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Relationship between manganese toxicity and waterlogging tolerance in *Zea mays* L. cv. Saracura

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ABSTRACT. The objective of this study was to evaluate the relationship between excess manganese and waterlogging tolerance in 18th selection cycle *Zea mays* L. cv. Saracura plants. Maize plants were transferred to plastic receptacles containing solutions with different concentrations of manganese. Leaves and roots were evaluated at the beginning of treatment and after 7, 14, and 21 days for chlorophyll content, biomass production and antioxidant metabolism. Mn was strongly translocated from the roots to the leaves, triggering a decrease in chlorophyll content. Excess Mn promoted an increase in reactive oxygen species that was accompanied by higher levels of antioxidative enzyme activity and lipid peroxidation. *Zea mays* L. cv. Saracura efficiently sequestered relatively large amounts of Mn in the leaves, with a significant impact on metabolism; however, we did not observe visual symptoms or a large decrease in biomass production.

Keywords: hypoxia, chlorophyll, oxidative stress.

Relação entre a toxicidade de manganês e a tolerância ao alagamento em plantas de *Zea mays* L. cv. Saracura

RESUMO. O objetivo desse trabalho foi avaliar a relação entre a toxicidade de manganês e a tolerância ao alagamento em plantas de *Zea mays* L. cv. Saracura do 18º ciclo de seleção. Plantas de milho foram transferidas para recipientes plástico contendo soluções com diferentes concentrações de manganês. Folhas e raízes foram coletadas antes dos tratamentos, 7, 14 e 21 após a imposição dos tratamentos para as análises de conteúdo de clorofilas e carotenoides, produção de biomassa e metabolismo antioxidante. O Mn foi fortemente translocado das raízes para as folhas desencadeando uma considerável redução no conteúdo foliar de clorofila. O excesso de Mn desencadeou uma aumento na produção de espécies reativas de oxigênio, o que foi acompanhado por uma elevação na atividade das enzimas do metabolismo antioxidante e da peroxidação lipídica. *Zea mays* L. cv. Saracura demonstrou ser eficiente no sequestro de uma grande quantidade de Mn nas folhas, com um significativo impacto no metabolismo, no entanto sem o aparecimento de sintomas visuais e sem uma grande redução no seu crescimento.

Palavras-chave: hypoxia, clorofila, estresse oxidativo.

Introduction

Soil waterlogging is a major abiotic stress affecting maize (*Zea mays* L.) grain yields (Yu et al., 2015). Waterlogging is typically caused by poor soil drainage combined with high levels of precipitation (Visser, Voesenek, Vartapetian, & Jackson, 2003) and is becoming increasingly frequent in many regions due to the changing climate. Long periods of soil inundation can have serious economic consequences for maize producers, inhibiting plant growth and resulting in severe yield loss (Bailey-Serres et al., 2012).

The main physiological consequence of waterlogging is oxygen deprivation, which restricts aerobic respiration (Fukao, & Bailey-Serres, 2004). When exposed to prolonged low-oxygen stress, plants typically overproduce reactive oxygen species (ROS), which can cause oxidative damage to plant cells at high concentrations (Shabala, 2011). This damage is the result of ROS reacting with macromolecules, such as proteins, lipids and nucleic acids, leading to a loss of enzyme activity, altered membrane fluidity and genomic damage (Mittler et al., 2004). Efficient antioxidant systems that involve both nonenzymatic and enzymatic molecules can provide some protection against the deleterious effects of ROS (Mittler et al., 2004). For example, superoxide dismutases (SODs) are (uniquely) capable of scavenging O_2^-, producing H_2O_2. Catalase (CAT) degrades H_2O_2 without any reducing power, providing plants with an energy-

efficient way to remove this compound. However, catalase is active only at relatively high concentrations of H_2O_2. At lower concentrations, H_2O_2 molecules are eliminated by ascorbate peroxidase (APX) and other peroxidases with the aid of various reductants, such as ascorbate and glutathione (Gechev, Van Breusegem, Stone, Denev, & Laloi, 2006).

In addition to reducing oxygen availability, water inundation leads a progressive decrease in soil redox potential (Zengin, 2013). Many metal oxides, including iron oxide III and manganese oxide IV, are utilized as alternative electron acceptors. Thus, the concentration of iron oxide II and manganese oxide II increases beyond that required by plants (Khabaz-Saberi & Rengel, 2010). Under these conditions, Mn is easily taken up by the roots, reducing plant growth and altering various physiological processes (Hauck, Paul, Gross, & Raubuch, 2003). For example, a significant increase in iron and manganese concentrations was reported in maize leaves growing in sandy loam soil subjected to 34 days of waterlogging (Ashraf & Rehman, 1999). Excess exposure to metals also leads to chlorophyll degradation, probably as a consequence of the action of ROS on cell membranes (Zengin, 2013). Excess Mn-induced Fe deficiency may also cause reduced chlorophyll concentrations in plants, as Fe is essential for chlorophyll biosynthesis (El-Jaoual & Cox, 1998). Fe is required for the conversion of protoporphyrin IX to protochlorophyllide in chlorophyll biosynthesis (Beale, 1999).

Zea mays L. is one of the most sensitive cultivated species to hypoxia, restricting its production to areas that are not subject to waterlogging. In 1997, the Embrapa (Brazilian Agricultural Research Agency) Maize and Sorghum program launched (after nine cycles of selection) the Maize variety BRS 4154, commonly known as "Saracura". The main characteristic of this cultivar is its high tolerance to waterlogging, principally due to the enhanced development of aerenchyma, which increases oxygen availability to the plant (Alves et al., 2002). Saracura is the result of plant breeding for waterlogging tolerance, which has traditionally targeted traits that increase oxygen availability, prevent oxygen loss from root tissues or improve oxygen transport and storage in the roots (Jackson, & Armstrong, 1999). In contrast, the impacts of ion toxicity caused by waterlogging stress have rarely been studied despite their demonstrated importance (Shabala, 2011).

Improving waterlogging tolerance by targeting plant tolerance to ion toxicities has yet to be fully accepted in the plant breeding community (Huang et al., 2015). Nevertheless, evidence is accumulating that this could be an effective strategy. For example, wheat genotypes with an improved ability to remediate the toxic effects of ions, such as Mn^{2+}, performed better than control genotypes in waterlogged soils (Khabaz-Saberi, Barker, & Rengel, 2012). To our knowledge, there have been no studies showing the adverse effects of the greater availability of manganese due to waterlogging in *Zea mays* L. cultivar Saracura. To address this research gap, we exposed maize plants (Saracura cultivar) to excess manganese and hypoxia and evaluated changes in biomass production, levels of photosynthetic pigments and antioxidant defense systems.

Material and methods

Zea mays L. (cv. Saracura) seeds were germinated on germination paper in a growth chamber (B.O.D. type) for ten days. The paper was first moistened with distilled water, another paper was placed on top, and both papers were made into a roll. The amount of distilled water was determined according Maia et al. (2012) in relation to the weight of the paper (2.5 mL g^{-1} paper). After selection for uniformity in size and vigor, plants were transferred to 10 L plastic containers (33x31x38 - WxHxD) containing a nutrient solution (Hoaglang, & Arnon, 1950). Plants were acclimated for 28 days, after which solutions with increasing concentrations were added in the following order: ¼ strength for 7 days, ½ strength for 7 days, and full strength for 14 days. Plants were then subjected to hypoxic conditions and two treatments: control and excess manganese. The original concentration of the nutrient solution was used for the both the control (2 μM Mn) and the excess Mn treatment, with the latter also exposed to manganese (500 μM Mn). The volume of the nutrient solution was replenished with deionized water on a daily basis. The pH of the solution was also adjusted daily to 5.5 ± 0.5 with NaOH solution (1 mol L^{-1}), and solutions were completely replaced on a weekly basis. All plants were maintained under hypoxia, by aeration suspension, throughout the experimental period.

Evaluations were performed on leaves and roots at the beginning of the experiment and after 7, 14 and 21 days. The experimental design was completely randomized (CRD) using a 2 x 4 factorial scheme: two treatments (control and excess Mn) and four time periods (0, 7, 14, and 21 days), for a total of 8 treatments with five replications. Each experimental plot consisted of five seedlings.

Leaf manganese content was determined according Malavolta, Vitti, and Oliveira (1989). Leaf chlorophyll and carotenoids were determined as described by Lichtenthaler and Buschmann (2001). The plants were divided into roots and shoots, the plant material was dried at 70°C to a constant weight, and the dry weight was measured. H_2O_2 was determined according Velikova, Yordanov, and Edreva (2000). Ascorbate concentration was determined as described by Arakawa, Tsutsumi, Sanceda, Kurata, and Inagaki (1981). SOD, CAT and APX were extracted according Biemelt, Keetman, and Albrecht (1998) and determined according to Giannopolitis, and Ries (1977), Havir, and McHale (1987) and Nakano, and Asada (1981), respectively. Lipid peroxidation was determined by quantification of thiobarbituric acid reactive species, as described by Buege, and Aust (1978).

The data were first assessed for normality using the Shapiro-Wilk test ($p \geq 0.05$): data considered normal were subjected to analysis of variance using the statistical program SISVAR 4.3 (System Analysis of Variance for Balanced Data) (Ferreira, 2011). Non-normal data were transformed and then subjected to analysis of variance, and means between treatments were compared using the Scott and Knott (1974) test at 0.05 probability.

Results and discussion

Zea mays L. plants cv. Saracura grown in nutrient solution for 21 days showed an increase in shoot (Figure 1A) and root (Figure 1B) biomass in both treatments. Nevertheless, 500 μM of Mn in the nutrient solution led to a significant decrease in biomass accumulation. On the twenty-first day, this reduction was 16% in the shoots and 22% in the roots. Thus, dry weight of the shoots and roots was similarly affected by excess Mn, generating a positive correlation of 0.98. Excess Mn had a negative impact on root dry weight by the seventh day. In shoots, this impact was only observable on the fourteenth day. Thus, the adverse effects of Mn toxicity were primarily noticeable in the roots (Figure 1).

Mn concentration (Table 1) increased in plants exposed to excess Mn, with greater accumulation in the shoots than roots. Leaf concentration of Mn was negatively correlated with total chlorophyll (-0.90) and carotenoid (-0.80) concentration and positively correlated with MDA concentration (0.86), APX (0.76) and CAT (0.73) activity. Mn concentration in the roots was positively correlated with MDA concentration (0.77) and CAT (0.76) and SOD (0.79) activity.

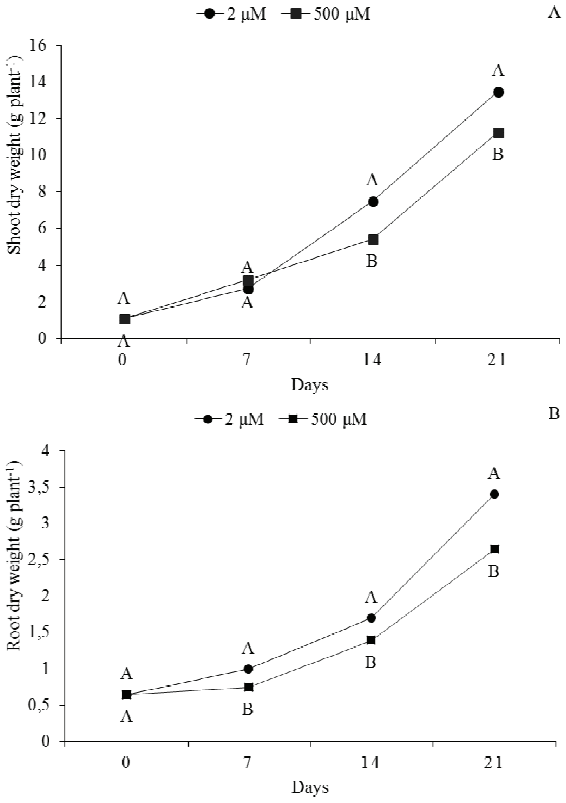

Figure 1. Effects of exposure to excess Mn and hypoxia on shoot (A) and root (B) biomass of maize (*Zea mays* L. cv. Saracura) plants. Different letters indicate significant differences between treatments (2 and 500 µM) in each experimental period ($p < 0.05$).

Table 1 Effects of exposure to excess Mn and hypoxia on manganese concentration in maize (*Zea mays* L. cv. Saracura) plants. Capital letters represent treatments (2 and 500 µM) in each experimental period, and lowercase letters represent plant organs (leaves and roots) for each experimental period.

		Mn concentration mg Kg^{-1} dry weight)	
Time (days)	Treatment	Leaves	Roots
0	2 μM Mn	147.7 ± 6.7 Aa	39.4 ± 2.4 Ab
7	500 μM Mn	147.7 ± 6.7 Aa	39.4 ± 2.4 Ab
14	2 μM Mn	166.6 ± 2.4 Ba	37.8 ± 0.3 Bb
21	500 μM Mn	270.5 ± 10.7 Aa	164.7 ± 3.9Ab
	2 μM Mn	122.5 ±1.4 Ba	38.2 ± 0.9 Bb
	500 μM Mn	612.5 ± 0.3 Aa	173.0 ± 0.6 Ab
	2 μM Mn	113.9 ± 8.1 Ba	37.4 ± 2.5 Bb
	500 μM Mn	622.0 ± 4.0 Aa	134.5 ± 0.7 Ab

Overall, chlorophyll (Figure 2A) and carotenoid (Figure 2B) concentrations were maintained throughout the experimental period in control plants. Plants exposed to excess Mn showed a 42% reduction in chlorophyll and a 40% reduction in carotenoids by the twenty-first day. However, no visual symptoms appeared.

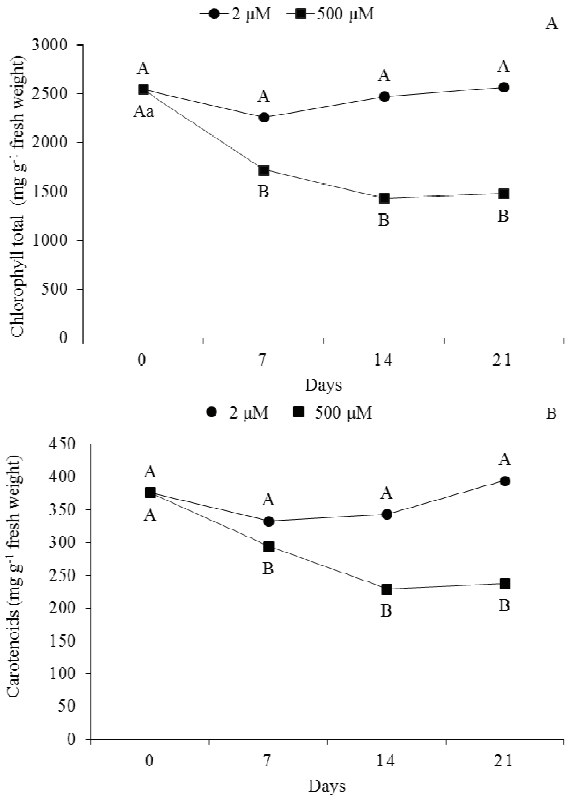

Figure 2. Effects of exposure to excess Mn and hypoxia on the leaf content of chlorophyll a (A), chlorophyll b (B) and carotenoids (C) in maize (*Zea mays* L. cv. Saracura) plants. Different letters indicate significant differences between the treatments (2 and 500 µM) in each experimental period ($p < 0.05$).

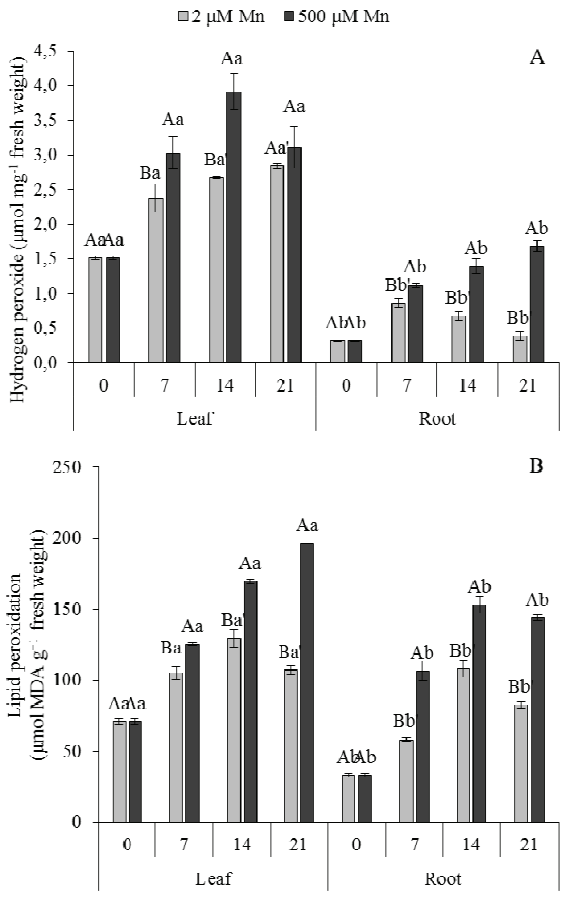

Figure 3. Effects of exposure to excess Mn and hypoxia on hydrogen peroxide concentration (A) and lipid peroxidation (B) in maize (*Zea mays* L. cv. Saracura) plants. Capital letters compare the treatments (2 and 500 µM) in each experimental period, and lowercase letters compare plant organs (leaves and roots) in each experimental period. Different letters indicate significant differences ($p < 0.05$).

Total chlorophyll concentration was strongly associated with leaf concentration of Mn, showing a negative correlation of -0.90. Moreover, a reduction in chlorophyll concentration was correlated with a higher concentration of H_2O_2 (-0.71) and subsequent increases in the activity of antioxidant metabolism enzymes (SOD -0.79, CAT -0.75 and APX -0.83) and MDA concentration (-0.83).

Plants exposed to excess Mn showed higher hydrogen peroxide (Figure 3A) and malondialdehyde (Figure 3B) levels than control plants after the seventh day of the experiment, with the highest concentrations in the leaves. In the roots, H_2O_2 concentration was positively correlated with the concentration of Mn (0.82). Both shoot and root MDA concentrations were positively correlated with Mn concentration (0.86 and 0.77, respectively). The positive correlation between H_2O_2 and MDA (0.80 for leaves and 0.82 for roots) confirms the impact of ROS on cellular components (e.g., cell membranes).

Plants exposed to excess Mn had greater superoxide dismutase activity (Figure 4A) than control plants. This increase occurred in roots after the seventh day and in leaves only from the fourteenth day. In roots, SOD activity was positively correlated with H_2O_2 (0.78), MDA (0.78) and Mn (0.79) concentrations.

Catalase activity (Figure 4B) was higher in plants exposed to 500 μM of Mn than those exposed to 2 µM of Mn. This increase occurred after the seventh day for both plant organs. However, the activity of this enzyme was generally greater in the leaves than in the roots. CAT activity was positively correlated with Mn concentration in both the leaves (0.73) and roots (0.76). In the roots, CAT was also correlated with SOD activity (0.80) and H_2O_2 concentration (0.86).

Leaves and roots of plants exposed to excess Mn showed greater ascorbate peroxidase activity (Figure 4C) than control plants. For both organs, this increase was observed from the seventh day;

however, the highest activity of this enzyme was observed in the roots. APX activity was positively correlated with Mn concentration in both the leaves (0.76) and roots (0.85). In the leaves, APX activity was also correlated with SOD (0.70) and CAT activity (0.73).

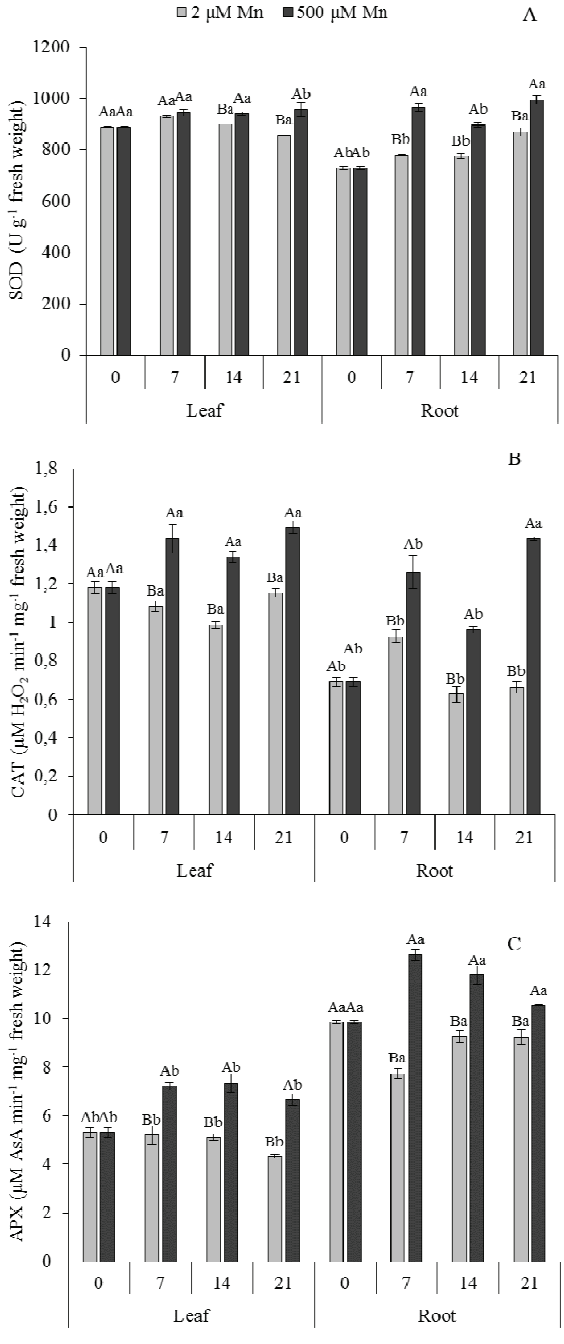

Figure 4. Effects of exposure to excess Mn and hypoxia on superoxide dismutase (A), catalase (B) and ascorbate peroxidase (C) activity in maize (*Zea mays* L. cv. Saracura) plants. Capital letters compare the treatments (2 and 500 µM) in each experimental period, and lowercase letters compare plant organs (leaves and roots) in each experimental period.

When combined with waterlogging, exposure to large amounts of Mn can be harmful to agricultural production (El-Jaoual, & Cox, 1998; Joselow et al., 1978; Sparrow, & Uren, 1987). This is because in waterlogged soils, there is more Mn available for uptake by roots (Shi, Zhu, He, Qian, & Yu, 2005), which can inhibit plant growth by interfering with normal cellular metabolism (Subrahmanyam, & Rathore, 2001; Fecht-Christoffers, Braun, Lemaitre-Guillie, Van-Dorsselaer, & Horst, 2003). We observed a significant growth inhibition in *Zea mays* L. plants cv. Saracura exposed to 500 μM Mn in a nutritive solution. Experimental plants showed a decline in biomass accumulation and an increase in Mn concentration over the course of the experiment, with greater Mn accumulation in the shoots than in roots. This is consistent with previous studies, which have reported that Mn is easily transferred to the shoots and typically has lower concentrations in the roots (Horst, 1983; Loneragan, 1988). Moreover, Mn-tolerant genotypes of *Triticum aestivum* L. (Burke, Watkins, & Scott, 1990), *Oryza sativa* L. (Wang, Wu, Wu, & Yan, 2002) and *Hordeum vulgare* (Huang et al., 2015) are characterized by higher Mn transportation from the roots to the leaves than Mn-intolerant genotypes, indicating the presence of internal tolerance mechanisms.

The observed reduction in plant growth might be due to Mn-induced inhibition of chlorophyll biosynthesis (Hauck et al., 2003). Mn-induced Fe deficiency has been identified as a possible cause for reduced chlorophyll content in plants, as Fe is essential for chlorophyll biosynthesis (El-Jaoual & Cox, 1998). Similar results have been found in *Phaseolus vulgaris* L., in which there was a 31.5% reduction in the chlorophyll content in plants exposed to 400 μM Mn (Zengin, 2013). Metal exposure also causes chlorophyll degradation, probably through an associated increase in ROS production (Zengin, 2013). Here, the increase in ROS generation was strongly correlated (-0.70) with a reduction in the levels of chlorophyll.

Stressful environmental conditions typically activate a physiological response involving overproduction of ROS (such as H_2O_2) in plant cells. One of the major consequences of metal stress may be the disruption of the equilibrium between ROS generation and detoxification, leading to oxidative stress. Indeed, increased production of ROS in response to various metals has been observed in many crop species (Wang et al., 2004). ROS cause oxidative damage to cellular components, such as membrane lipids, proteins, enzymes and pigments (Dat et al., 2000; Sharma, & Dubey, 2007). In this study, a positive correlation

was found between H_2O_2 and MDA (0.80 for leaves and 0.82 for roots), as well as a negative correlation between the concentrations of H_2O_2 and chlorophyll a (-0.70). These results support the negative impact of ROS on cellular components and photosynthetic pigments. Similarly, excess Mn elevated H_2O_2 levels and induced oxidative stress in *Hordeum vulgare* L. and *Cucumis sativus* plants (Demirevska-Kepova, Simova-Stoilova, Stoyanova, Holzer, & Feller, 2004; Shi et al., 2005).

ROS generation and detoxification are well regulated under normal conditions. However, when plants are exposed to excess metals, they can overproduce ROS, leading to oxidative stress and an imbalance in cellular antioxidants (Sharma & Dubey, 2007). Excess Mn has been shown to induce oxidative stress in many plant species and alter the activity of antioxidative enzymes (Demirevska-Kepova et al., 2004; Boojar, & Goodarzi, 2008).

ROS are scavenged enzymatically by a variety of antioxidant enzymes (Apel, & Hirt, 2004). Among the antioxidative enzymes, superoxide dismutase (SOD) is responsible for the conversion of superoxide radical into hydrogen peroxide and water. H_2O_2 is the substrate of the enzyme catalase. In this way, a positive correlation is anticipated between the activities of these enzymes. If there is an overproduction of H_2O_2 followed by poor neutralization by antioxidant systems, this will result in damage to cell membranes. Under this scenario, SOD activity must also be correlated with MDA levels. However, in our study, these correlations were only observed in the roots. This is because H_2O_2 in the leaves can be produced by other pathways in addition to dismutation of superoxide radicals. Indeed, there is increasing evidence that, at least partially, metal toxicity is due to oxidative damage (Xiong, Fu, Tao, & Zhu, 2010).

Increased SOD activity in response to Mn toxicity suggests induction of a protective mechanism against oxidative damage in Mn-stressed plants caused by $O_2^{\cdot-}$. Similar increases in SOD activity have been observed in *Cucumis sativus* L. and *Lycopersicon esculentum* Mill when exposed to excess Mn (Shi et al., 2005). The earlier SOD activity observed in roots was probably because the root is the first plant organ to come into contact with the excess Mn. Conversely, SOD activity was altered in leaves slightly later in response to Mn being translocated from the roots.

CAT and APX are involved in the metabolism of H_2O_2 produced in the cells (Apel, & Hirt, 2004). CAT has a poor affinity for H_2O_2 because two molecules of H_2O_2 must simultaneously enter the same active site. Therefore, its action occurs predominantly under high concentrations of the substrate. This is exactly what happens in the leaves, where higher concentrations of H_2O_2 cause high CAT activity under excess Mn. In contrast, APX has a much higher affinity for H_2O_2 than CAT and consequently functions in sites with low concentrations of its substrate (Ahmad, 2014). In the present study, lower H_2O_2 generation and higher APX activity were observed in the roots.

Conclusion

Zea mays L. cv. Saracura is adapted to low oxygen availability in soil through the formation of aerenchyma (Alves et al., 2002). In addition, this cultivar efficiently sequesters relatively large amounts of Mn in the leaves, although there is a significant impact on metabolism. Further work is necessary to determine the mechanisms underlying the increased shoot accumulation of Mn in this cultivar. Although there are relatively large amounts of Mn in leaves, there were no visual symptoms and only a moderate decrease in biomass production.

Acknowledgements

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