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Nutritional and biochemical changes induced by lead in sunflower (*Helianthus annuus* L.)

Distúrbios nutricionais e bioquímicos induzidos pelo chumbo em girassol (*Helianthus annuus* L.)

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Abstract

The aim of this study was to evaluate the effects of increasing concentrations of lead (Pb) in the nutrient solution on growth and macronutrient and organic solute contents of *Helianthus annuus* plants. The experimental design was completely randomized with four replications. The dry mass yield was not affected by the treatments up to a Pb concentration of 0.6 mM, indicating that *H. annuus* was tolerant to this Pb concentration in the growth medium. The reductions in leaf, stem and root dry masses at a Pb concentration of 0.8 mM were 74, 84 and 85%, respectively. Lead stress did not reduce the levels of nitrogen (N), phosphorus (P) and potassium (K), indicating that the growth reduction observed at 0.8 mM Pb could not be attributed to deficiencies of these nutrients. On the other hand, Pb significantly reduced calcium (Ca) and magnesium (Mg) contents in leaves, stems and roots, which might, at least in part, explain the Pb-induced growth reduction in the *H. annuus* plants. Pb increased soluble carbohydrate, free amino acid and proline contents in leaves, and soluble protein and proline contents in roots, showing stress-induced changes in cell metabolism. The data also suggest that Ca and Mg concentrations may be used as nutritional indicators and the proline content may be used as a biochemical indicator of Pb toxicity in *H. annuus*.

Key words: Lead. *Helianthus annuus.* Mineral nutrition. Heavy metal. Organic solutes.

Resumo

Objetivou-se com esse trabalho, avaliar os efeitos de doses crescentes de chumbo (Pb) na solução nutritiva sobre o crescimento e teores de macronutrientes e solutos orgânicos em plantas de *Helianthus annuus*, em casa de vegetação. O delineamento experimental foi o inteiramente casualizado, com quatro repetições. A produção de massa seca não foi afetada até o tratamento de 0,6 mM indicando que o *H. annuus* é tolerante até esta concentração de Pb no meio de cultivo. As reduções nas massas secas das folhas, caule e raízes foram de 74, 84 e 85%, respectivamente. O estresse por Pb não reduziu os teores de nitrogênio (N), fósforo (P) e potássio (K), indicando que a redução do crescimento induzida pelo Pb não pode ser atribuída às deficiências destes nutrientes. Por outro lado, o Pb reduziu significativamente os teores de cálcio (Ca) e magnésio (Mg) nas folhas, caule e raízes, o que pode, ao menos em parte,

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explicar a redução do crescimento induzida pelo Pb nas plantas de *H. annuus*. O Pb aumentou os teores de carboidratos solúveis, aminoácidos livres e prolina nas folhas e os de proteínas solúveis e de prolina nas raízes, evidenciando as alterações no metabolismo celular decorrentes do estresse. Os dados também sugerem que os teores de Ca e Mg podem ser utilizados como indicadores nutricionais e os de prolina como indicador bioquímico da toxidez por Pb em *H. annuus*.
Palavras-chave: Chumbo. *Helianthus annuus.* Nutrição mineral. Metal pesado. Solutos orgânicos.

Introduction

Heavy metal pollution has become one of the major environmental problems worldwide (MALAR et al., 2014). It is particularly difficult to remediate soil, water and air of metal pollutants because, unlike organic pollutants, which may be degraded into harmless small molecules, toxic elements, such as lead (Pb), mercury (Hg), cadmium (Cd), copper (Cu) and zinc (Zn), are immutable by biochemical reactions (MALAR et al., 2014). The heavy metals are defined as metals with a density higher than 5 g cm^{-3} (GASIC; KORBAN, 2006).

Excess lead in plants can cause various symptoms of toxicity, such as reduced growth, chlorosis, browning of the root system, inhibition of photosynthesis and changes in mineral nutrient concentrations (SHARMA; DUBEY, 2005). As a result of toxicity caused by the pollutant action, inhibition of cell activity or damage to the cell structure may occur due to heavy metal interference with some essential nutrients (COUTINHO; BARBOSA, 2007). Responses to stress range widely, depending on the plant species, the toxic element, and the environmental conditions (SOUZA et al., 2011).

At high soil concentrations, Pb replaces elements such as potassium (K) and calcium (Ca) in aluminosilicates, particularly feldspar, metals and sulfides (BOSSO; ENZWEILER, 2008). When soil contains large amounts of metals, their uptake by plants is mainly affected by the bioavailability fraction (VAMERALI et al., 2010). In the case of lead, the uptake occurs either in an ionic form (Pb^{2+}) or via a passive mechanism (KABATA-PENDIAS; PENDIAS, 2011).

Heavy metal ions are toxic to plants at the micromolar level and can induce a variety of physiological and biochemical changes, such as impairments of the membrane function, enzyme activity, hormonal balance, mineral nutrition, photosynthesis, translocation and water relations, which lead to metal-induced growth reduction (POSCHENRIEDER; BARCELÓ, 2006).

Thus, several authors have reported changes in macronutrient contents in plants grown under Pb stress (AKINCI et al., 2010; AUGUSTO et al., 2014; BERTOLI et al., 2011; HUANG; CUNNINGHAM, 1996; KIBRIA et al., 2009; LAMHAMDI et al., 2013; PAIVA et al., 2002, 2003) and in organic solutes in plants grown in the presence of Pb (BHARDWAJ et al., 2009; LAMHAMDI et al., 2013). However, little research has been conducted on the damage caused by Pb to physiological and biochemical processes in sunflower (*Helianthus annuus* L.).

H. annuus is a dicot of the family Compositae, originally from North America, which possesses important agronomic traits, such as tolerance to high and low temperatures and adaptation to different soil and climate conditions (CASTRO et al., 1997). The global production of *H. annuus* grain has grown in recent years, reaching in 2013-2014 42.9 million tons. This is due to sunflower seed and oil characteristics, which have a number of applications in the food industry. In 2013-2014, the cultivated area for sunflower was 145,700 ha, and the production reached 232,700 tons in Brazil (CONAB, 2014). The entire plant can be used for forage, silage, and green manure (EVANGELISTA; LIMA, 2001).

Determination of heavy metals in soil, plants, and human beings, and knowledge of their toxicity are of great importance for the utilization of food and economic crops (MACÊDO; MORRIL, 2008).

Thus, this study aimed to evaluate the effect of increasing concentrations of lead on growth and contents of macronutrients and organic solutes in *H. annuus* plants to gain a better understanding of the nutritional and biochemical effects of Pb on this crop.

Materials and Methods

Growth and treatment conditions

The experiment was carried out in a greenhouse of the Universidade Federal do Recôncavo da Bahia (UFRB), Cruz das Almas, Brazil, from April to May 2013.

Seeds from *H. annuus* genotype Olisun-05 were sown in 200-mL plastic cups containing washed sand and irrigated daily with distilled water. Seedlings (12-day-old) were transferred for acclimatization to trays containing 12 L of aerated Hoagland and Arnon (1950) nutrient solution. Treatments were applied five days later and consisted of a no-lead control and four different lead concentrations in the nutrient solution (0.2, 0.4, 0.6 or 0.8 mM), referred to thereafter as $Pb_{0.0}$ through $Pb_{0.8}$. Lead was applied as $Pb(NO_3)_2$, and each treatment contained four replicates, resulting in a total of 20 experimental plants. The experiment was performed in duplicate in order to obtain samples for biochemical and growth analyses, respectively. Lead added to the nutrient solution was chelated with EDTA to avoid precipitation with sulfate and phosphate ions. The volume of the nutrient solution was made up daily with distilled water, and the pH was maintained at 5.5 ± 0.5 by adding NaOH or HCl.

After 16 days of Pb stress, leaf and root samples of four plants from each group were cut, frozen in liquid nitrogen, and lyophilized for further biochemical analyses. The remaining four plants were harvested, separated into leaves, stems and roots, put in paper bags and dried in an oven at 65 °C for 72 h for the determination of leaf (LDM), stem (SDM) and root (RDM) dry masses using a semi-analytical balance. The dried plant material was ground to be analyzed for nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and lead (Pb).

Extract preparation and nutrient and biochemical analyses

For analysis of nutrient concentrations, extracts were prepared by wet acid digestion in a mixture of concentrated sulfuric acid (H_2SO_4) and 30% hydrogen peroxide (H_2O_2), as described by Jones (2001). The digested samples were adjusted to a volume of 100 mL with deionized water to carry out the analysis of N, P, K, Ca, Mg, and Pb.

Determination of N and P was performed spectrophotometrically by the phenol-hypochlorite (WEATHERBURN, 1967) and molybdo-vanadate (FAITHFULL, 2002) methods, respectively. Concentrations of Pb, K, Ca, and Mg were determined simultaneously by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Varian, VISTA-PRO).

For biochemical analysis, extracts were prepared by homogenization of 1.0 g of lyophilized leaf and root powders with a mortar and pestle in 5 mL of 100 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA. The homogenate was filtered through muslin cloth and then centrifuged at 12,000 ×*g* for 15 min. The supernatant fraction was kept in an ultra freezer (-80 °C) and used for the determination of organic solutes.

Soluble carbohydrates, free proline, free amino acids, and soluble proteins were determined according to the methods described by Dubois et al. (1956), Bates et al. (1973), Yemm and Cocking (1955), and Bradford (1976), respectively.

Experimental design and statistical analysis

The experimental design was completely randomized, with five treatments of four replicates

each. The treatment effects were evaluated by analysis of variance ($p < 0.05$) and, in the case of significance, a regression study was performed using the statistical program SISVAR (FERREIRA, 2003).

Results and Discussion

Growth

The dry mass production in all parts of the *H. annuus* plants remained unchanged up to a Pb concentration of 0.6 mM. However, it abruptly decreased in the treatment with 0.8 mM Pb, and the reductions in the LDM, SDM, and RDM were 74, 84, and 85%, respectively (Table 1). Compared with the other plant organs, the roots showed the highest reduction in the dry mass as a result of Pb stress, probably because they are the only part directly exposed to the toxic metal. The reduction in root growth can affect the growth of the whole plant due to limited water and nutrient absorption (PEREIRA et al., 2013).

Table 1. Leaf (LDM), stem (SDM) and root (RDM) dry mass yields of *H. annuus* plants after 16 days of growth in a greenhouse with different concentrations of $Pb(NO_3)_2$ in the nutrient solution. The values indicate the mean of four replicates ± SD. The values in parentheses represent the percentage of reduction relative to the control.

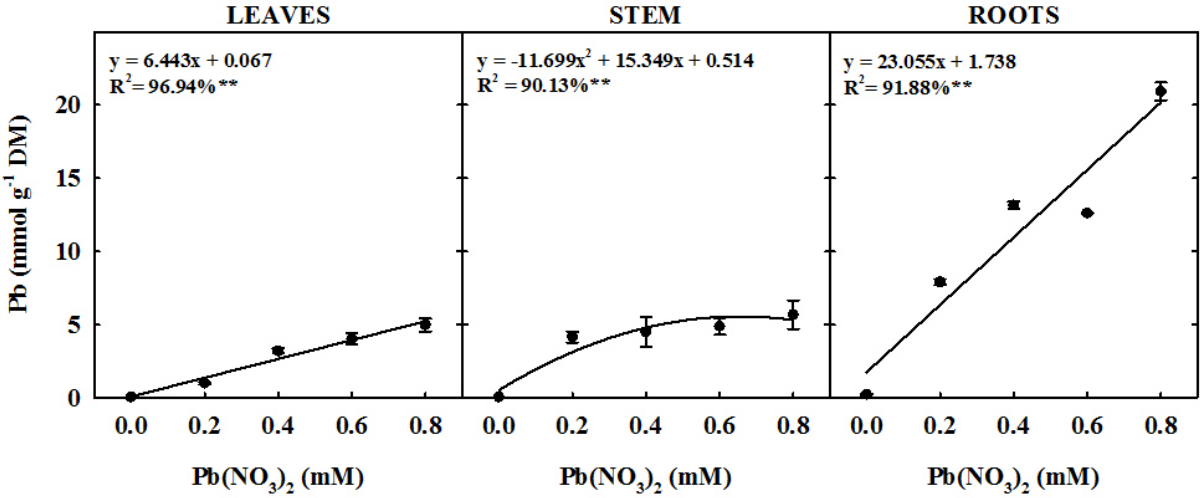
Levels of Pb (mM)	LDM	SDM	RDM
0.0	3.995 ± 0.811	2.832 ± 0.160	2.443 ± 0.463
0.2	3.649 ± 0.283 (9)	2.599 ± 0.150 (8)	1.827 ± 0.248 (25)
0.4	3.458 ± 0.494 (13)	2.383 ± 0.432 (16)	1.766 ± 0.482 (28)
0.6	3.647 ± 0.221 (9)	2.605 ± 0.253 (8)	1.965 ± 0.116 (20)
0.8	1.054 ± 0.225 (74)	0.455 ± 0.098 (84)	0.372 ± 0.069 (85)

Lead concentrations

The leaf and root Pb concentrations increased linearly, while in the stems they showed a quadratic relationship with the Pb levels in the nutrient solution (Figure 1). The Pb concentrations in the *H. annuus* leaves ranged from 0.067 ($Pb_{0.0}$) to 5.22 mmol g^{-1} of dry weight (DW, $Pb_{0.8}$). The stem concentrations varied from 0.51 ($Pb_{0.0}$) to 5.55 mmol g^{-1} DW ($Pb_{0.66}$), and the root concentrations ranged from 1.74 ($Pb_{0.0}$) to 20.18 mmol g^{-1} DW ($Pb_{0.8}$). Thus, the Pb concentrations in the leaves and roots exposed to 0.8 mM Pb were, respectively, 76 and 11 times higher than those found in the control. In the stems, the highest Pb concentration was detected at a Pb concentration of 0.66 mM in the solution, corresponding to a 9.78-fold increase relative to the control. Comparison of the Pb contents in different parts of the plant showed that the concentrations in the roots were about three times as high as those in the stems and leaves.

Greater accumulation of Pb in roots than in other parts of the plant suggests that roots play an important role in the lead storage (AZAD et al., 2011). It has been shown that lead can be retained in the root cell wall, particularly in the pyrophosphate form (MARSCHNER, 2012). Lead accumulation in roots is considered to be a factor that increases plant tolerance to Pb toxicity, because it prevents the metal translocation to leaves (AZAD et al., 2011). In this study, it could also be observed that the stem Pb concentrations showed a stabilization trend, starting from the treatment with 0.2 mM Pb, indicating a saturation of the Pb retention mechanism in the stem and, consequently, a finite capacity of this organ to act as a metal reservoir, preventing Pb transport to leaves.

Figure 1. Lead (Pb) content in leaves, stems, and roots of *H. annuus* plants after 16 days of growth in a greenhouse with different concentrations of $Pb(NO_3)_2$ in the nutrient solution. *Significant ($p \leq 0.05$); **significant ($p \leq 0.01$).



Greater Pb accumulation in roots compared to shoots was also reported by Romeiro et al. (2007) in *Canavalia ensiformis* and by Andrade et al. (2009) in *Paspalum notatum*. In general, apparent concentrations of Pb in aboveground tissues decrease as the transport distance from the root increases. This is due to a higher concentration of Pb in the root cell walls than in other parts of plants, showing that the transport is restricted by the root endodermis (SHARMA; DUBEY, 2005).

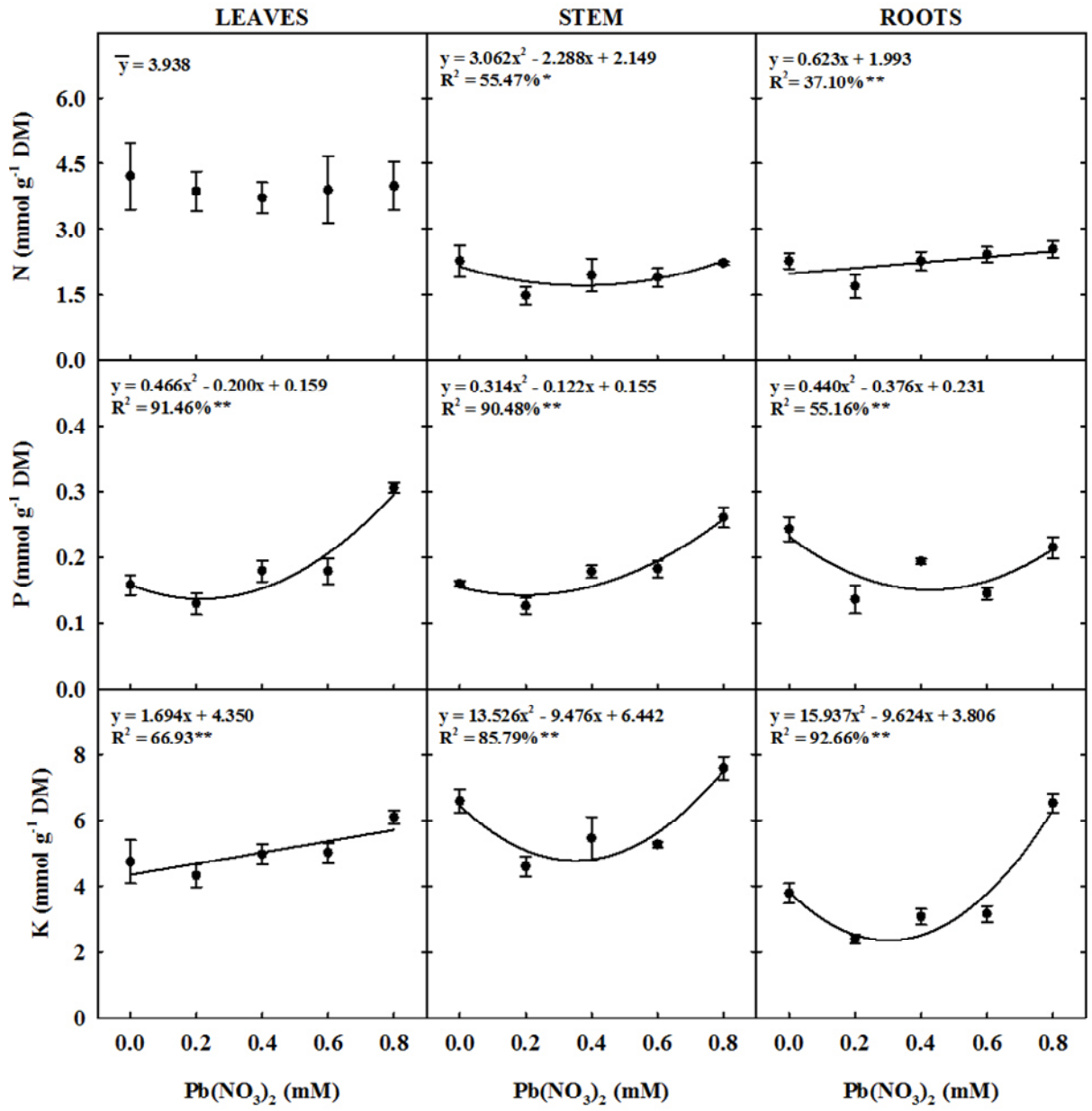
Macronutrient (N, P, K, Ca, and Mg) concentrations

Fig. 2 shows the macronutrient (N, P and K) levels in the leaves, stems, and roots of the *H. annuus* plants at different Pb concentrations in the nutrient solution.

It was found, using the regression equation, that Pb did not induce substantial changes in the N content in different plant parts (Figure 2). Nitrogen is a constituent of various components of the plant cell, including amino acids, nucleic acids, and chlorophylls (TAIZ; ZEIGER, 2013). It is absorbed by roots in the form of nitrate (NO_3^-) and ammonium (NH_4^+), with preferential uptake in the anionic form (nitrate). Considering that the NO_3^-/NH_4^+ ratio in Hoagland solution is 14:1 and that Pb is absorbed in a cationic form, it is likely that the absence of changes in N concentrations observed in this study resulted from the lack of competition between this nutrient and the toxic metal. This may explain why little information is available in the literature regarding changes in N concentrations in plants grown in the presence of Pb.

The leaf and stem P concentrations increased quadratically with the Pb concentration increase in the culture medium (Fig. 2). Thus, the estimated P concentrations in the leaves and stems of the plants exposed to 0.8 mM Pb were, respectively, 87 and 67% higher than those in the control. This increase in the P concentration in shoot tissues, in conjunction with the growth reduction, indicates a concentration effect (IMO, 2012). In contrast, the mathematical simulation indicated that the P content in the plant roots subjected to higher Pb concentration decreased by only 8% (Fig. 2), suggesting that Pb stress did not affect the P translocation from roots to shoots in the *H. annuus* plants. Contrasting results were reported by (BERTOLI et al., 2011; HUANG; CUNNINGHAM, 1996; PAIVA et al., 2003), respectively, for *Lycopersicon esculentum, Ambrosia artemisiifolia*, and seedlings of *Tabebuia impetiginosa*. These authors reported that Pb applications increased P concentrations in roots.

Figure 2. Nitrogen (N), phosphorus (P), and potassium (K) contents in leaves, stems, and roots of *H. annuus* plants after 16 days of growth in a greenhouse with different concentrations of $Pb(NO_3)_2$ in the nutrient solution. *Significant ($p \leq 0.05$); **significant ($p \leq 0.01$).



It has been suggested that increased root P concentrations might be due to the formation of a Pb-P (lead-phosphate) complex, an insoluble form of Pb unavailable for translocation from roots to shoots (HUANG; CUNNINGHAM, 1996). Formation of this complex is expected due to the tendency of the reaction between the Pb^{2+} cations and the $H_2PO_4^-$ anions, the predominant form of P uptake (KABATA-PENDIAS; PENDIAS, 2001). In this study, there was no increase in the P levels in the roots, which might be due to the application of Pb chelated with EDTA to avoid metal precipitation with P in the form of the Pb-P complex.

The stress caused by 0.8 mM Pb significantly increased the K content in all parts of the *H. annuus* plants; however, this effect was more pronounced in the roots. In the leaves, the increase was linear, while quadratic patterns were observed in both stems and roots (Figure 2). Thus, the equations indicated that in the treatment with 0.8 mM Pb the K content increased by 31, 17 and 65% in the leaves, stems and roots, respectively, compared to the control. The data from this study indicated that the increases in the potassium concentration were the result of a concentration effect (IMO, 2012), when taking into account the significant growth reduction in this treatment. Increases in the K content in two *Amaranthus* genotypes grown in the presence of high Pb concentrations were also reported by Kibria et al. (2009).

Considering that Pb stress did not affect the uptake and translocation of N, P, and K, the data from this study indicated that the Pb-induced growth reduction in the 0.8 mM Pb treatment could not be due to the changes in concentrations of these elements in different plants parts.

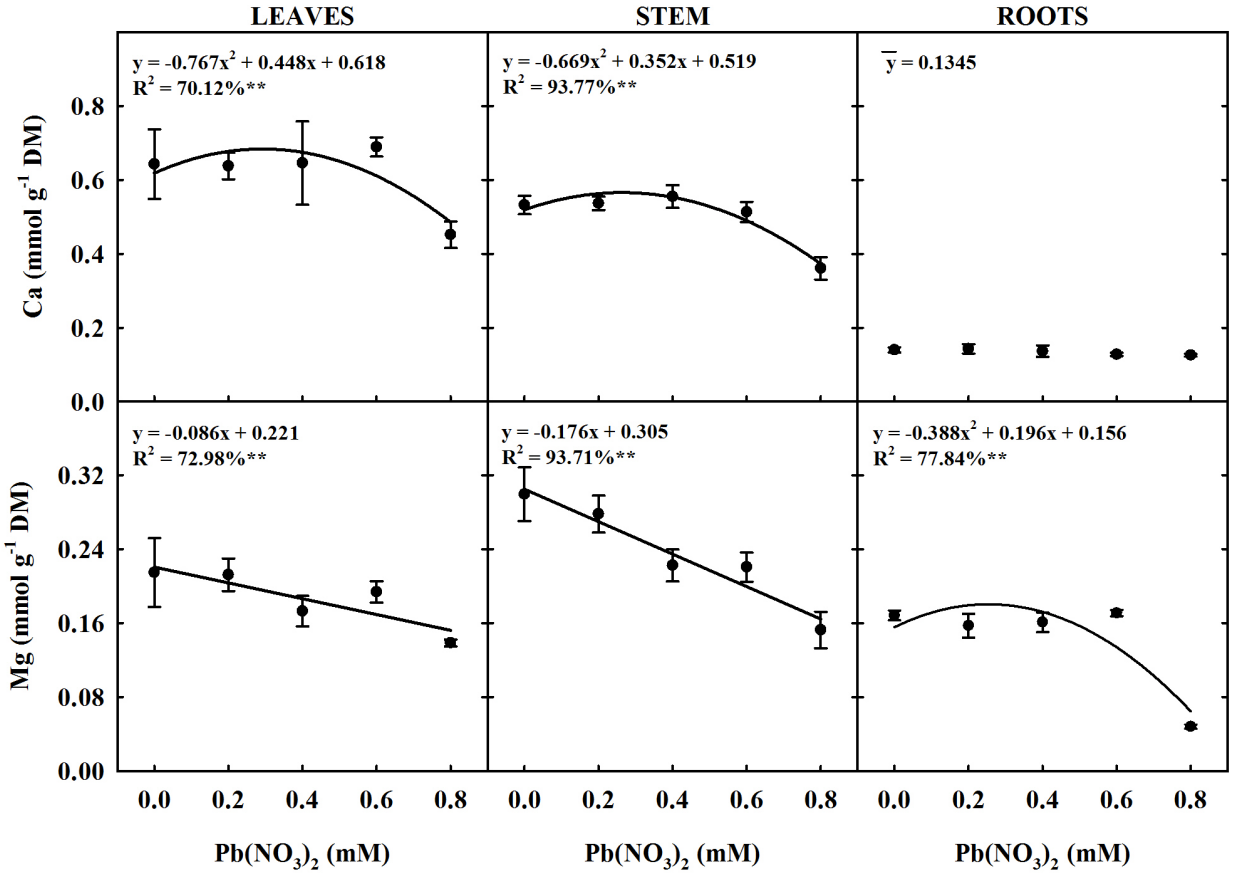
In Figure 3, it can be observed that the Ca concentrations in different plant parts did not change up to a Pb concentration of 0.6 mM. However, the Ca concentrations were reduced by 21 and 28% in the leaves and stems, respectively, of the plants treated with 0.8 mM Pb, showing a negative quadratic effect.

In *Zea mays*, Huang and Cunningham (1996) observed that 20 µM Pb reduced Ca concentrations by 12% in roots and more than 40% in shoots. However, no changes were observed in the Ca content of *Ambrosia . artemisiifolia* (HUANG; CUNNINGHAM, 1996). The application of 288 µM Pb decreased by 35.6 and 18.2%, respectively, Ca concentrations in roots and leaves of *Cedrela fissilis* seedlings (PAIVA et al., 2002), but did not affect levels of this nutrient in roots and stems of *Tabebuia impetiginosa* seedlings (PAIVA et al., 2003). Azad et al. (2011) observed that Pb stress decreased the Ca content in leaves of *H. annuus* seedlings. Augusto et al. (2014) reported that Ca contents in roots and leaves of *Brassica juncea* were significantly affected at lower Pb levels. Małkowski et al. (2005) found that a higher absorption of Ca occurred with increasing Pb accumulation in root tips of *Zea mays*.

According to Marschner (2012), divalent cations, such as Pb, can compete with other cations, such as Ca. The Pb inhibition of the Ca channel can be the result of either the Pb-blockage of the channel or competitive transport of these cations through the channel (MARSCHNER, 2012; SHARMA; DUBEY, 2005). Regardless of the cause, the data from this study indicated that in *H. annuus* the deleterious effect of Pb on Ca nutrition only occurred at Pb concentrations equal to or greater than 0.6 mM.

Calcium is a key element for maintaining cell integrity, because as a divalent ion Ca can form intramolecular complexes and bind molecules to form intermolecular complexes (PILBEAM; MORLEY, 2007). Pb-induced Ca deficiency may cause disturbances in cell division and elongation processes (SHARMA; DUBEY, 2005). Calcium is required for the formation of the mitotic spindle during cell division and for synthesis of the new cell wall in newly divided cells. It is also necessary for the physical integrity and normal function of membranes and, more recently, has been considered as a second messenger for various plant responses to hormonal and environmental signals (HOPKINS; HÜNER, 2009; TAIZ; ZEIGER, 2013). The data from this study suggested that reduced calcium levels could, at least partly, explain the Pb-induced growth reduction in the *H. annuus* plants.

Figure 3. Calcium (Ca) and magnesium (Mg) contents in leaves, stems, and roots of *H. annuus* plants after 16 days of growth in a greenhouse with different concentrations of $Pb(NO_3)_2$ in the nutrient solution. *Significant ($p \leq 0.05$); **significant ($p \leq 0.01$).



A linear negative effect of increasing Pb concentrations was also observed on the Mg contents in the leaves and stems; thereby, the levels of Mg were, respectively, 31 and 46% lower at 0.8 mM Pb than those in the control (Fig. 3). In the roots, the Mg contents showed a negative quadratic behavior and decreased dramatically (by 58%) only in the 0.8 mM Pb treatment. This reduction in the Mg levels could be related to a reduction in the levels of chlorophyll a and b, observed in several species grown in the presence of Pb (GOMES et al., 2014).

Similar to this study, treatment with Pb concentrations below 0.3 mM did not affect the Mg content in the roots of *T. impetiginosa* seedlings (PAIVA et al., 2003). Huang and Cunningham (1996) observed that Pb reduced Mg concentrations in shoots of *Z. mays* and *A. artemisiifolia* plants. Lamhamdi et al. (2013) reported that Pb concentrations higher than 1.5 mM significantly decreased Mg contents in shoots and roots of *Triticum aestivum* and *Spinacia oleracea* seedlings.

In plant cells, Mg ions have a specific role of activating a number of enzymes involved in respiration, photosynthesis and DNA and RNA synthesis (TAIZ; ZEIGER, 2013). In addition, they have a structural role in the chlorophyll molecule, stabilization of proteins, ribosomes, nucleic acids and membranes and are critical in the reactions involving ATP (HOPKINS; HÜNER, 2009). Since Mg is an integral component of chlorophyll and enzyme processes of energy metabolism, its deficiency directly affects the carbon assimilation

and energy transformations (MERHAUT, 2007). Considering that both growth and Mg contents were affected by Pb present in the culture medium, the reduction in the Mg content in all plant parts indicated an antagonistic effect between Mg and Pb at the absorption sites (IMO, 2012). Thus, it is likely that the reduction in the levels of this nutrient is an important factor affecting the metabolism of *H. annuus* plants and, consequently, reducing their growth.

Organic solutes

Changes in the soluble carbohydrate, free amino acid, soluble protein and free proline levels in the leaves and roots are shown in Figure 4.

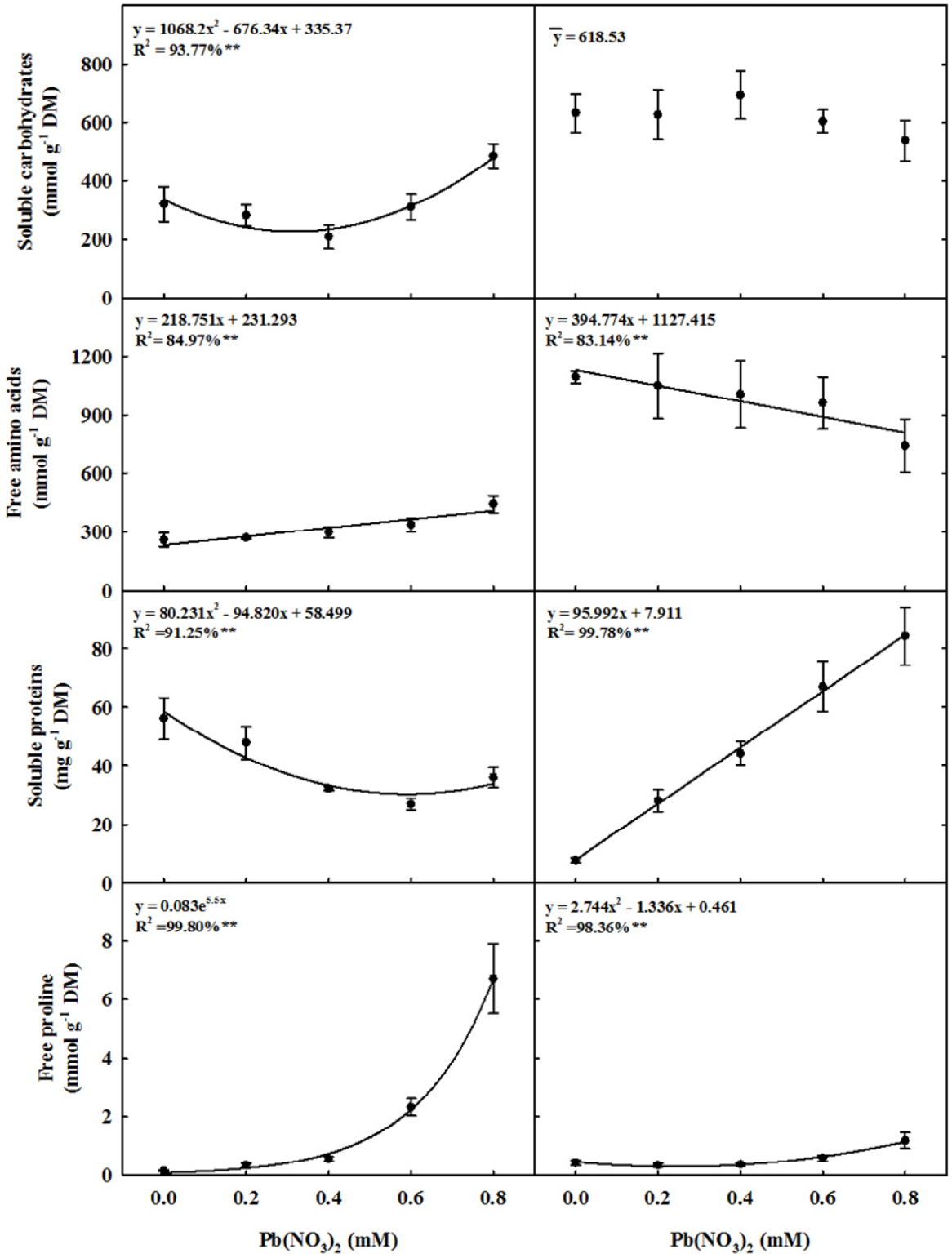
In the leaves, the soluble carbohydrate levels showed a positive quadratic response and increase by 42% at the 0.8 mM Pb level. In the roots, no effect of Pb was observed on the levels of these compounds. Free amino acids increased linearly in the leaves and decreased linearly in the roots. Therefore, at 0.8 mM Pb the equations indicated an increase (by 76%) of amino acids in the leaves and a reduction (by 28%) of amino acids in the roots. The soluble protein levels showed a quadratic behavior in the leaves and decreased by 42% at 0.8 mM Pb, in contrast with the roots where Pb stress induced a linear, about 10-fold, increase compared to the control. The free proline contents quadratically increased in the leaves and roots. Thus, the proline concentrations in the leaves and roots were, respectively, 80 and 1.5 times higher at a Pb concentration of 0.8 mM than those in the control.

Several studies have reported reduction of water content in plants grown under lead stress (PATRA et al., 2004; SHARMA; DUBEY, 2005). In this scenario, an exposure to potentially toxic metal ions induces an osmotic adjustment, mainly due to accumulation of soluble carbohydrates (CHAI et al., 2012; ELLOUMI et al., 2014) and free amino acids (BHARDWAJ et al., 2009). In this work, the stress-induced increases in leaf soluble carbohydrate, free amino acid and proline contents suggested an osmotic adjustment resulting from a decrease in water availability induced by Pb stress. In contrast, the observation that the levels of these solutes in the roots did not increase may indicate limited water absorption by the roots and its flux to the shoots, affecting the water balance in the Pb-stressed plants.

The increase in the leaf amino acid content also suggested a detoxification mechanism through Pb chelation by these compounds. Chelating agents contribute to detoxification and increase plant tolerance to toxic levels of metal ions (GASIC; KORBAN, 2006), and it is already well established that amino acids can be mobilized in response to Pb toxicity to form complexes with this metal (MAESTRI et al., 2010; MANARA, 2012; POURRUT et al., 2011).

The observation that the increase in leaf amino acids occurred simultaneously with the decrease in leaf soluble protein suggested the occurrence of Pb-induced proteolysis. Bhardwaj et al. (2009) also reported that increases of Pb and Cd decreased soluble protein concentrations in leaves of *Phaseolus vulgaris*. On the other hand, the increase in root soluble protein occurred in parallel with the decrease in root amino acids, suggesting that protein synthesis was increased in the roots. Considering that roots are directly exposed to the contaminant, it is likely that this increase is the result of the induction of stress proteins (LAMHAMDI et al., 2010). In this context, metallothioneins and phytochelatins represent the main classes of heavy metal-chelating peptides in plants (MANARA, 2012; POURRUT et al., 2011). Pb stress has also been shown to increase the protein content, including phytochelatins, in *Ceratophyllum demersum* L. (MISHRA et al., 2006).

Figure 4. Soluble carbohydrate, free amino acid, soluble protein, and free proline contents in leaves, stems, and roots of *H. annuus* plants after 16 days of growth in a greenhouse with different concentrations of $Pb(NO_3)_2$ in the nutrient solution. *Significant ($p \leq 0.05$); **significant ($p \leq 0.01$).



Among the organic solutes studied, the proline levels were increased most with increasing Pb concentrations in the nutrient solution, indicating that proline accumulation can be a good biochemical indicator of Pb stress in *H. annuus*. Increased proline biosynthesis has been considered to play an important role in plant tolerance to Pb stress (POURRUT et al., 2011; QURESHI et al., 2007; SHARMA; DUBEY, 2005). Lamhamdi et al. (2013) found that proline contents increased in leaves of *T. aestivum* and *S. oleracea* when the plants were exposed to increasing Pb concentrations. According to Verbruggen and Hermans (2008), proline contents may vary among species, and their values are up to 100 times higher in stressed plants than those in control ones. Proline accumulation is also related to stress tolerance, and concentrations of this amino acid are usually higher in tolerant than in sensitive plants (ASHRAF; FOOLAD, 2007).

There are different opinions regarding the potential mechanisms by which proline reduces the toxic effects of metals, including detoxification of excess ammonia (KAVI KISHOR et al., 2005), osmoprotection and stabilization of proteins (SHARMA; DUBEY, 2005), metal chelation (MANARA, 2012), inhibition of lipid peroxidation and removal of free radicals (WHITE, 2012). Proline can also contribute as an available source of carbon and nitrogen.

Our data showed that the presence of Pb in the nutrient solution significantly changed the concentrations of the major groups of cell organic solutes, indicating significant metabolic changes due to the presence of this toxic metal in the cellular environment. These changes in the metabolism may, at least in part, explain the Pb-induced growth reduction.

Conclusions

The results of this study showed that *H. annuus* tolerates Pb up to a concentration of 0.6 mM in the culture medium and may be a viable alternative for phytoremediation programs where soil contamination with Pb does not exceed this limit. The data also showed that Pb toxicity changed the nutritional balance of Ca and Mg and the concentrations of major groups of cell organic solutes, indicating that the plant growth reduction was the result of Pb-induced metabolic changes. Based on the results, Ca and Mg may be used as nutritional markers and proline may be used as a biochemical marker of lead toxicity in *H. annuus*.

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