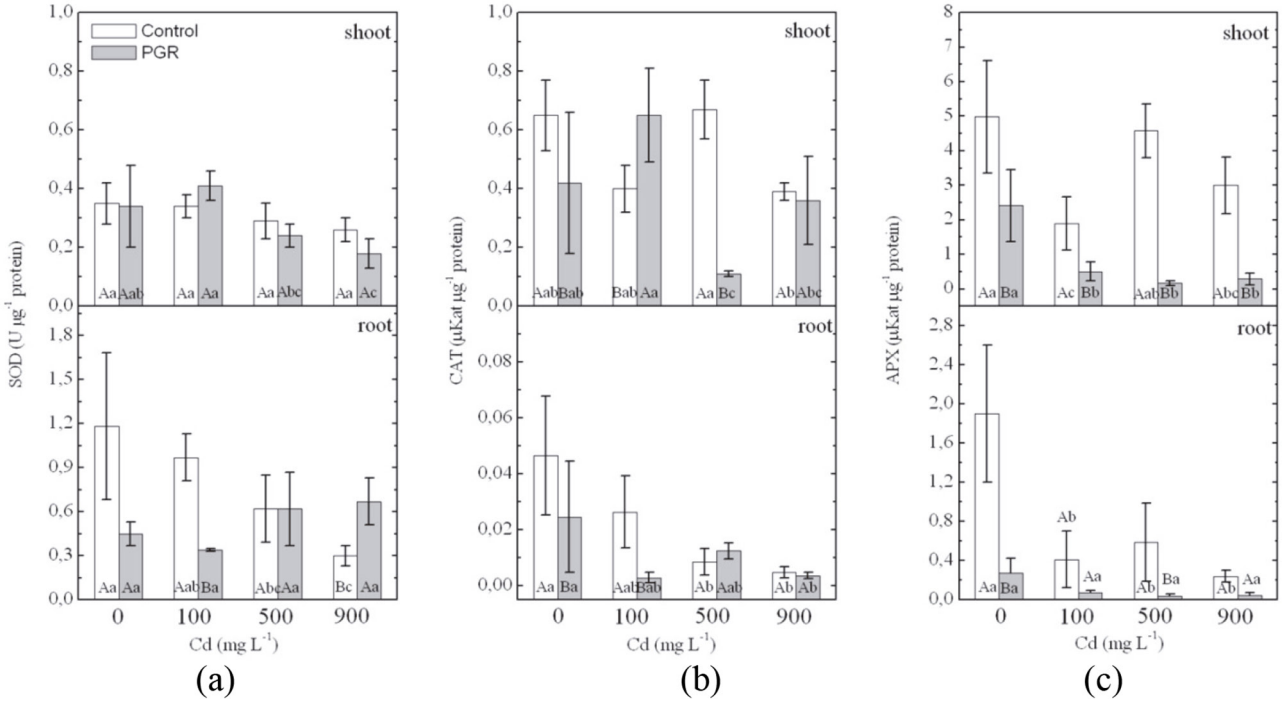

Figure 3. Effects (mean ± SD) of control and plant growth regulator (PGR) treatments on activities of (a) superoxide dismutase (SOD), (b) catalase (CAT) and (c) ascorbate peroxidase (APX) in shoot and root of seedlings exposed to 0, 100, 500 and 900 mg L^{-1} of Cd. Different capital and lowercase letters indicate $p \leq 0.05$ between control and PGR treatment and the Cd concentrations, respectively.

Considering the influence of the Cd levels on the APX activity of the shoot, it was observed that in both treatments (control and PGR) there was a significant decrease in the enzymatic activity in doses ≥100 mg L^{-1}, whereas in the root, the enzymatic activity was significantly reduced (p<0.05) in the control treatment. Under the PGR the APX activity showed no alteration in all the concentrations evaluated (p>0.05).

Principal component analysis (PCA) showed different responses in seedlings when evaluating the Cd levels and treatments (control and PGR). The PCA was performed considering all morphological and biochemistry parameters represented 58.3% of the total variation of the original data (Fig. 4). The component one (PC1) explained 41.5% of the variance, while the component two (PC2) accounted for 16.7%. The variables that contributed most to distinguish the groups in PC1 were proline, root length and APX activity in roots (eigenvector value >0.81), whereas for PC2 they were MDA content in roots, root area and CAT activity in roots (eigenvector values>0.55). The spatial ordination of the replicates allowed the visualization of a group composed by the control treatment seedlings at the dose 0 mg L^{-1} in the top-right quadrant and another group composed by PGR seedlings at doses 500 and 900 mg L^{-1} of Cd in the left quadrant. In the PC2 the replicates clustered in the bottom quadrant representing the PGR treatment at the dose Cd of 100 mg L^{-1}. Seedlings under control treatment at Cd doses of 100 mg L^{-1}, 500 mg L^{-1} and 900 mg L^{-1} and the seedlings of the PGR treatment at Cd doses of 0 mg L^{-1} were overlaid on the graph.

Discussion

The exposure of soybean plants to Cd was documented in previous studies. These responses may include decreases in plant length and biomass, increases in lipid peroxidation, leaf proline content, activity of antioxidant enzymes hydrogen peroxide and nitric oxide (Pérez-Chaca *et al*., 2014; Nasser Alyemeni *et al*., 2017; Marques *et al*., 2019). Nasser Alyemeni *et al*. (2017) showed that the biomass of soybean plants was reduced when grown under concentrations below 150 mg L^{-1} of Cd supplied to pots with sand and vermicompost. However, our results reveal that dry mass of seedling was not influenced by doses above 100 mg L^{-1} and by the PGR. Heavy metal-plant interactions are complex and depend on many factors. Among them are

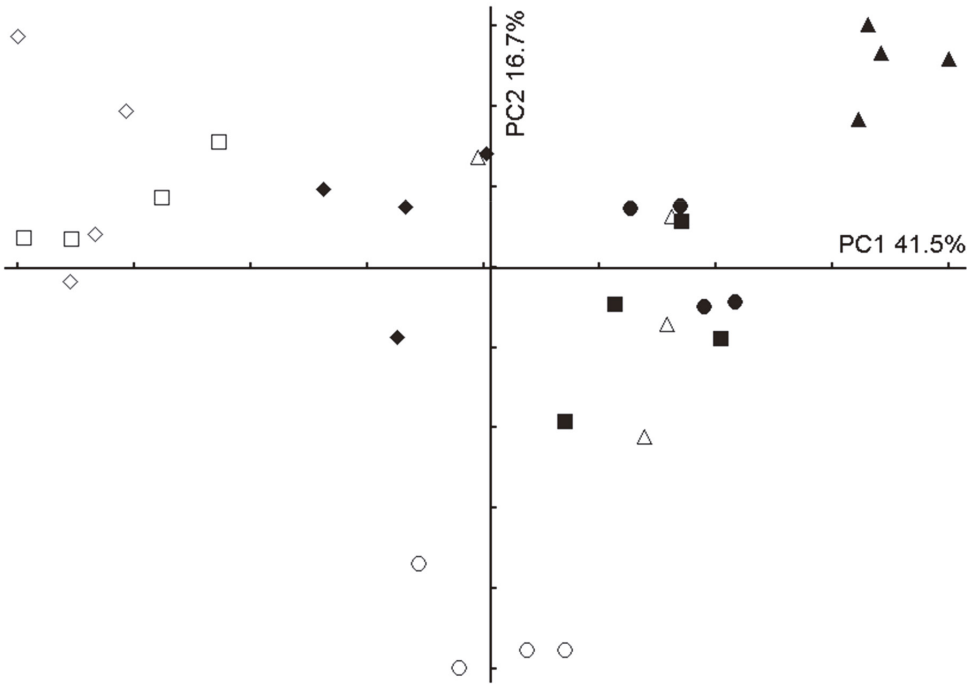

Figure 4. Principal component analysis with all parameters measured (n = 5). Closed symbols indicate control treatment and open symbol indicate plant growth regulator (PGR) treatment. Triangles, circles, squares and diamonds represent 0, 100, 500 and 900 mg L^{-1} $CdCl_2$, respectively. The percentages that explain the data variations in each component are showed on axis. The descending order of three more important traits to group clustering in principal component 1 (PC1) was proline (eigenvector = 0.91), root length (eigenvector = 0.81) and ascorbate peroxidase activity in the roots (eigenvector = 0.81); whereas in principal component 2 (PC2) was malondialdehyde content in roots (eigenvector = -0.57), root area (eigenvector = -0.55) and catalase activity in roots (eigenvector = 0.55).

the cultivar sensibility, the stage of plant development, the chemistry of the metal, including its concentration (Prasad & Strzalka, 2002; Liu L *et al*., 2018). The lack of biomass decreases on our study suggests that soybean in seedling stage is tolerant to the presence of Cd, but it is important take into account that the growing medium (gerbox) may have been another factor influencing this biomass response.

Although seedling dry mass was not reduced by exposure to Cd, an increase tendency in root length, volume and area at the Cd dose of 100 mg L^{-1} compared to the 0 mg L^{-1} was observed in the control treatment. When the seedlings were exposed to 900 mg L^{-1} these same parameters were reduced compared to 100 mg L^{-1}. Other studies have documented this phenomenon, named as "hormesis", defined as low dose stimulation and high dose inhibition, and it has been observed in several organisms (Kumar & Prasad, 2004; Jain *et al*., 2007; Cornu *et al*., 2016) including soybean (Liu *et al*., 2018). In the present work, the hormesis phenomenon also occur in seedlings under PGR treatment, however the inhibition by the Cd occurred in 500 mg L^{-1}.

The application of PGR increased the development of the roots when they were not exposed to the Cd (0 mg L^{-1}). The use of plant growth regulators (as used in our study) were documented for increasing the biomass accumulation and the root system of tomato plants (Cato *et al*., 2013), cotton (Vieira & Santos, 2005) and the productivity of soybeans (Albrecht *et al*., 2012). However, when seedlings of our study were exposed to the 500 mg $L^{-1}$of Cd, the root development was decreased compared to the control, suggesting that the physiological responses to the PGR and the oxidative stress caused by heavy metal were synergistic.

Often photosynthetic capacity can be reduced under stressful conditions, which results in an increase in the electron transport rate of photosystems to O_2 (Souza *et al*., 2004; Yan *et al*., 2012). This response, in turn, may triggers the increase in the production of ROS resulting to lipid peroxidation and an enhance in the lipid peroxidation products, as MDA (Zayneb *et al*.; 2015; Yan *et al*., 2016). MDA has cytotoxic action and when found at high levels indicate an increase in oxidative stress at the cellular level. Our results indicated that the increment in Cd levels increased the lipid peroxidation in the shoot until the dose of 500 mg L^{-1} in both treatments (control and PGR) (Fig. 2). However, under the 900 mg L^{-1} dose, seedlings in the control treatment decreased the MDA content, whereas in PGR treatment this same parameter increased. Additionally, it was observed that the MDA content in both shoots and roots was higher in the PGR treatment. These results indicate that lipid peroxidation, besides being increased by the increment in Cd levels, was also potentiated by the PGR.

Plants are able to develop different survival strategies when exposed to heavy metals (Monni *et al*., 2001a, b). They may be protected against these elements by form a mutualistic symbiosis with mycorrizal fungi that immobilize the metal in the root system (Rask *et al.,* 2019), or can tolerate high concentrations through specific physiological mechanisms such as the antioxidant enzymes SOD, CAT and APX (Prasad & Strzalka, 2002; Emamverdian *et al*., 2015). Among these enzymes, SOD and APX play an important role in the process of cleaning superoxide radicals. However, SOD activity has been recognized to be more important in the regulation of plant cell function on oxidative stress (Nasser Alyemeni *et al*., 2017). Our results showed that under the control treatment, the SOD activity in the roots was inhibited by the exposure to Cd, which did not happen in the shoots, once the reduction of the activity in this organ was observed with the increase of Cd (Fig. 3). On the other hand, under PGR the shoots showed a reduction of the SOD activity with the increment of the Cd doses, whereas in the roots, the activity of the enzyme was not influenced by the metal. These results indicate that plant growth regulators influenced the organ (shoot and root) and the level of SOD activity in response to the Cd levels tested. The reduction of SOD activity in response to the increase of Cd has already been documented in pine roots (Schützendubel *et al*., 2002) and common beans (Somashekaraiah *et al*., 1992).

The reduction in CAT and APX activities on shoots with the increasing of Cd levels (900 mg L^{-1}in control treatment and ≥500 mg L^{-1} in PGR), and the lower values of CAT and APX of roots under the treatment with the PGR, suggest that the plant growth regulator acted antagonistically to the antioxidant activity. These results corroborate the negative influences of the plant growth regulator on the responses to oxidative stress caused by heavy metal as described above in the root morphology and MDA content. The CAT and APX activity were reported to reduce in wheat (Milone *et al*., 2003) and *Brassica juncea* (Nouairi *et al*., 2006) treated with Cd.

Previous studies have shown that exogenous application of plant growth hormones (also called as phytohormones or plant hormones) can improve protection against heavy metal toxicity (Masood *et al*., 2012; Zhu *et al*., 2012; Agami & Mohamed, 2013; Khan *et al*., 2018). Exogenous applications of both natural and synthetic auxins under controlled concentrations show enhanced antioxidant system activity (Piotrowska-Niczyporuk & Bajguz, 2014). Some recent approaches showed that interaction between heavy metal and auxin

can be used as a protective mechanism against toxicity in crop plants or as a useful tool in phytoremediation programs for detoxification of polluted areas (Bücker-Neto *et al*., 2017). Tandon *et al*. (2015) showed that exogenous application of natural and synthetic auxins increased phytoremediation efficiency in waste-water treatment. Pandey & Gupta (2015) demonstrated that exogenous application of auxin in rice seedlings reduced the stress caused by selenium (Se) over the morphological and biochemical characteristics. The toxicity to Cd manifested as a negative effect on seed germination and seedling growth is reported to be relieved by exogenous application of cytokinin which results in an increase of antioxidant enzymes activity (Gangwar *et al*., 2010, 2014; Khan *et al*., 2018). On the other hand, exogenous application of GA in low concentrations counteracts some of the adverse effects of chromium phytotoxicity with the increased levels of antioxidants, whereas high concentrations of GA showed apparently reverse effects under Cr phytotoxicity (Gangwar *et al*., 2011). These studies reveal that the exogenous application of plant hormones to prevent growth inhibition and increase heavy metal tolerance rely on several processes, and are still poorly understood, especially when these substances are applied concomitantly.

One possible explanation for the variations in antioxidants enzymes activity, in response to Cd increment in our study, may be related to the exposure time of the seedlings to the metal. Studies have shown that antioxidant activity may play an effective role in short-term stress, but not in the long term (Dazy *et al*., 2008; Yan *et al*., 2016). Stroiński & Kozłowska (1997) observed that SOD, CAT and APX activity can be reduced after one hour of exposure to Cd. The decreases observed at high Cd concentrations can be interpreted as a cytotoxicity sign due to overproduction of ROS (Dazy *et al*., 2008). Another possible explanation is the excess of superoxide radicals that disturb the signal transduction and triggers the genes related to the production of antioxidant enzymes (Lamb & Dixon, 1997). On the other hand, Romero-Puertas *et al*. (2004) showed that the CAT transcripts may be inducted by Cd while de CAT activity is decreased. They assumed that Cd induce posttranslational modifications of CAT.

A third possible explanation for the antioxidant activity reduction is the binding of heavy metal ions to the enzyme active center. The Cd may can substitute Fe ions in active site of SOD and CAT decreasing the enzyme activity (Stroiński & Kozłowska, 1997; Štolfa *et al*., 2015). In addition, Cd probably interact with the thiol group in SOD structure, inactivating them (Shah & Nongkynrih, 2007; Štolfa *et al*., 2015; Nasser Alyemeni *et al*., 2017). The limited antioxidant activity caused by different concentrations of Cd has been observed in *Petroselinum crispum* seedlings (Ulusu *et al*., 2017), wheat (Dey *et al*., 2007) and roots of *Pinus sylvestris* (Schützendubel *et al*., 2002).

Once the enzymatic antioxidants can be activated under short-term Cd stress, non-enzymatic processes may have a long-term effect in scavenging for ROS (He *et al*., 2011). The increase of compatible organic osmolytes in the cytosol has been recognized as a tolerance response of plants to environmental stresses by mediating the maintenance of the cell water content (Tester, 2003). High proline content plays a key role in osmotic adjustment, cellular sub-structures stabilization (*e.g*., membranes and proteins), scavenging free radicals and buffering cellular redox potential (Kavi Kishor *et al*., 2005; Hayat *et al*., 2012). As a response to stress and oxidative damage, the proline content in our study was increased as Cd levels enhanced which, in turn, may have contributed to reduce the lipid peroxidation of shoot under control treatment and root under PGR treatment. The accumulation of proline in plants tolerant to high doses of Cd has already been documented for other species including soybean (He *et al*., 2011; Nasser Alyemeni *et al*., 2017). However, the role of free proline in the maintenance of metabolic activities of soybean seedlings under Cd stress must be further investigated once this amino acid is associated with the ROS scavenger and as a heavy metal chelator (Hayat *et al*., 2012).

Principal components analysis performed using all growth parameters reinforces the synergistic effect of the bioregulator and oxidative stress caused by Cd. The PCA indicated that PGA treatment in seedlings which were not exposed to Cd and exposed to the minimum dose of Cd (100 mg L^{-1}) promoted responses similar to seedlings of intermediate and maximum levels of Cd (500 mg L^{-1} and 900 mg L^{-1}) in the control treatment. While the seedlings under PGR treatment in intermediate and maximum doses of Cd were those that showed the highest levels of toxicity to this heavy metal.

Bioregulators play an important role in regulating the physiology of stress. However, plant tolerance to Cd appears to be regulated not only by a single hormone, but by the complex combination of multiple plant hormones (Yan *et al*., 2016). In addition, the components of the bioregulator tested in this study (IBA, gibberellin and cytokinin) may have interacted with each other, and also with other endogenous hormones produced by the seedlings. Our results indicated that the combination of these substances may have affected synergistically the oxidative stress caused by Cd exposure, although proline levels were increased

with the PGR treatment. Signaling of plant growth regulators to stress responses is possibly linked to redox reactions (Jiang & Zhang, 2001), but the relationship of these substances with ROS and antioxidant enzymes in signal-transduction cascades is unclear.

This study showed that exposure of Cd at different concentrations influenced the morphological and physiological parameters of soybean seedlings. Although some of the parameters have shown "hormesis" responses to Cd increase (antioxidant activity and root morphology parameters), the results indicated that the increase in proline content may have played a key role in the maintenance of metabolic activities and maintenance of biomass. Variations in root development (length, volume and area) indicated a structural reorganization of the tissues according to the concentration of Cd. In addition, the results showed that the use of the bioregulator behaved synergistically to the toxicity responses caused by the Cd doses, invalidating our hypothesis. Stress indicators such as MDA content and antioxidant activity in different organs (root and shoot) of soybean seedlings may present a high complexity regarding the use of products based on plant growth hormones in environments contaminated with Cd.

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