BLESSING JUMOKE KELECHI

EVALUATION OF IRON AND MANGANESE TOXICITY IN LETTUCE (*Lactuca sativa* L.): BIOACCUMULATION AND OXIDATIVE DAMAGE

Dissertation submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Juraci Alves de Oliveira

VIÇOSA - MINAS GERAIS
2023

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
K29e
2023

Kelechi, Blessing Jumoke, 1988-
Evaluation and toxicity of iron and manganese in lettuce (*Lactuca sativa* L.): bioaccumulation and oxidative damage / Blessing Jumoke Kelechi. – Viçosa, MG, 2023.
1 dissertação eletrônica (41 f.): il. (algumas color.).

Texto em inglês.
Orientador: Juraci Alves de Oliveira.
Dissertação (mestrado) - Universidade Federal de Viçosa, Departamento de Biologia Vegetal, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.192
Modo de acesso: World Wide Web.

1. Alface - Efeito dos metais pesados. 2. Ferro - Testes de toxicidade. 3. Manganês - Testes de toxicidade. 4. Estresse oxidativo. 5. Antioxidantes. I. Oliveira, Juraci Alves de, 1965-. II. Universidade Federal de Viçosa. Departamento de Biologia Vegetal. Programa de Pós-Graduação em Fisiologia vegetal. III. Título.

CDD 22. ed. 635.52

Bibliotecário(a) responsável: Bruna Silva CRB-6/2552

FICHA CATALOGRÁFICA A SER PREPARADA
PELA BIBLIOTECA CENTRAL DA UFV

Solicitar pelo site https://www3.dti.ufv.br/bbt/ficha/autenticacao

BLESSING JUMOKE KELECHI

EVALUATION OF IRON AND MANGANESE TOXICITY IN LETTUCE (*Lactuca sativa* L.): BIOACCUMULATION AND OXIDATIVE DAMAGE

Dissertation submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

APPROVED: March 1, 2023.

Assent:

Blessing Jumoke Kelechi
Author

gov.br
Documento assinado digitalmente
JURACI ALVES DE OLIVEIRA
Data: 17/04/2023 15:55:21-0300
Verifique em https://validar.iti.gov.br

Juraci Alves de Oliveira
Adviser

My Parents, my children and my Husband for their sacrifice.

I dedicate.

ACKNOWLEDGEMENTS

To God Almighty, Lord of all things who brought me this far, THANK YOU MY CREATOR.

To the Universidade Federal de Viçosa, for the opportunity to become a member of a welcoming community, in this institution of recognized quality.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

To Forum for Agricultural Research in Africa (FARA) for bringing this platform and opportunity for me to be one of the Arifa Fellows.

To Tertiary Trust Fund (TETFUND) Nigeria, for granting the scholarship and the financial assistance to make this Program a success.

To the Postgraduate Program in Plant Physiology, for providing access to a course of excellence in the University and Brazil as a whole.

To my Husband, for his support, love and care in making sure this program is a success, making sure I arrive Brazil timely, the encouragement is really appreciated. I LOVE YOU.

To my Family, my Parents and siblings for the prayers, support and care throughout my Absence in Nigeria, taking care of my kids, I do not take it for granted. Thank you, my people.

To my in-law (The Onyegbula's) I thank you all for the prayers and encouragement. I appreciate you all.

To all my friends I met during my stay here in Vicosa, the presbyterian church family (Flavia, Aline, Elisa, Vivi, Rejane, Andreza), Renata a very good friend that answers whenever I beckon her, my Medical Doctors (Joao and Leticia). Thank you so much for making my stay beautiful.

To my Laboratorio de Biofisica Ambiental colleagues, (Ana, Ari, Vinni, Taline, Pedro, Lais, Jordan, Daniel, Darlielva, Esneider) I appreciate you all for the support, love and team work in making this research a success.

To my supervisor and father here in Brazil, Professor Juraci Alves de Oliveira, for all the teachings, listening ears, support, love, continuous encouragement and for all the efforts to make our projects and this thesis work possible, even sometimes in the midst of commitments and restrictions because of my Baby. Thank you so much PROF. I do not take this for granted.

To the Professors of PPG Plant Physiology, especially Prof. Fabio da Matta, Prof Cleberson, Prof. Wagner Araujo, Prof. Dimas, Prof. Adriano Nunes, Prof. Samuel Martins, I appreciate the support from you all.

To All the Arifa Fellows here in Brazil.

I will never fail to acknowledge my GOD GIVEN GIFT here in Brazil, my son MARVELOUS, for being a good baby that allows mummy to carry out all the academic activities. I love you, Son.

Thanks to everyone who somehow contributed to make this step possible and become a reality. I LOVE YOU ALL.

ABSTRACT

KELECHI, Blessing Jumoke, M.Sc., Universidade Federal de Viçosa, March, 2023. **Evaluation and toxicity of iron and manganese in lettuce (*Lactuca sativa* L.): bioaccumulation and oxidative damage.** Advisor: Juraci Alves de Oliveira.

The disaster with the Fundao dam in Mariana, MG, Brazil, launched tons of iron ore tailings into the environment, which elevated the levels of iron and manganese in the mining area. Thus, it is imperative to understand how these metallic elements pollutant can buildup in lettuce and also the oxidative damage this pollutant can cause to the lettuce plant. Therefore, we investigated the bioaccumulation, the toxicity symptoms and the oxidative damage of this elements on lettuce. The specimens were subjected to four treatments: control (nutrient solution only); Fe (5 mM Fe-EDTA); Mn (4 mM $MnCl_2$); Fe + Mn, which were assessed at 2^{nd} day of exposure to treatments. Physiological and biochemical related analysis were performed. The results showed that lettuce plants cannot undergo Fe and Mn metal stress without showing toxicity symptoms. Although the toxicity exhibited by Fe treated plants was more severe than Mn treated plant. Also, the accumulation of the elements in the plant was not altered by their association, it has cumulative effects on the plant and are not competitive in the absorption process. The translocation of Fe from the roots to the leaves was high meanwhile Mn translocation to the leaves was low. Furthermore, a remarkable antioxidant enzymes activity was observed in all treatments but the ROS produced due to the oxidative stress could not be scavenge which led to oxidative damage especially in the Fe isolated and combined treated plants compared to Mn treated plant.

Keywords: Reactive oxygen species. Oxidative stress. Antioxidant enzymes. Mining residue.

RESUMO

KELECHI, Blessing Jumoke, M.Sc., Universidade Federal de Viçosa, março de 2023. **Avaliação da toxicidade de ferro e manganês em alface (*Lactuca sativa* L.): bioacumulação e danos oxidativos.** Orientador: Juraci Alves de Oliveira.

O desastre da barragem de Fundão, em Mariana, MG, lançou no meio ambiente toneladas de rejeitos de minério de ferro, elevando os níveis de ferro e manganês na área da mineração. Assim, é imperativo entender como esses elementos metálicos poluentes podem se acumular na alface e, também, os danos oxidativos que esse poluente pode causar à planta de alface. Portanto, investigamos a bioacumulação, os sintomas de toxicidade e os danos oxidativos desses elementos em alface. Os espécimes foram submetidos a quatro tratamentos: controle (somente solução nutritiva); Fe (5 mM Fe-EDTA); Mn (4 mM $MnCl_2$); Fe + Mn, que foram avaliados no 2º dia de exposição aos tratamentos. Análises fisiológicas e bioquímicas relacionadas foram realizadas. Os resultados mostraram que as plantas de alface não podem sofrer estresse dos metais Fe e Mn sem apresentar sintomas de toxicidade. A toxicidade exibida pelas plantas tratadas com Fe fosse mais severa do que a planta tratada com Mn. Além disso, o acúmulo dos elementos na planta não foi alterado pela sua associação, têm efeitos cumulativos na planta e não são competitivos no processo de absorção. A translocação de Fe das raízes para as folhas foi alta, enquanto a translocação de Mn para as folhas foi baixa. Além disso, uma atividade notável das enzimas antioxidantes foi observada em todos os tratamentos, mas as espécies reativas de oxigênio produzidas devido ao estresse oxidativo não puderam ser eliminadas, o que levou aos danos oxidativos, especialmente nas plantas tratadas com Fe isolado e combinado, em comparação com as plantas tratadas com Mn.

Palavras-chave: Espécies reativas de oxigênio. Estresse oxidativo. Enzimas antioxidantes. Resíduo de mineração.

LIST OF FIGURES

LIST OF TABLES

SUMMARY

1. INTRODUCTION

Vegetables are basic eating routine taken by populations all through the world, being wellsprings of fundamental supplements, antioxidants agents and metabolites. However, both essential and toxic components are available in vegetables over an extensive variety of concentrations as they are said to be great absorber of metals from the environment (Shakya and Khwaounjoo, 2013). Increased heavy metal uptake by vegetables could impact the quality and safety of food if there is an excessive buildup of heavy metals in agricultural soils through the use of agrochemicals and other sources (Reyes-Gutierrez *et al*., 2007).

Heavy metal contamination constitutes a major threat to human life and this is a recognized environmental problem on a global scale (Khan *et al*., 2008). Over the past few decades, heavy metal concentrations in the environment and crop cultivation soils have increased, drawing a lot of attention from ecologists throughout the world. Man's activities, including mining operations over time has produce an adverse impact on various ecosystems and cause significant environmental harm (Pandey *et al*., 2016). The pollution of water, soil, animals, and plants by metals such iron (Fe), cadmium (Cd), lead (Pb), zinc (Zn), and manganese (Mn) and metalloids such as arsenic (As) are one of the main problems (Sun *et al*., 2018). Particularly when tailings dams are breached, there is a risk to public health and a potential environmental hazard because the discharge can end up in waterways and places hundreds of kilometers away from the mining location (Kossoff *et al*., 2014).

To meet the food needs of the populace, it is a common practice in many developing nations to plant vegetables along the banks of rivers, streams, and channels that run through urban areas. However, the waters of these rivers, streams, and channels are frequently reported to be polluted by heavy metals (Kashem and Singh, 1999; Othman, 2001). Studies have shown that heavy metals are absorbed by vegetables and accumulate in their edible components (Bahemuka and Mubofu, 1991), with leafy vegetables accumulating heavy metals more readily than grain or fruit crops (Mapanda *et al*., 2005). When animals and humans consume these metal-rich plants, the quantities that accumulate could be large enough to induce clinical issues (Alam, 2003).

On November 5, 2015 a rupture of the Fundão tailing dam occurred in Mariana, Minas Gerais, belonging to Samarco Mineração S.A., resulted in about 34 million m^3

of iron ore mining waste (IOMW) being launched into the environment, covering 650 km along the states of Minas Gerais (MG) and Espírito Santo (ES), a riverside area of 1176.6 ha, and carrying away 457.6 ha of the Atlantic Forest (Omachi *et al*., 2018). Mining activities have a localized impact but the tailing components, when solubilized, can easily reach watercourses and affect areas hundreds of kilometers removed from the mining site, which poses a threat to human health and a constitutes a potential environmental hazard (Salomons, 1995; Hashemi *et al*., 2015).

This event spilt out tonnes of oxides and hydroxides of Fe and Mn along the course of water bodies and soils (Vergilio *et al*., 2020; Quaresma *et al*., 2021). There is a risk of biomagnification after the entry of metals into the food chain, leading to excessive consumption by humans and animals, which can cause significant clinical problems (Blanc, 2018; Andrew *et al*., 2003). Analysis of the metal content of plant tissues used for animal and human consumption is therefore critical for the health of populations living in environments with some level of contamination (Peralta-Videa *et al*., 2009).

Manganese is a trace element that plays a role in a number of physiological processes in plants, including photosynthesis, redox reactions, and PSII enzyme co-factoring, respiration, scavenging of reactive oxygen species (ROS), pathogen defense, and hormone signaling (Fernando *et al*., 2015). Plants can readily absorb the soluble form of Mn in soil, hence there is a direct correlation between the amounts of soluble Mn in plants and soils. Increased Mn levels result in phytotoxicity, which is mediated by the inhibition of superoxide dismutase, catalase and peroxidase, crucial antioxidant enzymes involved in the reduction of free radicals. High levels of Mn in plants also lead to oxidative stress due to the antagonistic interactions between metals with similar structural characteristics, which results in a lack of enzyme cofactors necessary for antioxidant activities (Fernando *et al*., 2009).

Mn is a crucial constituent of Mn-superoxide dismutase (Mn-SOD), a major antioxidant enzyme (Lidon *et al*., 2004). Mn in plants also participates in carbohydrate and lipid biosynthesis. Besides, this Mn also serve as a cofactor of many enzymes, like Mn-catalase, Mn-peroxidases, TCA cycle decarboxylases, RNA polymerases and numerous glycosyltransferases (Lidon *et al.,* 2004). High concentration of Mn, greater than the required amount, cause ROS production via the Fenton mechanism (Heine *et al*., 2011).

Iron is an essential mineral nutrient element for plant growth and development, synthesis of chlorophyll and deoxyribonucleic acid, activation of a number of respiratory enzymes, transport of oxygen, and also play significant roles in the physiological processes of photosynthesis, respiration, nitrogen metabolism and redox system of the plasma membrane (Connorton *et al*., 2017). About 80% of the Fe in plants is found in photosynthetic cells. Fe competes with other transition metals such as Cu, Zn, and Mn in its uptake, transport and chemical reaction within plant cells (Rout and Sahoo, 2015).

Iron toxicity can result from environmental disasters promoted by human activities associated with the iron processing makes Fe excess an environmental problem (Xing *et al*., 2010; Araújo *et al*., 2014; Cordeiro *et al*., 2019; Valeriano *et al*., 2019). Excess Fe in the plant is a potential oxidative stress inducer (Lapaz *et al*., 2020) and can reflect a decrease in gas exchange traits and chlorophyll content, deactivation of PSII reaction center and a decrease in saturated fatty acids and increase unsaturated fatty acids in chloroplast membrane (Xu *et al*., 2015). Due to its potential toxicity, Fe is translocated through the plant body associated with chelating molecules and under the proper control of redox states between the ferrous and ferric forms (Kobayashi and Nishizawa, 2012). Fe^{2+}-nicotinamide (NA) complex is mainly involved in the subcellular distribution and inter-organ partitioning of Fe by the phloem, while Fe^{2+}-citrate is considered the main form in which Fe is transported by the xylem (Kobayashi *et al*., 2019).

Mutation in Fe transporter IRT1 demonstrated the role of IRT1 in Mn transport, and it uncouples with Mn and Fe transport (Rogers *et al.,* 2000). Broad substrate specificity of the majority of Mn translocation transporters (like NRAMPs) substantially influences the Mn homeostasis (Socha and Guerinot, 2014). Furthermore, the ZIP transport family has also been advocated to have a broad substrate specificity for Fe^{2+}, $Mn^{2+}$and Zn^{2+}. The CDFs/MTP family proteins have been identified to efflux Mn^{2+}, Zn^{2+}, and Fe^{2+} into subcellular compartments or out of the cytoplasm by acting as act proton antiporters (Gustin *et al.,* 2011). These findings suggest complex crosstalk exists for the regulation of metals in cells and these pathways are interconnected to regulate metal homeostasis to maintain a stable metabolism.

Lettuce (*Lactuca sativa* L.) is a major vegetable crop in Brazil, with broad social and economic importance. They are significant edible vegetables that are a crucial component of the human diet and are typically consumed as vegetables because of their nutritional worth (Deribachew *et al*., 2015; Hang *et al*., 2016; Rumteke *et al.,* 2016). Due to its accumulating characteristic, it is subject to contamination by heavy

metals dissolved in the soil solution (Eissa and Negim, 2018; Silva *et al*., 2019). Due to their role as a source of nutrients, vegetables play a significant role in human diets as a source of protein, vitamins, iron, calcium, and other minerals (Arai, 2002). Heavy metals and other harmful compounds have an innate propensity to be taken up by plants, where they are then passed through the food chain (Singh *et al.,* 2010). Because they absorb these metals through their roots, leafy vegetables cultivated on heavy metal-contaminated soils acquire higher levels of metals than those grown in uncontaminated soils (Marshall *et al.,* 2007; Sharma *et al.,* 2007).

According to estimates from the World Health Organization (WHO), extended exposure to environmental pollution is a contributing factor in roughly 25% of the diseases that affect people today (Prüss-Üstün and Kimani, 2007). One of the main health issues around the world is heavy metal contamination of the environment, even at low levels, this can have long-term, cumulative consequences on health. Since they are not biodegradable, heavy metals are known to remain for a very long time in both terrestrial and aquatic settings (Oluyemi *et al*., 2008). Pollutants may negatively impact crop development and may also infiltrate the food chain by integrating with plants, creating a significant exposure pathway for people who consume these products (Khan *et al.,* 2009).

On the other hand, it is not yet well documented if lettuce plants are able to accumulate high concentrations of Fe and Mn in the leaves without showing visual symptoms of toxicity and if elements Fe and Mn have cumulative effects on plants and are not competitive in the absorption process, Therefore our objectives of this research were to evaluate the toxic effects of high levels of Fe and Mn on the lettuce plants, the accumulation of Fe and Mn in roots and leaves and the translocation factor of Fe and Mn from the roots to leaves, in addition to assessing the toxic effects on the antioxidant metabolism.

2. MATERIAL AND METHODS

2.1. Cultivation, acclimatization and application of treatments

Lettuce seeds (*Lactuca sativa* L.), cultivar Elba, were germinated in foam based on phenolic resin, sterile and inert, with 2x2 cm per block, and kept in polyethylene trays, being moistened with Clark's nutrient solution (1975), with ½ ionic strength, under aeration, for 40 days. After selection for size and uniformity of shoot and root (plants with at least 4 fully expanded leaves and roots with a length of at least 10 cm), were transferred to polyethylene pots (1 plant/pot), containing 275 mL of Clark's nutrient solution (1975), with pH 6.5 adjusted daily, aerated and kept in a plant growth room at a temperature of 25 ± 2 ºC, irradiance of 230 µmol m^{-2} s^{-1} and light photoperiod of 16 hours, for a period of 5 days for acclimatization.

After the acclimatization period, the plants were submitted to the following treatments: control (nutrient solution only); Fe, Mn and Fe + Mn with five repetitions. Iron was supplied at 5 mM ethylenediaminetetraacetic acid iron (III) sodium salt hydrate (Fe-EDTA) and manganese as 4 mM manganese (II) chloride ($MnCl_2$). The concentrations of Fe and Mn were determined based on preliminary experiments. The pH was adjusted to 6.5 daily. The plants remained for two days in the treatments for further evaluations.

2.2. Visual symptomatology

The photographic record of visual symptoms was performed at the end of the exposure period to the treatments (48 h), using a digital camera.

2.3. Determination of the concentration of Fe, Mn and translocation factor

Samples of 0.1 g of dry material, leaves and roots, were homogenized and mineralized in a heating block, with controlled temperature (210 ºC), using the nitro-perchloric mixture, in the proportion of 3:1, until all the material was plant being oxidized (Marin *et al*., 1993). The mineral extract was diluted to 25 mL with deionized water and the concentration of Fe and Mn determined by atomic absorption spectrophotometry (Model AA-6701F, Shimadzu Corporation), and the results expressed as µg g^{-1} dry mass (MS).

The translocation factor (TF) was calculated using the equation $TF = C_S / C_R$. The terms C_S and C_R mean element concentration in shoot and root (μg g^{-1} MS), respectively.

2.4. Determination of the concentration of chloroplast pigments

Samples of 0.1 g of leaves were macerated in liquid nitrogen and then homogenized with 2 mL of 80% (v/v) acetone and 10 mg of calcium carbonate. The mixture was vortexed for 30 seconds and centrifuged at 3000 xg for 15 min at room temperature. The supernatant was collected and the absorbance measured at 663, 646 and 470 nm (Lichtenthaler and Wellburn, 1983) in a microplate reader (Multiskan GO, Thermo Scientific). Pigment contents were calculated according to the formulas: chlorophyll a (μg mL^{-1}) = (12.21 x (Abs_{663})) – (2.81 x (Abs_{646})); chlorophyll b (μg mL^{-1}) = (20.13 x (Abs_{646})) – (5.03 x (Abs_{663})); total carotenoids (μg mL^{-1}) = (1000 x [Abs_{470}]) – (3.27 x [Chlorophyll a]) – (104 x [Chlorophyll b]) / 229. Results were expressed as μg g^{-1} of fresh mass (FW).

2.5. Biochemical analyzes of the oxidative metabolism

2.5.1. Determination of the concentration of hydrogen peroxide (H_2O_2)

Samples of 0.3 g of leaves and roots were macerated in liquid nitrogen and homogenized in 2 mL of extraction medium consisting of 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM hydroxylamine, and centrifuged at 10,000 xg for 15 min at 4 °C (Kuo and Kao, 2003).

Aliquots 20 μL of supernatant was added to the reaction medium containing 80 μL of 250 μM ferrous ammonium sulfate in 25 mM sulfuric acid, 50 μL of 250 μM xylenol orange and 50 μL of 100 mM sorbitol. The mixture was homogenized and kept in the dark for 30 min, and the absorbance was determined at 560 nm and the concentrations of H_2O_2 were estimated based on a calibration curve, previously prepared with H_2O_2 standards, and the results were expressed in nmol g^{-1} FW.

2.5.2. Lipid peroxidation

The evaluation of lipid peroxidation was performed through the concentration of malondialdehyde (MDA), a reactive species of thiobarbituric acid (TBARS), in 0.2 g samples of leaves and roots, macerated in liquid nitrogen and homogenized in 2 mL of 80% (v/v) ethanol, followed by centrifuging at 10,000 xg, 4 °C, for 10 min. An aliquot

of 0.5 mL of the supernatant will be added to 1.5 mL of 0.65% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid (TCA) and another added to only TCA 20% (w/v). The mixture was incubated at 95 °C for 30 min, followed by immersion in an ice bath for 10 min, and subsequent centrifugation at 3,000 xg, 4 °C, for 10 min. The supernatant was used to measure the absorbance at 440, 532 nm and 600 nm in a Hitachi UV/visible spectrophotometer, model U-5100. The concentration of MDA was calculated using the following equation and the results expressed as nmol MDA g^{-1} FW (Hodges *et al*., 1999):

1) $[(Abs_{532+TBA} - Abs_{600+TBA}) - (Abs_{532-TBA} - Abs_{600-TBA})] = A$

2) $[(Abs_{440+TBA} - Abs_{600+TBA})\ 0.0571] = B$

3) MDA (nmol mL^{-1}) = (A - B) /157000).10^6.

2.5.3. Analysis of enzymes

To determine the activity of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidase (POX, EC 1.11.1.7) enzymes, 0.3 g samples of leaves and roots were macerated in liquid nitrogen and homogenized in 2 mL of extraction buffer consisting of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w/v) polyvinylpyrrolidone (PVPP) (Peixoto *et al*., 1999). The homogenate centrifuged at 12,000 xg for 15 min, at 4 °C, and the supernatant used as an enzyme extract, activities being determined by adding it to the following reaction media:

- 50 mM potassium phosphate buffer, pH 7.8, containing 13 mM methionine, 75 µM p-nitro blue tetrazolium (NBT), 0.1 mM EDTA and 2 µM riboflavin, for SOD (Giannopolitis and Ries, 1977);
- 50 mM potassium phosphate buffer, pH 7.0 and 12.5 mM H_2O_2, for CAT (Havir and Mchale, 1987);
- 25 mM potassium phosphate buffer, pH 6.8, 20 mM H_2O_2 and 20 mM pyrogallol for POX (Kar and Mishra, 1976).

SOD activity was conducted at 25 °C, in a reaction chamber under illumination for 5 min and measured at 560 nm. One unit of SOD will be defined as the amount of enzyme required to inhibit NBT photoreduction by 50% and the results were expressed as U min^{-1} mg^{-1} protein (Beauchamp and Fridovich, 1971). For CAT, the activity was determined by measuring the decrease in absorbance, in the first minute of reaction,

at 240 nm, at 30 °C and this was calculated using the molar extinction coefficient of 36 M^{-1} cm^{-1} (Anderson *et al*., 1995) and the results were expressed in µmol H_2O_2 min^{-1} mg^{-1} protein.

For POX activity, the increase in absorbance was measured during the first minute of reaction at 420 nm, at 25 °C, and the activity was calculated using the molar extinction coefficient of 2.47 mM^{-1} cm^{-1} (Chance and Maehley, 1955) and the results were expressed in µmol min^{-1} mg^{-1} protein.

2.5.4. Protein determination

Protein determination in enzymatic extracts was performed by the Bradford method (Bradford *et al*., 1976), using 20 µL of each enzymatic extract and 200 µL of Bradford reagent, read at 595 nm using BSA as a standard.

All biochemical analyses, with the exception of SOD and lipid peroxidation, which was performed on a UV/visible spectrophotometer (Hitachi, U-5100), were performed on a microplate reader (Multiskan GO, Thermo Scientific).

2.6. Experimental design and statistical analysis

The experiment was performed in a completely randomized design with 4 four treatments (Control, Iron, Manganese and Iron+ Manganese) in four repetitions, totaling 16 experimental plots. The experimental unit consisted of one plant per pot. The experimental data were submitted to the Shapiro-Wilk ($p>0.05$) and Bartlett ($p>0.05$) tests for basic verification of residual normality and homoscedasticity, respectively.

Parametric data were analyzed by ANOVA - analysis of variance ($p>0.05$) with Tukey mean comparison test ($p>0.05$). In this model, bar plots were used to represent the results. Non-parametric data were analyzed by Kruskal-Wallis test ($p>0.05$). A boxplot was used to represent the non-parametric results. For data analysis, the R language and environment (R. CORE TEAM, 2016) were used.

3. RESULTS

3.1. Visual symptoms of toxicity

The toxicity symptoms of iron and manganese were observed in the leaves and roots of the lettuce plants subjected to high concentration of these elements. The Fe-treated plant showed necrosis progressing to the leaf margin browning, whereas yellowing of leaves were observed in Mn- treated plant. For the combined treatment, the characteristics toxic symptoms of each element were manifested on the plant, necrosis and yellowing of leaves, browning of leaf margin were manifested with plants treated with Fe + Mn combination. In roots of the plants, reduction of the root volume was observed in all treatment apart from the control. Browning of roots was also observed especially in the combined Fe + Mn treated plants (**Fig. 1**).

a b c d

Figure 1. Appearance of lettuce plants growing for two days in a solution containing 5 mM of Fe-EDTA or 4 mM $MnCl_2$ isolated or in combinations. Control (a), Fe (b), Mn (c), Fe + Mn (d). Blue arrows indicate toxicity symptoms such as yellowing of leaves, dark spots, and necrosis.

3.2. Concentration of Fe, Mn and translocation factor

The Mn concentration in the leaves of plant treated with Fe + Mn combination was drastically increased, followed closely by Mn treated plant which has high concentration with much variations like the Fe + Mn treated plant. While the Fe treated plants showed Mn concentration in the leaves similar to the control (**Fig. 2A**).

In the roots, Mn concentration increased up to 4,000 ug g^{-1} DW in treatments with Mn and Fe + Mn combination, and the Fe presence didn´t interfere with Mn accumulation. Lettuce plants subjected to Fe treatment presented high Mn concentration than control plants cultivated in conventional nutritive solution, indicating beneficial effect of Fe on Mn absorption (**Fig. 2B**)

The Fe treated plants has increased concentration of Fe in their leaves, followed closely by the Fe + Mn combination treated plant, the concentration of Fe remained stable in the Mn treated plant and the control plants (**Fig. 3A**).

The concentration of Fe in the root was increased in Fe single and Fe + Mn combined treatments with high variation compared to Mn single treatment and control which had low or no concentration of Fe (**Fig. 3B**). The translocation of Fe in both Fe isolated and combination increases while the translocation of Mn decreases in the Mn isolated and combination treated plants (**Table1**).

Table 1. Translocation factor in lettuce plants subjected to Fe, Mn and Fe + Mn concentrations

	Translocation factor	
Treatment	**Fe**	**Mn**
Control	0,09 ± 0,0093 b	0,90 ± 0,0847 a
Fe	0,23 ± 0,0183 a	0,26 ± 0,0129 a
Mn	0,08 ± 0,0026 b	0,13 ± 0,0230 b
Fe + Mn	0,20 ± 0,0148 a	0,18 ± 0,0325 b

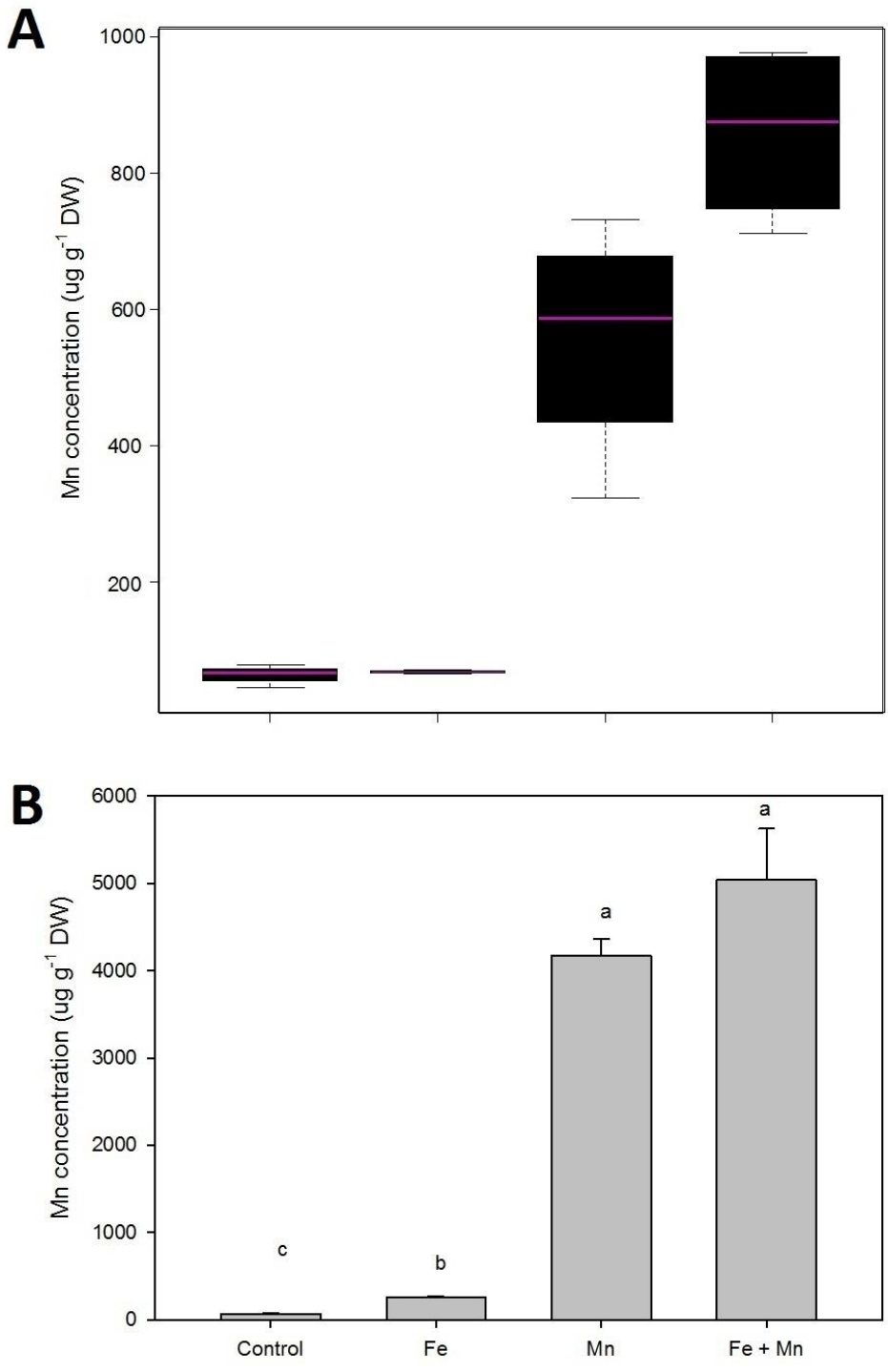

Figure 2. Concentration of Mn in the leaves (A) and roots (B) of lettuce plants treated with Fe, Mn and Fe + Mn concentrations. Same letter does not differ from each other by the Tukey test ($p > 0.05$) according to one-way analysis of variance (ANOVA) or nonparametric Kruskal-Wallis's test.

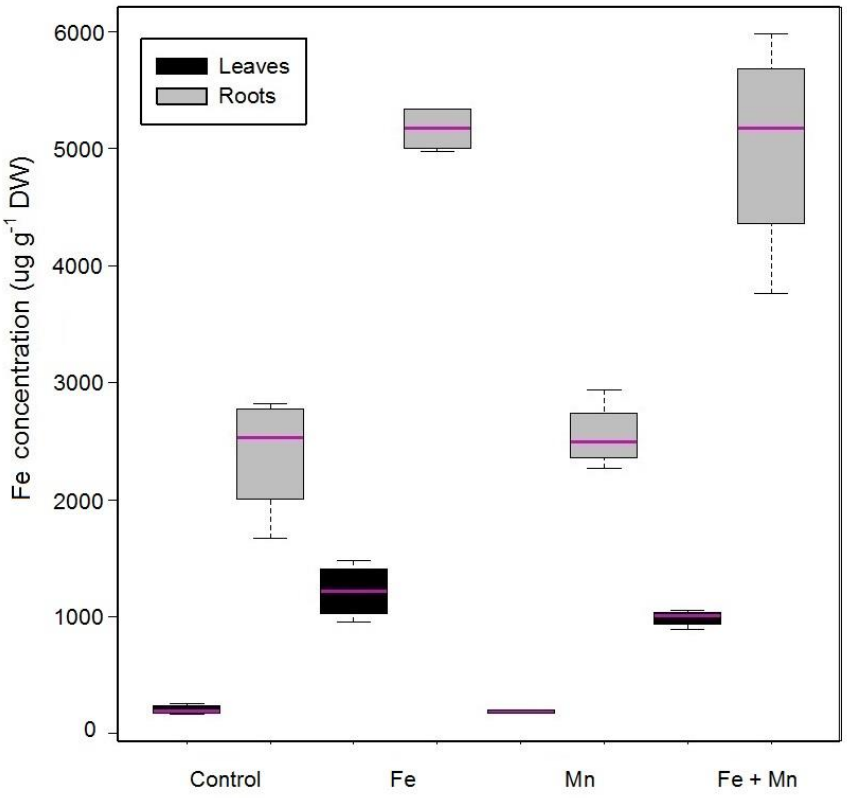

Figure 3. Iron concentration in the leaves and roots of lettuce plants treated with Fe, Mn and Fe + Mn concentrations. Same letter does not differ from each other by the Tukey test ($p > 0.05$) according nonparametric Kruskal-Wallis's test.

3.3. Concentration of chloroplast pigments

The concentration of chlorophyll *a* was significantly decreased in the leaves of plant treated with Fe isolated and Fe + Mn combinations compared to other treatment (control, and Mn single treatment) which were not affected and not significantly different from each other (**Fig. 4A**). It was also observed that chlorophyll *b* (**Fig. 4B**) and carotenoids contents (**Fig. 4C**) in the leaves were not affected by Fe, Mn, and Fe + Mn combination treatments because they were not significantly different to the control plants.

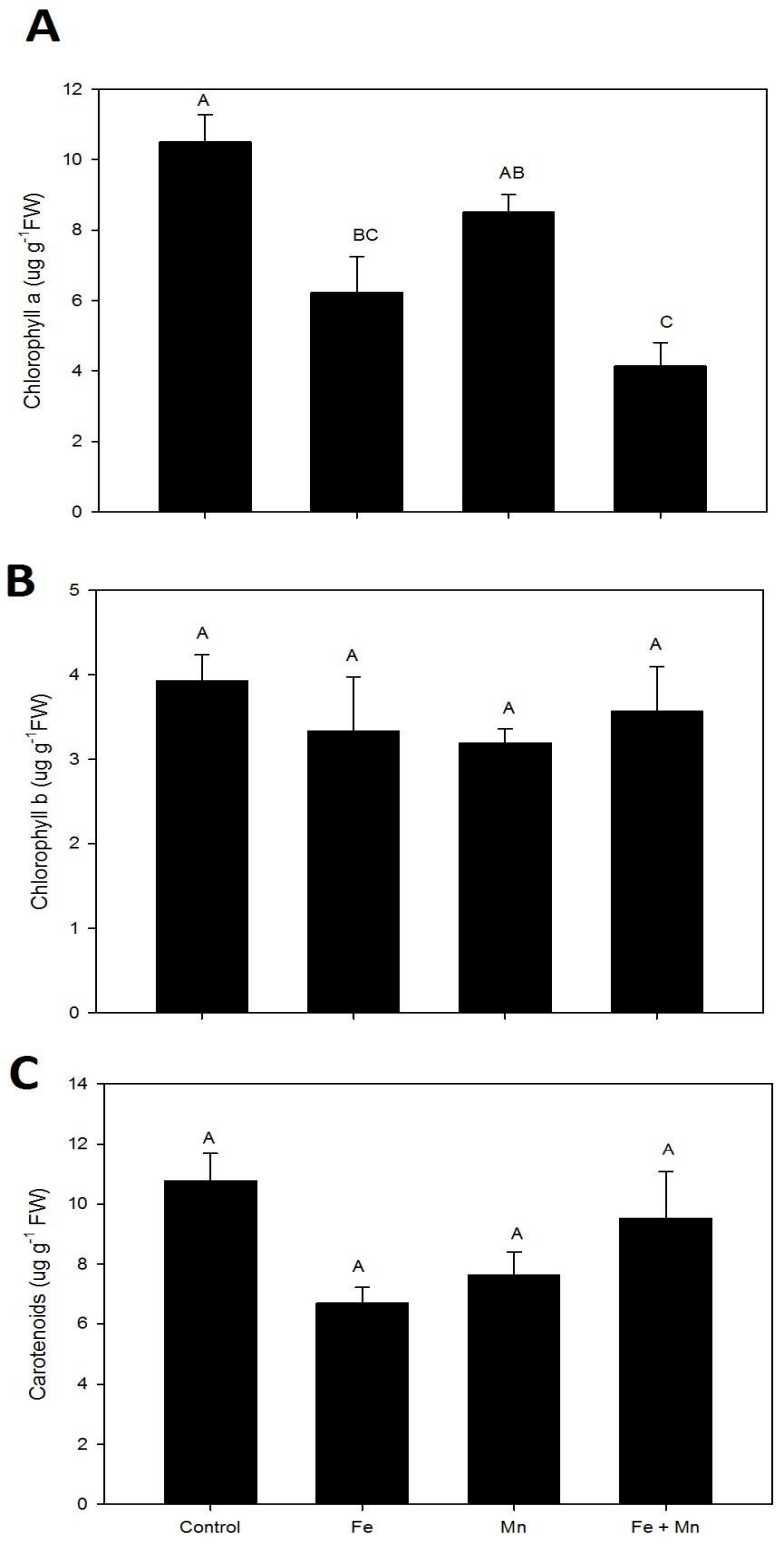

Figure 4. Chlorophyll a (A), chlorophyll b (B) and carotenoids (C) contents of lettuce plants treated with Fe, Mn and Fe + Mn concentrations. Same letter does not differ from each other by the Tukey test ($p > 0.05$) according to one-way analysis of variance (ANOVA).

3.4. Oxidative metabolism

3.4.1. Concentration of hydrogen peroxide

High hydrogen peroxide concentrations were observed in the leaves of the lettuce plants, mainly in the Fe + Mn treatment, which produced the highest amount of hydrogen peroxide but it is not different statistically of the other treatments and plants control.

In the roots, the hydrogen peroxide increased in the Fe treated plant and the Fe + Mn combination treated plant which are statistically the same with control but different from the Mn treated plant where hydrogen peroxide decreased (**Fig. 5**).

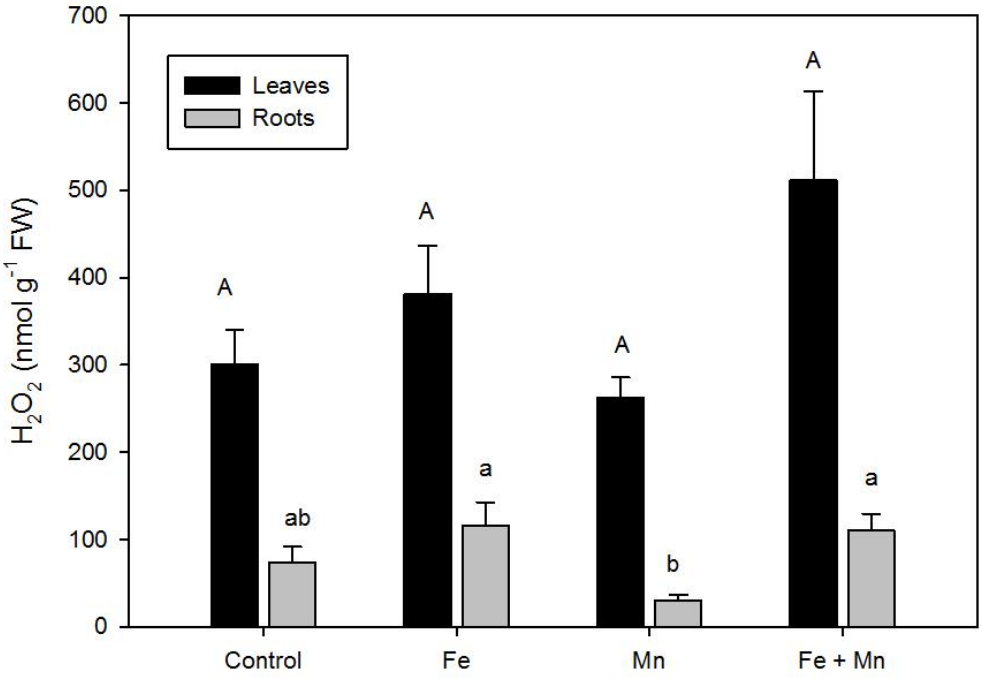

Figure 5. Hydrogen peroxide production of lettuce plants treated with Fe, Mn and Fe + Mn concentrations. Same letter does not differ from each other by the Tukey test ($p > 0.05$) according to one-way analysis of variance (ANOVA).

3.4.2. Lipid peroxidation

The level of lipid peroxidation was measured in terms of TBARS production to represent damage in membrane. The TBARS values were higher in the leaves of the plants treated with Mn, isolated and in combination with Fe. The TBARS content was higher in Fe treated lettuce leaves compared to the control. In the roots, in turn, TBARS content increased in Fe treated plant, followed closely by the combined treatment of

Fe + Mn which was statistically the same. Mn treated plants exhibited the lowest content of TBARS in the roots compared to the control which was statistically the same with combination of Fe + Mn (**Fig 6**).

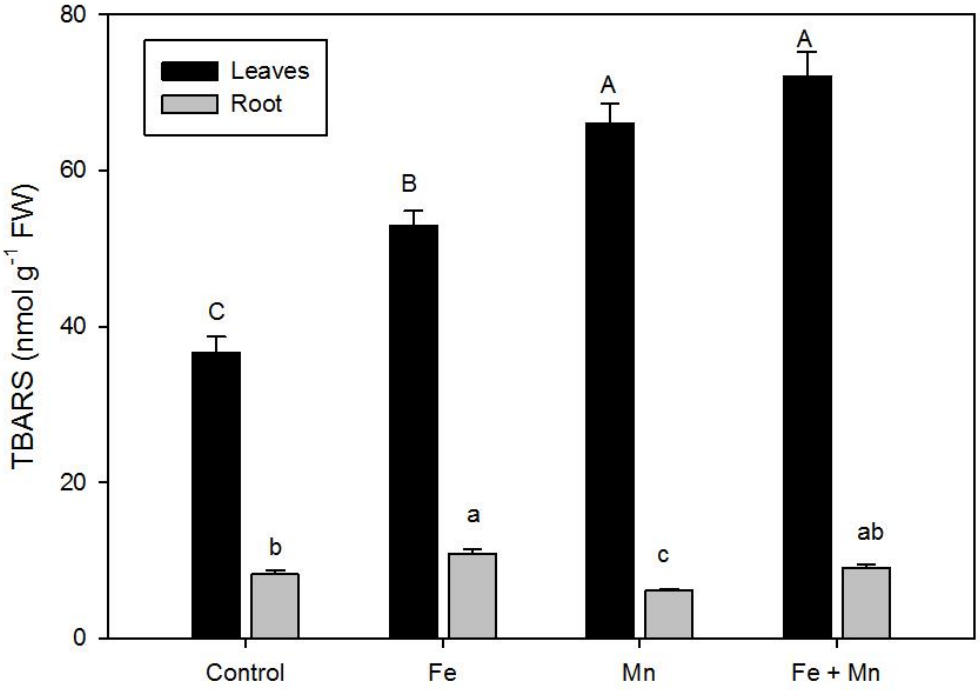

Figure 6. Lipid peroxidation, as TBARS concentration, of lettuce plants treated with Fe, Mn and Fe + Mn concentrations. Same letter does not differ from each other by the Tukey test ($p > 0.05$) according to one-way analysis of variance (ANOVA).

3.4.3. Enzymatic activity

The results showed no effects of the Fe and Mn, isolated, on the catalase activities (CAT) in the leaves. This activity decreased in plants treated with Fe + Mn combination compared to the control and other treatments. The CAT activities in the roots were increased in the Fe+ Mn combined treatment, it was also observed that the activity of the catalase was the same statistically in Fe treated plant and Mn treated plant compared to the control where there was a decrease in the catalase activity (**Fig. 7A**).

The peroxidase activities (POX) in the leaves were statistically the same for all the treatment applied, although the increase tendency in Fe and Fe + Mn treated plants compared to Mn treated plant and control. The roots presented high peroxidase activities in the Fe treatments, isolated and in combination com Mn, compared to Mn treatment and control plants (**Fig. 7B**).

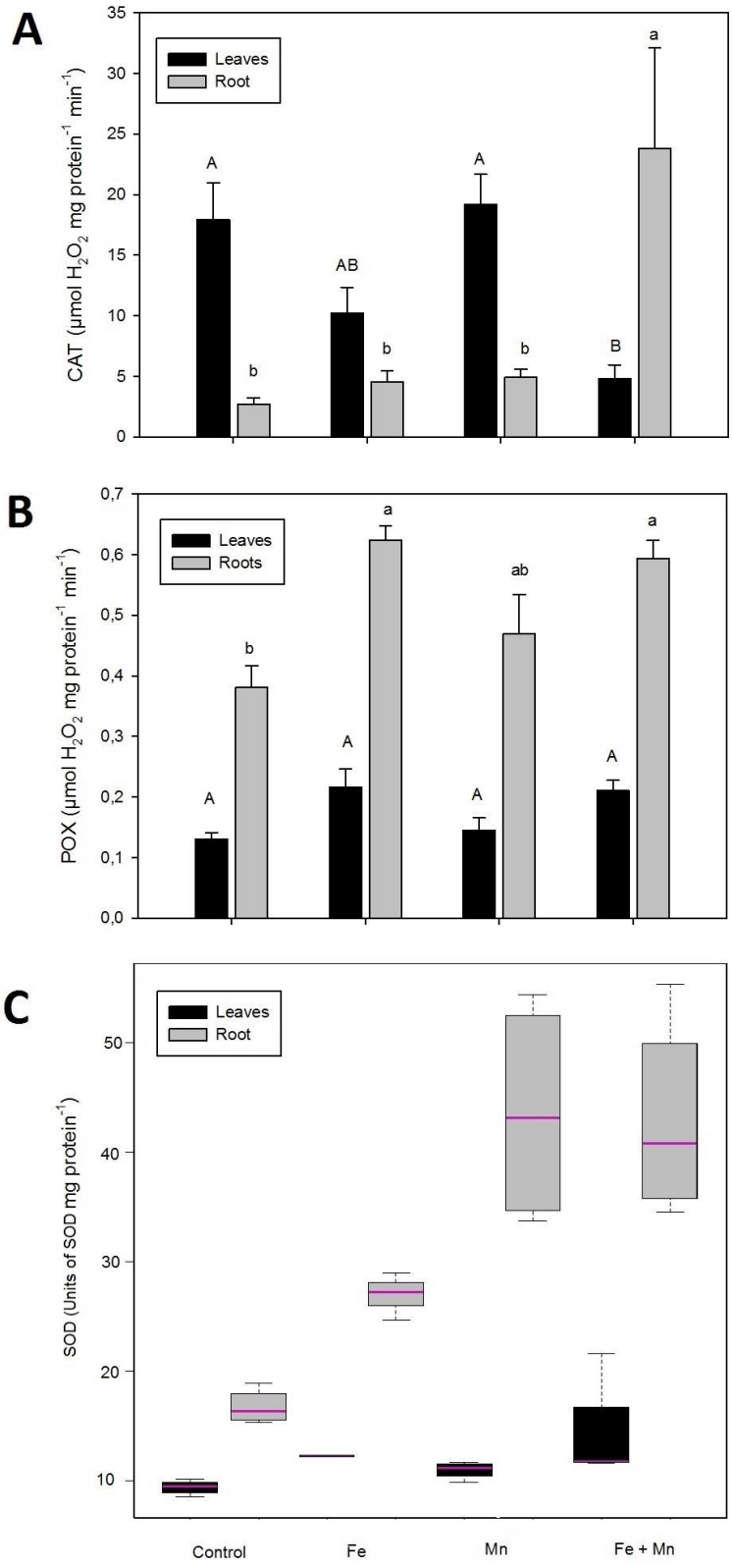

Figure 7. Enzymes activities in the lettuce plants: catalase (A), peroxidase (B) and superoxide dismutase (C), treated with Fe, Mn and Fe + Mn concentrations. Same letter does not differ from each other by the Tukey test ($p > 0.05$) according to one-way analysis of variance (ANOVA) or nonparametric Kruskal-Wallis's test.

The superoxide dismutase activities (SOD) in the leaves increased in all treatments, mainly in the Fe + Mn combined treated plants. In the Mn treated plants the variation of the enzyme activity was higher compared to the Fe treated plants, where there was little or no variation but the enzyme activities was higher (**Fig. 7C**). In the roots, the higher SOD activities occurred in the Mn treatments, isolated and in Fe + Mn combination, but with high variation in these treatments. The Fe treatment resulted in values higher than control plants, with low variations (**Fig. 7C**)

4. DISCUSSION

In the environment, most pollutants are composed of mixtures that contain more than one metallic element, pollutant mixtures vary in their effects on living organisms, being additive, synergistic, or inhibitory (Ramakrishnan *et al*., 2011). In this study, lettuce (*Lactuca sativa* L) plants were subjected to Fe and Mn concentrations, as single treatments and as combinations, for 48 hours to have a better understanding of the single as well as combined effects of the elements on the plants.

The most visible signs of toxicity observed in the aerial plant parts of lettuce were yellowing of leaves and necrosis (**Fig 1**). Fe-treated plants display dark spots progressing to browning of leaf margins and also in roots, a common coloration caused by phenols accumulation (Wu *et al*., 2014). The yellowing of leaves in plants treated with Mn only or the combination of Fe + Mn, are likely related to the decrease observed in chlorophyll *a* content. It was also previously demonstrated that chlorosis and necrosis might be the result of Fe and Mn stress, mainly due to the formation of reactive oxygen species in the region of the cell wall and within the cell (Mascher *et al*., 2002). These changes affect membrane permeability, enzyme and photosynthetic activity, and could cause chlorosis and necrosis. Similarly, to leaves, morphological changes due to Fe and Mn toxicity were observed in lettuce roots, as the observed reduction in secondary root emission, also been described in bean specimens subjected to Fe exposure (Singh *et al*., 2007).

Lettuce plants represents a highly promising target for enrichment with metallic elements (Smoleń *et al*., 2014). Manganese and iron were easily taken up from the growing medium and subsequently accumulated and translocated to aerial organs of plants, which are edible parts of leafy vegetables, similar to that observed in soybean (Lavres Junior *et al*., 2010). Although Fe and Mn share some of the influx transporters

(Tripathi *et al*., 2018), the association of the elements did not decrease bioaccumulation of the elements in the plant. On the other hand, Mn translocation decreased compared to Fe (**Table 1**). These effects are probably due to the Mn/Fe competitive inhibition for the xylem loading (Green and Rogers, 2004). Furthermore, it is an interesting result looking into food safety aspect, since less Mn is being translocated to the leaves.

It was observed that the association or interaction between the Fe and Mn did not affect the concentration of the elements in the plant, (**Fig 2 and 3**) there was no competition in the absorption process of the metallic elements in the treatments applied to the lettuce plant, therefore the metals were not inhibitory in their absorption process, as observed in rice plants by Dokiya *et al*. (1968). This is in contrast with the report that the antagonism between Mn and Fe is a well-known interaction in barley (Kobayash, 1964).

Heavy metals have been found to decrease the chlorophyll and carotenoids contents in various plants in most cases (Aggarwal *et al.,* 2012), being considered one of the primary toxic events and used as parameters to the bioindication of oxidative stress caused by heavy metals (Macfarlane and Burchett, 2001). This provides evidence for the loss of the photosynthetic apparatus and changes in photosynthetic ability. Data showed that Fe single treatment and Fe + Mn combination treatment was more effective in the reduction of chlorophyll *a* compared to Mn single treatment. (**Fig 4**) Iron is a cofactor for redox enzymes such as cytochrome (Cyt) oxidase, peroxidase, catalase, iron-sulfur proteins, and ferredoxin (Guerinot, 1994). Decreasing chlorophyll pigments under Fe conditions was previously reported in pepper (Roosta and Mohsenian, 2012), tomato (Machold and Stephan, 1969), pea (Mahmoudi *et al*., 2005), and strawberry (Pestana *et al*., 2012). This decline in photosynthetic pigments is most probably due to the inhibition of the reductive steps in the biosynthetic pathways of photosynthetic pigments due to the high redox potential of many heavy metals (Elloumi *et al*., 2007).

In this present study, chlorophyll *b* was not affected by the treatments which might be as a result of short time exposure of the plant to the treatments. Chlorophyll *b* was less sensitive to heavy metal stress, as reported by Appenroth *et al*. (2010). Carotenoids contents were not decreased by the treatments because contents were similar to the control, but fairly affected by Fe. As an interesting result, compared to the control plants, the results of chlorophyll *b* and carotenoids in lettuce were non-significant under the treatments. (**Fig 4**)

The maintenance of hydrogen peroxide concentration in the leaves, in all treatments, although the concentration of Fe was higher compared to other treatments, this might be because Fe is well known for its reactivity with hydrogen peroxide thereby generating the highly reactive hydroxyl radical through Fenton chemistry. Also in the roots, elevated levels of ROS were recorded in all treatments, except for the Mn single treatment, whose concentration seems to be low. Elevated ROS concentrations were recorded in leaves in all treatments, except for Mn single treatment (**Fig 5**). Manganese is a transition metal involved in the production of ROS via Fenton reaction (Fitsanakis *et al*., 2010), even though it is less likely than Fe to undergo spontaneous redox cycling (Gregus, 2008). High production of O_2^- increased levels of H_2O_2 and thiobarbituric acid reactive substances in Mn and Fe treated plants (Srivastawa and Dubey, 2011).

Most of the stressful conditions of the environment activate a common response involving the overproduction of ROS such as O_2^- and H_2O_2 in plant cells. Our results showed increased levels of H_2O_2 in the leaves of lettuce exposed to Fe single and Mn single and combined treatment. Similar results showing increased production of ROS due to toxicity of various metals have been shown in many crop species (Wang *et al*., 2004; Shi *et al*., 2005; Maheshwari and Dubey, 2009; Sandalio *et al*., 2009). These overproduced ROS can cause oxidative damage to the biomolecules such as membrane lipids, proteins, chloroplast pigments, enzymes and nucleic acids.

As result of the lipid peroxidation, MDA content significantly increased in both Fe and Mn single and combined treatments. (**Fig 6**) The excess accumulation of MDA indicates damage in the functional and structural integrity of biological membranes (Upadhyay and Panda, 2009). The increase in TBARS concentration in the leaves and roots of the plants with Fe, Mn single treatment and Fe + Mn combination indicates the occurrence of lipid peroxidation, probably due to the increased production of H_2O_2 (Päivöke and Simola, 2001). Heavy metals like Cu, Zn, Mn, Fe caused elevated level of H_2O_2 and induced oxidative stress in barley, *Cucumis sativus* and *Populus cathayana* plants (Demirevska-Kepova *et al*., 2004; Shi *et al*., 2006; Lei *et al*., 2007).

Plants possess efficient antioxidative defense system comprising of non-enzymatic and enzymatic components that protect them from destructive oxidative reactions. An enhanced level of antioxidative components is often correlated with increased stress tolerance of plants (Fecht Christoffers *et al*., 2003; Shi *et al*., 2005).

Reactive oxygen species are scavenged enzymatically through a complex and elaborate coordination of antioxidative enzymes (Apel and Hirt, 2004). Among these

enzymes SODs play important role in scavenging O_2^- by catalyzing the dismutation of two molecules of O_2^- into O_2 and H_2O_2 and serve as first line of defense against toxic O_2^- (Wang *et al*., 2005). The plants subjected to Mn, single and in combination Fe, presented increase in the activities of SOD and suggests induction of a protection mechanism in Mn-stressed plants to protect the cells from oxidative damage caused by O_2^-. Similar increase in SOD activity was observed in common bean, cucumber and tomato plants on Mn exposure (Shenker *et al*., 2004; Shi and Zhu, 2008).

The SOD decreased in the roots of plants subjected to Fe only probably because the high production of H_2O_2 inactivated this enzyme due to conversion of the superoxide radicals, which is a reaction catalyzed by SOD itself. Inactivation of this enzyme may also be due to the inactivation of other enzymes involved in the degradation of these compounds (Khan *et al*., 2009). Iron is a constituent of some of the key antioxidant enzymes associated with detoxification like catalase, ascorbate peroxidase, superoxide dismutase. Therefore, iron stress makes plants more prone to chlorosis and less efficient in reducing the toxic effects of ROS, as a result more oxidative damage increasing the severity of oxidative stress. (Tripathi *et al*., 2018).

The POX activity is an important mechanism in defending against oxidative stress, mainly by eliminating H_2O_2 (Sinha *et al*., 2005). In the roots of lettuce plants POX activity was increased by all treatment compared to the control. Peroxidases play important role in scavenging H_2O_2 in plants however, under excess Mn, its function becomes more complex. The oxidation of Mn^{2+} by a H_2O_2 consuming peroxidase has been proposed to be the key reaction leading to Mn toxicity symptoms (Shi *et al*., 2005).

CAT enzyme showed increased activity in response to oxidative stress and participates in the elimination of H_2O_2, a product of SOD activity (Choi *et al*., 2004). Catalase activities increased in Mn single treatment, as demonstrated by Demirevska-Kepova (2004) in barley plants, but there was a decrease in Fe single and Fe + Mn combinations treated plant. Treatment Fe + Mn significantly decreased CAT activity in lettuce leaves, and resulted in a lower efficiency in scavenging H_2O_2 which showed that CAT might not be a key enzyme in removing H_2O_2. The different effects of Mn toxicity on CAT activity as reported in different studies may be due to the differences in plant species, treatment time and tissues. (Gonnelli *et al*., 2001; Verma and Dubey, 2003; Kim *et al*., 2005).

Catalase and peroxidase are the enzymes involved in the decomposition of H_2O_2 produced in cells due to higher SOD activity (Apel and Hirt, 2004). Different observations have been reported for alterations in CAT activity under abiotic stresses. The activity of CAT increased in plants subjected to salinity and toxicity of certain heavy metals, allowing active scavenging of H_2O_2 (Kim *et al*., 2005), whereas other results showed decline in CAT activity (Gonnelli *et al*., 2001; Verma and Dubey, 2003; Sharma and Dubey, 2007). No definite pattern of alteration in CAT activity could be noticed when rice seedlings were subjected to Ni treatment (Maheshwari and Dubey 2009). Decline in CAT activity in metal exposed plants could be attributed to either inactivation of enzyme due to its direct interaction with ions or ROS (Dat *et al*., 2000) or due to its decreased synthesis or impaired protein assembly (Ushimaru *et al*., 1999).

5. CONCLUSIONS

The result of this study demonstrated that the lettuce plants (*Lactuca sativa* L) cannot undergo Fe and Mn stress without showing toxicity symptoms. The high accumulation of Fe and Mn in the lettuce plant indicated that there was a beneficial effect of Fe on Mn absorption. Also, the absorption and accumulation of Fe and Mn was not inhibitory. The translocation of Fe from the roots to the leaves was high meanwhile Mn translocation to the leaves was low which is interesting in terms of food safety because lettuce is a vegetable eaten by man.

There was an increase in the enzymes activities of the antioxidant system especially the Mn isolated treated plants which was able to scavenge the ROS production and lipid peroxidation thereby preventing oxidative damage to the isolated Mn treated plant. However, Fe treated plant both isolated and combined produced more reactive oxygen species (H_2O_2), high lipid peroxidation which could not be scavenge by the enzymes probably because of the short period of exposure.

6. REFERENCES

Aggarwal A, Sharma I, Tripati B.N, Munjal A.K, Baunthiyal M, Sharma V. (2012). Metal toxicity and photosynthesis. In: Photosynthesis: overviews on recent progress & future perspectives. 1st ed. New Delhi: I K International Publishing House Pvt. Ltd; p. 229–236.

Alam M.G, Snow E.T, Tanaka A. (2003). Arsenic and heavy metal contamination of vegetables grown in Samta village, Bangladesh. Sci. Total Environ 308: 83- 96

Anderson M.D, Prasad T.K, Stewart C.R. (1995). Changes in isozyme profiles of catalase, peroxidase, and glutathione reductase during acclimation to chilling in mesocotylus of maize seedlings. Plant Physiology 109: 1247-1257.

Andrew A.S, Warren A.J, Barchowsky A, Temple K.A, Klei L, Soucy N.V, O'Hara K.A, Hamilton J.W (2003). Genomic and proteomic profiling of responses to toxic metals in human lung cells. Environment Health Perspective 111:825–835.

Anjum N, Singh H.P, Khan M.I.R, Masood A, Per T.S. (2015). Too much is bad - an appraisal of phytotoxicity of elevated plant-beneficial heavy metal ions. Environmental. Science. Pollution. Research: 22, 3361–3382.

Apel K, Hirt H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Rev Plant Biol 55:373–399.

Appenroth KJ, Krech K, Keresztes A, Fischer W, Koloczek H (2010). Effects of nickel on the chloroplasts of the duckweeds Spirodela polyrhiza and Lemna minor and their possible use in biomonitoring and phytoremediation. Chemosphere 87: 3 (216-23).

Arai S. (2002). Global view on functional foods: Asian perspectives. British J. of Nutrition, 88(2), S139-S143.

Araújo T.O, Freitas-Silva L, Santana B.V.N, et al. (2014). Tolerance to iron accumulation and its effects on mineral composition and growth of two grass species. Environmental Science and Pollution Research: 2777–2784.

Baghaie A.H, Fereydoni M. (2019). The potential risk of heavy metals on human health due to the daily consumption of vegetables. Environmental Health Engineering and Management J., 6:11-16.

Bahemuka T.E, Mubofu E.B. (1991). Heavy metals in edible green vegetables grown along the sites of the Sinza and Msimbazi Rivers in Dar Salaam,Tanzania. Food Chemistry 66: 63-66.

Beauchamp C, Fridovich I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry 44: 276–287.

Blanc P.D. (2018). The early history of manganese and the recognition of its neurotoxicity, 1837–1936. Neurotoxicology 64:5–11.

Bonjoch NP, Tamayo PR (2003). Protein content quantification by Bradford method. Handbook Plant Ecophysiol. Tech. pp. 283-295.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochemistry 72:248–254.

Carvalho M.S. de, Ribeiro K.D, Moreira R.M, Almeida, A.M de. (2017). Concentração de metais no rio Doce em Mariana, Minas Gerais, Brasil. Acta Bras.1:37.

Carvalho G.O, Pinheiro A.A, Sousa D.M, Padilha J.A, Souza J.S, Galvão P.M, Paiva T.C, Freire A.S, Santelli R.E, Malm O. (2018). Metals and arsenic in water supply for riverine communities affected by the largest environmental disaster in Brazil: The dam collapse on Doce River. Orbital 10: 299–307.

Chance B, Maehley A.C (1955). Assay of catalase and peroxidase. Met. in Enzymes 2: 755 764

Choi, DG., Yoo, NH., Yu, CY., de Los Reyes, B. and Yun, SJ., (2004). The activities of antioxidant enzymes in response to oxidative stresses and hormones in paraquat-tolerant Rehmannia glutinosa plants. Journal of Biochemistry and Molecular Biology, vol. 37, no. 5, p. 618-624. http://dx.doi.org/10.5483/ BMBRep.2004.37.5.618. PMid:15479627

Connorton J.M, Balk J, Rodriguez-Celma J. (2017). Iron homeostasis in plants - a brief overview. Metallomics. 9: 823.

Cordeiro M.C, Garcia G.D, Rocha A.M, et al. (2019). Insights on the freshwater microbiomes metabolic changes associated with the world's largest mining disaster. Science of the Total Environment 654: 1209–1217.

Dat J, Vandenabeele S, Vranova E, Van Montagu M, Inze D, Van Breusegem F. (2000). Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci 57:779–795.

Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Halzer R, Feller U. (2004). Biochemical changes in barley plants after excess supply of copper and manganese. Environmental and Experimental Botany, 52,253-266.

Deribachew B, Amde M.Z, Nigussie-Dechassa R, Taddesse A.M. (2015). Selected Heavy Metals in Some Vegetables Produced Through Wastewater Irrigation and Their Toxicological Implications in Eastern Ethiopia African Scholarly Science Trust15: 3

Dokiya, Y, Owa, N, & Mitsui, S. (1968). Comparative physiological study of iron, manganese and copper absorption by plants. Soil Science and Plant Nutrition, 14(5), 169–174. doi:10.1080/00380768.1968.10432761

Eissa, M.A, Negim, O.E. (2018). Heavy metals uptake and translocation by lettuce and spinach grown on a metal contaminated soil. J. of Soil Science and Plant Nutrition, 18:1097-1107.

Elloumi N, Ben F, Rhouma A, Ben B, Mezghani I, Boukhris M. (2007). Cadmium-induced growth inhibition and alteration of biochemical parameters in almond seedlings grown in solution culture. Acta Physiologiae Plantarum 29:57–62.

Fecht-Christoffers M.M., Braun H-P., Lemaitre-Guillier Ch. (2003). Effect of manganese toxicity on the proteome of the leaf apoplast in cowpea. Plant. Physiol., 133(4): 1935-1946.

Fernando D.R, Baker A.J.M, Woodrow I.E. (2009). Physiological responses in *Madacadami integrifolia* on exposure to Mn treatment. Australian J. of Botany 57: 406–413.

Fernando N.P, Healy M.A, Aslam M, Davis S.S, Hussein A. (2015). Lead poisoning and traditional practices: the consequences for world health. A study in Kuwait. Public Health, 95: 250-60.

Fitsanakis V.A, Zhang N, Garcia S. Aschner M. (2010). Manganese (Mn) and iron (Fe): interdependency of transport and regulation. Neurotox. Res., 18: 124-125

Gay C, Gebicki J.M. (2000). A critical evaluation of the effect of sorbitol on the ferric-xylenol orange hydroperoxide assay. Anal Biochemistry 284: 217–220.

Giannopolitis C.N, Ries S.K. (1977). Superoxide dismutases: I. Occurrence in higher plants. Plant Physiology 59: 309–314.

Gonnelli C, Galardi F, Gabbrielli R. (2001). Nickel and copper tolerance and toxicity in three tuscan populations of Silene paradoxa. Physiol Plant 113:507–514.

Green Laura S, Rogers Elizabeth E. (2004). FRD3 Controls Iron Localization in Arabidopsis. Plant physiology, Volume 136, Issue 1, p. 2523-2531.

Gregus Z. (2008). Mechanisms of toxicity. In: Casarett and Doull's Toxicology: The Basic Science of Poison. Klassen C.D. (ed), McGraw-Hill Medical, New York, USA.

Guerinot, M.L. (1994). Microbial iron transport. Annu. Rev. Microbiol. doi:10.1146/annur

Guerra M.B.B, Teaney B.T, Mount B.J, Asunskis D.J, Jordan B.T, Barker R.J, Santos E.E, Schaefer C.E.G.R. (2017). Post-catastrophe analysis of the Fundão tailings dam failure in the Doce River system, Southeast Brazil: Potentially toxic elements in affected soils. Water Air Soil Pollution 228:252.

Gustin, J.L, Zanis, M.J, Salt, D.E, (2011). Structure and evolution of the plant cation diffusion facilitator family of ion transporters. BMC Evol. Biol. doi:10.1186/1471-2148-11-76.

Hang Z, Wen-Tao Y, Xin Z, Li L, Jiao-Feng G, Wen-Lei W, Jia-35 Ling Z, Tao T, Pei-Qin P, Bo-Han L. (2016). Accumulation of Heavy Metals in Vegetable Species Planted in Contaminated Soils and the Health Risk Assessment: Int. J. Environ. Res. Public Health13 :289.

Hashemi S.A, Alinejad F, FallahChay M. (2015). Analyzing lead concentration in the sycamore tree species in high- and low-traffic areas of Rasht. Iran. Toxicology. Ind. Health 31: 542–545.

Hatje V, Pedreira R.M, Rezende C.E, Schettini C.A.F, Souza G.C, Marin D.C, Hackspacher P.C. (2017). The environmental impacts of one of the largest tailing dam failures worldwide. Sci. Rep 7:10706.

Havir E.A, McHale N.A. (1987). Biochemical and Developmental Characterization of Multiple Forms of Catalase in Tobacco Leaves. Plant Physiology 84: 450–455.

Heine, G, Max, J.F.J, Führs, H, Moran-Puente, D.W., Heintz, D, Horst, W.J, (2011). Effect of manganese on the resistance of tomato to Pseudocercospora fuligena. J. Plant Nutr. Soil Sci. doi:10.1002/jpln.201000440.

Ismael M. A, Elyamine A. M, Moussa M. G, Cai M, Z hao X, Hu C. (2019). Cadmium plants: uptake, toxicity, and its interactions with selenium fertilizers. *Metallomics* 11:255–277. 10.1039/c8mt00247a [PubMed] [CrossRef] [Google Scholar]

Kar M, Mishra D. (1976). Catalase, Peroxidase, and Polyphenoloxidase Activities during Rice Leaf Senescence. Plant Physiology 57: 315–319.

Kashem M.A, Singh B.R. (1999). Heavy metal contamination of soil and vegetation in the vicinity of industries in Bangladesh. Water Air and Soil Pollution, 115:347-361.

Khan, I., Ahmad, A. and Iqbal, M., (2009). Modulation of antioxidant defense system for arsenic detoxification in Indian mustard. Ecotoxicology and Environmental

Safety, vol. 72, no. 2, p. 626-634. http://dx.doi.org/10.1016/j.ecoenv.2007.11.016. PMid:18262648

Khan S, Cao Q, Zheng Y.M, Huang Y.Z, Zhu Y.G. (2015). Health risk of heavy metals in contaminated soils and food crops irrigated with waste water in Beijing, China. Environmental Pollution Series, 152(3):686-692.

Kim, J.C, Simmins, P.H, Mullans, B. P, Pluske, J.R. (2005). The Digestible energy value of wheat for pigs, with special reference to the post weaned animal. Anim. Feed Sci. Technology, 122(34):257-287.

Kimani N. G. (2007). Implications of the Dandora Municipal Dumping Site in Nairobi, Kenya. Environmental Pollution and Impacts on Public Health. UNEP

Kobayashi M. (1964): Bulletin of the Gunma Agricultural Experiment Station, No.5

Kobayashi T, Nishizawa N.K. (2012). Iron uptake, translocation, and regulation in higher plants. Annual Review of Plant Biology 63: 131–152.

Kobayashi T, Nozoye T, Nishizawa N.K. (2019). Iron transport and its regulation in plants. Free Radical Biology and Medicine 133: 11–20.

Kossoff D, Dubbin W.E, Alfredsson M, et al. (2014). Mine tailings dams: characteristics, failure, environmental impacts, and remediation Appl. Geochem., 51:229-245.

Kuo M.C, and Kao C. (2003). Aluminum effects on lipid peroxidation. Biol Plant 46: 149–152.

Lapaz A.M, Camargos L.S, Yoshida C.H.P, et al. (2020). Response of soybean to soil waterlogging associated with iron excess in the reproductive stage. Physiology and Molecular Biology of Plants 26: 1635–1648.

Lavres Junior, J, Reis A.R, Rossi M.L, Cabral C.P, Nogueira N.L, Malavolta E. (2010). Changes in the ultrastructure of soybean cultivars in response to manganese supply in solution culture. Sci. Agric. 67, 287-294.

Lei Y, Yin C, Ren J, Li C (2007). Effect of osmotic stress and sodium nitroprusside pretreatment on proline metabolism of wheat seedlings. Biol Plant 51:386–390.

Li C, Zhou K, Qin W, Tian C, Qi M, Yan X, Han W. (2019). A Review on heavy metals contamination in soil: effects, sources, and remediation techniques. Soil Sediment Contamination. 28: 380–394.

Lichtenthaler H.K, Wellburn A.R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemistry. Soc Trans 11:591–592.

Lidon, F.C, Barreiro, M.G, Ramalho, J.C, (2004). Manganese accumulation in rice: implications for photosynthetic functioning. J. Plant Physiol. Doi 10.1016/j.jplph.2004.02.003

MacFarlane, GR. and Burchett, MD, (2001). Photosynthetic pigments and peroxidase activity as indicators of heavy metal stress in the grey mangrove, Avicennia marina (Forsk.) Vierh. Marine Pollution Bulletin, vol. 42, no. 3, p. 233-240. http://dx.doi.org/10.1016/S0025-326X (00)00147-8. PMid:11381878

Machold O., Stephan U. (1969). The function of iron in porphyrin and chlorophyll biosynthesis. – Phytochemistry 8: 2189-2192.

Magris R. A, Marta-Almeida M, Monteiro J. A, Ban N. C. A. (2019). Modelling approach to assess the impact of land mining on marine biodiversity: Assessment in coastal catchments experiencing catastrophic events (SW Brazil). Science of The Total Environment, 659: 828-840.

Maheshwari R, and Dubey R.S (2009). Nickel induced oxidative stress and the role of antioxidant defense in rice seedlings. Plant Growth Regulator 59:37–49.

Mahmoudi H., Ksouri R., Gharsalli M. et al. (2005). Differences in responses to iron deficiency between two legumes: lentil (Lens culinaris) and chickpea (Cicer arietinum). – J. Plant Physiol. 162: 1237-1245.

Mapanda F, Mangwayana E.N, Nyamangara J, Giller K.E. (2005). Impacts of sewage irrigation on heavy metals distribution and contamination. Environ Intern 31: 05- 812.

Mapanda F, Mangwayana E.N, Nyamangara J, Giller K.E. (2005). The effects of long-term irrigation using water on heavy metal contents of soils under vegetables. Agriculture, Ecosystem and Environment, 107:151-156.

Marin A.R, Pezeshki S.R, Masschelen P.H, Choi H.S. (1993). Effect of dimethylarsenic acid (dmaa) on growth, tissue arsenic, and photosynthesis of rice plants. J. Plant Nutrition 16:865–880.

Marshall F.M, Holden J, Ghose C, Chisala B, Kapungwe E, Volk J, Agrawal R, Sharma R.K. Singh R.P. (2007). Contaminated Irrigation Water and Food Safety for the Urban and Peri-urban Poor. Appropriate Measures for Monitoring and Control from Field Research in India and Zambia. University of Sussex.

Mascher, R, Lippmann, B, Holzinger, S. and Bergmann, H, (2002). Arsenate toxicity: effects on oxidative stress response molecules and enzymes in red clover plants. Plant Science, vol. 163, no. 5, p. 961-969. http://dx.doi.org/10.1016/ S0168-9452(02)00245-5

Oluyemi E.A, Feujit G, Oyekunle J.A.O, Ogunfowokan A.O. (2008). Seasonal variations in heavy metal concentrations in soil and some selected crops at a landfill in Nigeria, African Journal of Environmental Science and Technology, 2 (5):89-96.

Omachi C.Y, Siani S.M, Chagas F.M, Mascagni M.L, Cordeiro M, Garcia G, Thompson C.C, Siegle E, Thompson F.L. (2018). Atlantic Forest loss caused by the world's largest tailing dam collapse (Fundão Dam, Mariana, Brazil). Remote Sens Appl Soc Environ 12:30–34

Othman O.C. (2001). Heavy Metals in Green Vegetables and Soils from Vegetable Gardens in Dar Es Salaam, Tanzania. Tanzania J. of Science, 27: 37-48.

Päivöke, A.E.A, and Simola, L.K, (2001). Arsenate toxicity to Pisum sativum: mineral nutrients, chlorophyll content, and phytase activity. Ecotoxicology and Environmental Safety, vol. 49, no. 2, p. 111-121. http://dx.doi.org/10.1006/eesa.2001.2044. PMid:11386724

Pandey B, Agrawal M, Singh S. (2016). Assessment of air pollution around coal mining area: Emphasizing on spatial distributions, seasonal variations and heavy metals, using cluster and principal component analysis. Atmospheric Pollution Research, 5:79-86.

Peixoto P.H.P, Cambraia J, Sant'Anna R, Mosquim P.R, Moreira M.A. (1999). Aluminum effects on lipid peroxidation and on the activities of enzymes of oxidative metabolism in sorghum. Rev. Brásil Fisiologial Vegetal. 11: 137–143

Pendias K.A. (2010). Trace elements in soils and plant 4th ed. USA: CRC Press.

Peralta-Videa J.R, Lopez M.L, Narayan M, Saupe G, Gardea-Torresdey J. (2009). The biochemistry of environmental heavy metal uptake by plants: Implications for the food chain. Int.

Pestana M., Correia P.J., Saavedra T. et al. (2012) Development and recovery of iron deficiency by iron resupply to roots or leaves of strawberry plants. – Plant Physiol. Bioch. 53: 1-5.

Prüss-Üstün A. Kimani C. (2007), Preventing disease through healthy environments Towards an estimate of the environmental burden of disease. Geneva: WHO 2006 Publication.

Quaresma V.S, Aguiar V.M, Bastos A.C, Oliveira K.S, Vieira F. V, Sá F, Baptista Neto J.A. (2021). The impact of trace metals in marine sediments after a tailing dam failure: the Fundão dam case (Brazil). Environ. Earth Sci. 80.

Rai S, Singh P.K, Mankotia S, Swain J, Satbhai, S.B. (2021). Iron homeostasis in plants and its crosstalk with copper, zinc, and manganese. Plant Stress 1.

Ramakrishnan, B, Megharaj, M, Venkateswarlu, K, Sethunathan, N, Naidu, R, (2011). Mixtures of environmental pollutants: effects on microorganisms and their activities in soils, in: Reviews of Environmental Contamination and Toxicology. pp. 63–120. https://doi.org/10.1007/978-1-4419-8011-3_3

Reyes-Gutiérrez L.R, Romero-Guzmán E.T, Cabral-Prieto A, Rodríguez-Castillo R. (2006). Characterization of Chromium in Contaminated Soil Studied by SEM, EDS, XRD and Mössbauer Spectroscopy. J. of Minerals & Materials Characterization & Engineering;7(1):59-70.

Rogers, E.E., Eide, D.J., Guerinot, M. Lou, (2000). Altered selectivity in an Arabidopsis metal transporter. Proc. Natl. Acad. Sci. U.S.A. doi:10.1073/pnas.210214197.

Roosta H.R., Mohsenian Y. (2012). Effects of foliar spray of different Fe sources on pepper (Capsicum annum L.) plants in aquaponic system. – Sci. Hortic. -Amsterdam 146: 182-191.

Rout, G.R, Sahoo, S, (2015). Role of iron in plant growth and metabolism. Rev. Agric. Sci. doi:10.7831/ras.3.1.

Rumteke S, Sahu B.L, Dahariya N.S, Patel K.S, Blazhev B, Matini L. (2016). Heavy Metal Contamination of Leafy Vegetables. J. Environ. Protect., 7:996-1004.

Salomons W. (1995). Environmental impact of metals derived from mining activities: processes, predictions, prevention. J. Geochemical Explor. 52:5–23.

Sandalio L.M, Rodriguez-Serrano M, del Rio LA, Romero-Puetas M.C (2009). Reactive oxygen species and signaling in cadmium toxicity. In: del Rio LA, Puppo A (eds) Reactive oxygen species and plant signaling. Springer, Berlin, pp 175–189.

Sankhla M.S, Kumari M, Nandan M, Kumar R, Agrawal P. (2016). Heavy Metals Contamination in water and their hazardous effect on human health- a review. International J. Curri. of Microbiology.Appl.Sci.5:759–766.

Shakya P.R, Khwaounjoo N.M. (2013). Heavy metal contamination in green leafy vegetables collected from different market sites of Kathmandu and their associated health risks. Science World J. 11: 37-42.

Sharma R.K, Agrawal M, Marshall F.M. (2007). Heavy metals contamination of soil and vegetables in suburban areas of Varanasi, India. Ecotoxicology and Environmental Safety, 66:258-266.

Shenker M, Plessner O.E, (2004). Manganese nutrition effects on tomato growth, chlorophyll concentration, and superoxide dismutase activities. pp. 105

Shi Q H, Zhu Z J, Al-aghabary K, Liu H Y, Yu J Q. (2005). Effects of iso-osmotic salt stress on the activities of antioxidative enzymes, H+-ATPase and H+-PPase in tomato plants. Journal of Plant Physiology and Molecular Biology, 30,311-316.

Shi QH, Zhu Z (2008). Effects of exogenous salicylic acid on manganese toxicity, element contents and antioxidative system in cucumber. Environ Exp Bot 63:317–326.

Silva W.R, Salomão L.C, Pereira D.R.M, Oliveira H.F.E, Pereira A.I.A, Cantuario F.S. (2019). Irrigation levels and use of hydro retainer polymer in greenhouse lettuce production. Revista Brasileira de Engenharia Agrícola e Ambiental, 23:406-412.

Singh A, Sharma R.K, Agrawal M, Marshall F.M. (2007). Risk assessment of heavy metal toxicity through contaminated vegetables from waste water irrigated area of Varanasi, India. International Society for Tropical Ecology, 51:375-387.

Singh, HP, Batish, DR., Kohli, RK. and Arora, K, (2010). Arsenic-induced root growth inhibition in mung bean (Phaseolus aureus Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. Plant Growth Regulation, vol. 53, no. 1, p. 65-73.

Sinha, S., Saxena, R. and Singh, S, (2005). Chromium induced lipid peroxidation in the plants of Pistia stratiotes L.: role of antioxidants and antioxidant enzymes. Chemosphere, vol. 58, no. 5, p. 595-604. http://dx.doi.org/10.1016/j.chemosphere.2004.08.071. PMid:15620753

Smoleń S., Kowalska J., Sady W. (2014). Assessment of biofortification with iodine and selenium of lettuce cultivated in the NFT hydroponic system. Sci Hortic., 166: 9-16.

Socha, A.L, Guerinot, M. Lou, (2014). Mn-euvering manganese: the role of transporter gene family members in manganese uptake and mobilization in plants. Front. Plant Sci. doi:10.3389/fpls.2014.00106.

Srivastava S., Dubey R.S. (2011). Manganese-excess induces oxidative stress, lowers the pool of antioxidants and elevates activities of key antioxidative enzymes in rice seedlings. Plant Growth Regul., 64: 1-16.

Sun Z, Mou X, Tong, C., Wang C, Xie Z, Song H, Sun W, Yingchun L. (2018). Spatial Variations and Bioaccumulation of Heavy Metals in Intertidal Zone of the Yellow River Estuary, China. CATENA., 126:43- 52.

Takabayashi, A, Kishine, M., Asada, K., Endo, T. and Sato, F, (2005). Differential use of two cyclic electron flows around photosystem I for driving CO2-concentration

mechanism in C4 photosynthesis. Proceedings of the National Academy of Sciences of the United States of America, vol. 102, no. 46, p. 16898-16903. http://dx.doi.org/10.1073/pnas.0507095102. PMid:16272223

Tripathi, D.K, et al, (2018.) Acquisition and homeostasis of Iron in higher plants and their probable role in abiotic stress tolerance. Front. Environ. Sci. doi:10.3389/fenvs.2017.00086.

Upadhyay H, Panda K. (2004). Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. Journal of Plant Nutrition, 27, 1-15.

Ushimaru T, Kanematsu S, Shibasaka M, Tsuji H (1999). Effect of hypoxia on antioxidant enzymes in aerobically grown rice (Oryza sativa) seedlings. Physiol Plant 107:181–187

Valeriano C.M, Neumann R, Alkmim A.R, et al. (2019). Sm–Nd and Sr isotope fingerprinting of iron mining tailing deposits spilled from the failed SAMARCO Fundão dam 2015 accident at Mariana, SE-Brazil. Applied Geochemistry 106: 34–44.

Varotto C, Maiwald D, Pesaresi P, Jahns P, Salamini F, Leister D. (2002). The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in Arabidopsis thaliana. Plant J.31:589–599.

Vergilio C. dos S, Lacerda D, Oliveira B.C.V. de, Sartori E, Campos G.M, Pereira A.L. de S, Aguiar D.B. de, Souza T. dá S, Almeida M.G. de, Thompson F, Rezende, C.E. de. (2020). Metal concentrations and biological effects from one of the largest mining disasters in the world (Brumadinho, Minas Gerais, Brazil). Sci. Rep.10:1–12.

Verma S, and Dubey R.S (2003). Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci 164:645–655.

Xing W, Li D, Liu G. (2010). Antioxidative responses of Elodea nuttallii (Planch.) H. St. John to short-term iron exposure. Plant Physiology and Biochemistry 48: 873–878.

Xu S, Lin D, Sun H, Yang X, Zhang X. (2015). Excess iron alters the fatty acid composition of chloroplast membrane and decreases the photosynthesis rate: a study in hydroponic pea seedlings. Acta Physiology Plantarum 37:212.

Wang Y, Fang J, Leonard S.S, Rao K.M.K. (2004). Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic Biol Med 36:1434–1443

Wu S (1994). Effect of manganese excess on the soybean plant cultivated under various growth conditions. J Plant Nutr 17:993–1003