ALINE APARECIDA SILVA PEREIRA

UNVEILING HOW CADMIUM AND MANGANESE INTERACT SPATIO-TEMPORALLY DURING SUNFLOWER PLANTS DEVELOPMENT

LAVRAS- MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, para obtenção do título de Doutor.

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UNVEILING HOW CADMIUM AND MANGANESE INTERACT SPATIO-TEMPORALLY DURING SUNFLOWER PLANTS DEVELOPMENT

REVELANDO COMO O CÁDMIO E O MANGANÊS INTERAGEM ESPAÇO-TEMPORALMENTE DURANTE O DESENVOLVIMENTO DAS PLANTAS DE GIRASSOL

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fisiologia Vegetal, para obtenção do título de Doutor.

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2022

DEDICATION

Às minhas mães, Nair e Antônia, minhas fontes de inspiração, força, fé e incentivo.

Dedico

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RESUMO

Devido ao aumento das atividades industriais, a poluição ambiental tem crescido exponencialmente nas últimas décadas. Este fato está diretamente associado à exposição os ecossistemas a altas concentrações de oligoelementos. Plantas tolerantes utilizadas na fitorremediação de ambientes contaminados tornaram-se uma excelente opção econômica para a remediação destas áreas. O girassol (*Helianthus annuus* L.) é uma planta com alto potencial hiperacumulador devido à sua capacidade de resistir a diferentes condições edafoclimáticas, acumular altas concentrações de elementos traços e à alta produção de biomassa. Embora as plantas fitorremediadoras sejam conhecidas por seus mecanismos de tolerância a concentrações elevadas de oligoelementos, o aumento destes elementos nos diferentes tecidos vegetais pode promover alterações morfofisiológicas e bioquímicas, modificando assim o crescimento e desenvolvimento dessas espécies. Assim, este trabalho visa entender como as plantas de girassol lidam com a disposição conjunta do elemento não essencial cádmio (Cd) e do elemento essencial manganês (Mn) em altas concentrações no solo, bem como os mecanismos de resposta relacionados a essa interação e como eles afetam a síntese de metabólitos e fotoassimilados. Para isso, as plantas foram cultivadas em seis condições: testemunha (T1); 1,3 mg. Kg^{-1} de Cd (T2); 5mg. Kg^{-1} de Cd (T3); 400 mg.Kg^{-1} de Mn (T4); 1,3 mg.Kg^{-1} de Cd e 400 mg.Kg^{-1} de Mn (T5); 5 mg.Kg^{-1} de Cd e 400 mg.Kg^{-1} de Mn (T6). Parâmetros bioquímicos, fotossintéticos e a fluorescência da clorofila *a* foram avaliados nas fases vegetativa e reprodutiva (estágios V4, V8, R4 e R7, de acordo com a escala fenológica BBCH). Ao final, foram quantificadas a biomassa, teor de óleo de aquênios e as concentrações de Cd, Mn e P no solo, raiz, folhas e aquênios. O potencial de bioacumulação, taxa de translocação e tolerância do elemento também foram calculados. É possível inferir que o girassol é tolerante às concentrações avaliadas de Cd, que se acumulam majoritariamente nas raízes como mecanismo de tolerância e alívio do estresse. Quando dispostos de maneira conjunta no solo, a planta foi capaz de tolerar Cd e Mn em ambas as concentrações avaliadas. Nessas situações, as respostas à condição estressante estão relacionadas a ajustes metabólicos, sem prejuízo significativo ao aparelho fotossintético. Além disso, o Mn aumenta a extração de Cd e este afeta negativamente à absorção de Mn. Ao contrário do que ocorre em outras espécies, o Cd não interferiu na absorção do elemento essencial fósforo (P). Alterações no metabolismo fotossintético foram observadas em altas concentrações de Mn com redução na taxa de assimilação de CO2, condutância estomática e relação Ci/Ca. Curiosamente, as concentrações de Cd não causaram alterações fotossintéticas, mas, quando disponíveis em conjunto com o Mn, as taxas fotossintéticas, condutância estomática e Ci/Ca aumentaram como mecanismo de ajuste. Esses eventos afetaram diretamente a matéria seca total e razão raiz/parte aérea, com o tratamento contendo 400 Mn apresentando os menores valores, seguido pelos tratamentos contendo Cd e Mn. Também houve atraso no desenvolvimento em plantas cultivadas em 400Mn e com disposição conjunta de Cd e Mn, principalmente no período reprodutivo, embora todas as plantas tenham completado o biociclo.

Palavras-chave: *Helianthus annuus* L.. Elementos traço. Fitorremediação. Condição de estresse. Respostas metabólicas.

ABSTRACT

Due to the increase in industrial activities, environmental pollution has grown exponentially in recent decades. This fact is directly associated with the exposure of ecosystems to high concentrations of trace elements. Tolerant plants used in the phytoremediation of contaminated environments have become an excellent economic option for the remediation of these areas. Sunflower (*Helianthus annuus* L.) is a plant with high hyperaccumulator potential due to its ability to resist different soil and climate conditions, accumulate high concentrations of trace elements and high biomass production. Although phytoremediation plants are known for their mechanisms of tolerance to high concentrations of trace elements, the increase of these elements in different plant tissues can promote morphophysiological and biochemical changes, thus modifying the growth and development of these species. Thus, this work aims to understand how sunflower plants deal with the joint disposition of the non-essential element cadmium (Cd) and the essential element manganese (Mn) in high concentrations in the soil, as well as the response mechanisms related to this interaction and how they affect the synthesis of metabolites and photoassimilates. For this, the plants were cultivated under six conditions: control (T1); 1.3 mg. Kg^{-1} of Cd (T2); 5 mg. Kg^{-1} of Cd (T3); 400 mg.Kg^{-1} of Mn (T4); 1.3 mg.Kg^{-1} of Cd and 400 mg.Kg^{-1} of Mn (T5); 5 mg.Kg^{-1} of Cd and 400 mg.Kg^{-1} of Mn (T6). Biochemical and photosynthetic parameters and chlorophyll a fluorescence were evaluated in the vegetative and reproductive phases (stages V4, V8, R4 and R7, according to the BBCH phenological scale). At the end, the biomass, oil content of achenes and concentrations of Cd, Mn and P in soil, roots, leaves and achenes were quantified. The bioaccumulation potential, translocation rate and element tolerance were also calculated. It is possible to infer that sunflower is tolerant to the evaluated concentrations of Cd, which accumulate mostly in the roots as a mechanism of tolerance and stress relief. When placed together in the soil, the plant was able to tolerate Cd and Mn in both concentrations evaluated. In these situations, the responses to the stressful condition are related to metabolic adjustments, without significant damage to the photosynthetic apparatus. In addition, Mn enhances Cd extraction and Cd negatively affects Mn uptake. Contrary to what occurs in other species, Cd did not interfere with the absorption of the essential element phosphorus (P). Changes in photosynthetic metabolism were observed at high Mn concentration with reduction in CO_2 assimilation rate, stomatal conductance and Ci/Ca ratio. Interestingly, Cd concentrations did not cause photosynthetic changes, but, when available together with Mn, photosynthetic rates, stomatal conductance and Ci/Ca increased as an adjustment mechanism. These events directly affected the total dry matter and root/shoot ratio, with treatment containing 400 Mn presenting the lowest values, followed by the treatments containing Cd and Mn. There was also a delay in development in plants grown in 400Mn and with the joint disposition of Cd and Mn, mainly in the reproductive period, although all plants have completed the biocycle.

Keywords: *Helianthus annuus* L.. Trace elements. Phytoremediation. Stress condition. Metabolism response.

SUMMARY

1. INTRODUCTION

The occurrence and high concentration of potentially toxic elements, with no defined physiological function for plants and animals, in the environment is one of the main negative impacts arising from human activities (ALI; KHAN OLHAI, 2019). With the development of industrialization and the intensification of urbanization, the abundance of pollutants in the environment has increased exponentially in recent decades (SUMAM et al., 2018; ASHRAF et al., 2019), generating major environmental and public health concerns (ABDELHAFEZ; LI, 2014). Among these pollutants, trace elements and/or heavy metals require greater attention due to their non-biodegradable characteristic by any biological or physical process and also because of their persistence in the environment, being considered a long-term threat (SUMAN et al., 2018).

According to the role of elements in biological systems, they are grouped into essential, when they are necessary for physiological and biochemical processes throughout the life cycle of plants and animals, or non-essential, which are highly toxic in small concentrations and do not present known functions (CEMPEL; NIKEL, 2006; FASANI et al., 2018). However, even with defined functions, in excess the essential elements can become toxic. Among the potentially toxic elements, cadmium (Cd), lead (Pb), arsenic (As) and chromium (Cr) stand out because they have a high potential for toxicity to plants even at low concentrations. These elements originate from natural sources, such as volcanoes and rocks (KLEIN; HOEHNE, 2015), or anthropogenic sources, such as the use of phosphate fertilizers (HAMZAH et al., 2016), sewage sludge (FARAHAT; LINDERHOLM, 2015), mining and metal smelting (CHEN et al., 2016).

Cadmium stands out to its high mobility in the soil and a high degree of toxicity to plants and animals, in addition to high solubility (CHANEY, 2015; KABATA-PENDIAS, 2011). As it is considered a non-essential element, plant species do not have specific transport channels, however, due to its electronic configuration, Cd can compete and be absorbed by plants by root absorption channels of other essential divalent elements (QIN et al. ., 2020). Cadmium accumulation in plants is affected by several factors, ranging from soil types and plant nutritional status to the levels of phytoavailable minerals in the soil (SARWAR et al., 2010).

The geochemical behavior of Cd in soil is very similar to that of essential metallic elements such as manganese (Mn) (BOLAN et al., 2003). Mn is a naturally occurring element in nature and is widely found in soils, rocks, air and plants, but it can also occur as a result of anthropogenic sources such as mining (WHO, 2017). As an essential trace element, Mn has defined physiological functions for plants (PEREZ et al., 2016), in addition to being one of the divalent elements constituting several plant metabolism enzymes. Among the trace elements, despite being an essential element, in large concentrations it results in toxic effects direct and indirect for plants, altering the absorption of nutrients such as Fe, N and P, reducing the activity of key enzymes of plant metabolism and productivity (LAVRES JUNIOR et al., 2010; YU et al., 2019).

Due to the similarity between some essential and non-essential elements, in environments where there is a high concentration of these ions, the nutritional status of plants can be negatively affected (MUSZYNSKA; LABUDDA, 2019). On the other hand, for hyperaccumulator plants, which are characterized by the ability to accumulate high concentrations of non-essential elements in different organs and tissues without harming metabolism, the similarity between the chemical elements allows the use of these species as an ecologically viable technique for remediation of contaminated areas. By changing the occurrence and concentration of potentially toxic elements in the environment and increasing the availability of essential elements, the development and application of ecologically friendly remediation methodologies can promote a balance of environmental, social and economic (HOU et al., 2018; JIA et al., 2019).

Phytoremediation is considered an ideal and efficient method to reduce soil ions through the absorption, transfer, extraction and/or fixation of these potentially toxic elements during plant growth and development, and is also described as a low-cost activity (WAN et al. 2016; YU et al., 2019). Plants have developed different strategies that allow them to tolerate and maintain their growth in areas contaminated by trace elements, either by excluding or restricting the absorption of elements by the roots or by absorbing and translocating these to the shoot (ARAUJO et al., 2020). Hyperaccumulator plants are capable of accumulating high concentrations of trace elements in different plant organs, a characteristic that can be applied in soil decontamination and also in providing information on the mechanisms of plant tolerance to the elements (FERNANDO et al., 2013).

Stressful conditions such as high concentrations of trace elements in the soil can have a negative impact on plant growth and development, affecting the functioning of metabolism and promoting significant losses in yield (BORGHI et al., 2019). However, to withstand these conditions described as adverse, plants have developed different mechanisms of perception and signaling that culminate in the phenomenon of acclimatization and consequent survival of the species (BAILEY-SERRES et al., 2019). However, for acclimation to occur in order to guarantee the survival of plants under stress conditions, it is necessary to regulate the responses efficiently, covering the different organs and tissues in a systemic way, which may have the cost of reducing growth, development and productivity, however, enabling survival (BAILEY-SERRES et al., 2019; KOLLIST et al., 2019). In addition, factors such as exposure time and intensity of the stressful condition, in addition to the plant development stage, can directly interfere with plasticity and responses at different levels (LOBO et al., 2000).

The physiological and structural changes in plants caused by the absorption and accumulation of trace elements non-essential such as Cd and high concentrations of essential elements such as Mn, involves an imbalance of nutrient homeostasis, changes in enzymatic activity and gas exchange, promoting oxidative stress , among others (RIBEIRO et al., 2015; ZANG et al., 2017). In this way, understanding the interactions between the environment, the elements that constitute it, in their different concentrations and oxidation states, and plant species is essential to perceive the different metabolic responses and the acclimation of plants to these conditions. Thus, the analysis of this interface can support the understanding of environmental ecology (LOWE et al., 2017) and evolutionary changes that occur at the population level from these correlations between the biotic and abiotic components of the environment.

Sunflower (*Helianthus annuus* L.) belongs to a select group of plant species with the ability to tolerate and develop in areas contaminated with high concentrations of potentially toxic elements (CUTRIGHT et al., 2010; GOVARTHANANA et al., 2018), including cadmium (JUNIOR et al., 2014). In this way, the present work evaluates the hypothesis that synergistic availability of Cd and Mn in the soil alters the phytoremediation potential and triggers morphophysiological responses that alter the synthesis of photoassimilates, the growth and the content of fatty acids in the seeds of sunflower plants cultivated in this stressful condition.

2. THEORETICAL REFERENCE

2.1 Trace elements

According to the function of the elements in the biological system of plants, they were grouped as essential and non-essential. The essential elements, whether in small or large concentrations, have defined physiological functions in the growth and development of plants

(BOUTLÉ; JAILLAIS, 2020; ZOUARI et al., 2020) while the non-essential elements have a high potential for toxicity even in small concentrations (SILVA , 2014). The term trace element applies to chemical substances that have a relatively high density and are present in low concentrations (ZULFIQAR et al., 2019). These elements occur naturally in the soil, and their concentration and form vary according to geochemical processes (BOUTTÉ; JAILLAS, 2020; CHANDRASEKARAN et al., 2015), however, with the advancement of the industrial sector and population growth, anthropogenic sources such as fertilizers, mining, and wastewater have exponentially increased the occurrence of these elements.

The class of trace elements includes heavy metals and others with high phytotoxic potential such as arsenic (As), chromium (Cr), lead (Pb) and cadmium (Cd) and even the essential that, in high concentrations, can become toxic such as iron (Fe), zinc (Zn) and manganese (Mn) (KIMPOUR et al., 2018). In addition to occurrence and concentration, an extremely important factor is the bioavailability of trace elements. Three categories were recognized in relation to ions in the soil according to the bioavailability of the elements, which can be presented as readily bioavailable (Cd, Zn, Se), moderately available (Mn, Mo, Cu) and poorly available (Pb, Cr, As) (KABATA-PENDIAS, 2011). The dynamics of trace elements in the soil is controlled and affected according to soil properties, soluble oxide contents, pH, cation exchange capacity, organic matter content, redox potential, microbial activity and the occurrence and concentration of the elements present there (VIGG et al., 2003; NYSTRAND et al., 2015).

Bioavailability can directly affect the absorption of these elements at trophic levels, however, the toxicity and non-biodegradable nature of these trace elements allows them to accumulate over time (KASTRATOVIC et al., 2014; WU et al., 2018). Changes in natural soil conditions may allow their biomagnification in the environment (BONANNO et al., 2018; RADIC et al., 2018), thus, toxicity, availability and reaction power depend directly on their form and on the environmental conditions where they are inserted (FORTE et al., 2017).

Once bioavailable, the uptake and translocation of trace elements by plants is mediated by ion transporters that can transport specific elements across the cell membrane or mediate the influx - efflux of translocation of these elements and also by complexing agents such as amino acids (DALCORSO et al., 2019). Due to the similarity shown by some essential and non-essential elements, such as Cd and Mn in their bivalent and more soluble form (PITTMAN et al., 2005), the relationship between these two groups can affect the nutritional status of plants (MUSZYNSKA; LABUDDA, 2019) and also favor the absorption of non-essential elements. In this way, this group of elements is subject to great investigation and concern, mainly due to the mobility and concentration levels at which toxic levels are manifested (LI et al., 2014).

When absorbed and accumulated, trace elements can trigger morphophysiological changes impairing plant growth and development, as well as changes in physiological processes such as photosynthesis (SENEVIRATNE et al., 2019). The mechanism of toxicity in plants involves a complex mobilization by the roots and translocation to the shoot. The ascent of these metals to the aerial part of plants can trigger physiological changes at a structural level in membranes, pigments, reduction in gas exchange, anatomical and morphological changes, in addition to the possibility of contamination of occupants of higher trophic levels (SHARMA; DUBEY, 2005; HOSSAIN et al., 2011). It is known that species described as hyperaccumulators do not show phytotoxicity symptoms to certain non-essential trace elements (LATA et al., 2019). Once these ions are absorbed, systemic tolerance strategies are developed by the plant to deal with the toxic potential.

Due to the intensification of activities liable to contamination of water and soil with potentially toxic elements, guidelines and regulations have been developed in order to promote quality control in these areas and establish reference values for the disposal of elements in the soil. Resolution number 420 of the National Council for the Environment (BRASIL, 2009), provides for criteria and values that guide soil quality regarding the presence of chemical substances and establishing guidelines for the environmental management of areas contaminated by these substances as a result of human activities. . Among the established values, there are the prevention values, which provide for the limit concentrations in the soil that are capable of sustaining its main functions, and the investigation values, which describe the amount above which there are direct or indirect potential risks due to of the different land uses and occupations.

A reference value is not defined for the non-essential element cadmium, which is defined by each State individually. In this Resolution, the values of 1.3mg/kg were defined as the prevention value and the concentration of 5.0mg/kg as the investigation value for this element. As for manganese, only the concentration of 400mg/kg was defined as an investigation value.

2.1.1 Cadmium (Cd)

Cadmium is a non-essential trace element recognized for being highly toxic, even in small concentrations, to living organisms and for inducing abnormalities related to plant growth and development (CHELLAIAH, 2018) The presence of cadmium in the soil occurs naturally and through anthropogenic sources (MOHAMMADI et al., 2015; KUMAR et al., 2018) such as mining, phosphate fertilizers, metallurgy, sewage sludge and others (KUBIER et al., 2019). The dominant form of this element in soil is as Cd^{2+} and its physicochemical properties allow its accumulation by binding to soil components (SMOLDERS et al., 1999; REN et al., 2015). From this aggregation of soil particles, Cd is one of the most bioavailable trace elements for absorption by plants and for adsorption on soil particles (LIN et al., 2016).

Cadmium is highly mobile and easy to assimilate, thus, once in the soil, this element is absorbed by the roots through non-specific transport channels and can be translocated to the shoot by vascular bundles (DONG et al., 2019). In general, the behavior of this element in the plant is that it accumulates in the roots and only a small fraction is transported to the shoot, having sequentially roots, leaves, fruits and grains as the order of translocation (KUBIER et al., 2019). Once absorbed, the magnitude and effects caused by Cd on plant development are dependent on the concentration of the element in the tissue, the physicochemical and biological properties of the medium, the plant development stage, the exposure period and the genotype (CARVALHO et al., 2018).

In the soil, in addition to competition with other elements, several factors can interfere with the solubility and bioavailability of cadmium such as soil pH, organic matter content (ABBAS et al., 2017), microbial activity (WANG et al., 2016) , the existence of other ions, due to complexation, exchange sites with the root surface (SAWAR et al., 2010), the redox potential (HASAN et al., 2009), the exudates by the roots and the macro and micro elements (JUNG, 2008). Cations such as Ca^{2+}, Zn and Mn^{2+} compete with Cd for sorption sites in the soil and for transport channels in the absorption by the roots, having been demonstrated the interaction of Cd in the storage and use of P and K elements (TYRAN; POPOVA, 2013). To date, the relationship between the effects of Cd on the nutritional effect of plants is mainly associated with the changes induced by Cd in the activities carried out by transport channels, either by influx or efflux (MIGOCKA et al., 2015) and also by competition with elements essential compounds such as P, Mn and Fe (SINGH et al., 2016).

Cd toxicity in plants has been identified by the reduction and negative impact on the absorption of essential nutrients and by reducing photosynthetic rates, thus promoting a drop in crop production rates (RIZWAN et al., 2016). In leaf tissues, Cd promotes disorganization of the photosystem and thylakoids in chloroplasts, in addition to reducing the number of photosynthetic reaction centers, inhibition of electron transfer rates to PSII (POMPEU et al., 2017) and reduction in chlorophyll content (GRATÃO et al., 2015). As visual symptoms, changes in the growth pattern and occurrence of chlorosis are characteristic of Cd damage (JALI et al., 2016) in addition to variations in root hair formation (BAHMANI et al., 2016). Cadmium exposure also induces ROS overproduction in plants and results in membrane and biomolecule damage (ABBAS et al., 2017). In addition, it has been widely studied on the ratio between Cd toxicity and impacts on crop productivity (FAROOQ et al., 2020), flowering time and consequently fruit maturation (BARMAN et al., 2020).

2.1.2 Manganese (Mn)

Micronutrients are trace elements that have a physiological function and play a central role in plant growth and development, in addition to being central parts in resistance mechanisms (SHAHZAD; AMTMANN, 2017). Among the microelements, iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) are required in low and optimal concentrations, becoming toxic at levels described as supra-optimal (SHINGLEAS et al., 2004). Mn is the second most abundant trace element in the earth's crust and is widely distributed in soil and sediments (GESZVAIN et al., 2012). In soil, Mn is present in several oxidation states, being Mn (II), Mn (III), Mn (IV), Mn (VI) and Mn VII), with the divalent form being the most soluble and available in soil. (GESZVAIN et al., 2012; KABATA-PENDIA, 2011). Anthropogenic activities such as mining and metallurgy result in the deposition of high concentrations of Mn in the soil (XIAO et al., 2020). The excess of Mn and consequently the toxicity caused by this element is considered a limiting factor for plant growth (MORA et al., 2009).

As an essential element, Mn is involved in the photosynthetic process of plants, in enzymatic reactions, redox activity (FERNANDO; LYNCH, 2015; ALEJANDRO et al., 2017), synthesis of fatty acids and proteins (MILLALEO et al., 2010; GRAHAM; WEBB, 2018). Regarding enzymatic activity, Mn acts as a cofactor of numerous enzymes, being considered an activator of responses to stress, such as for MnSOD, oxalate oxidase and in the maintenance of metabolism, acting in the OEC (Oxygen Envolving Center) complex of the PSII responsible for water photolysis (ALEJANDRO et al., 2020). In addition, another important process related to photosynthesis deals with the possible replacement of Mg molecules by Mn at the active site of RUBISCO (BLOOM; LANCASTER, 2018).

Mn deficiency occurs mostly in sandy soils, with a pH above 6 and heavily weathered (DUCIC; POLLE, 2007). At ideal concentrations, Mn in the soil can be absorbed by the roots through the low-affinity Mn transport system, mostly by non-specific transporters, being directly related to the transport of other divalent elements such as Fe, Zn and Cd (PITTMAN, 2005; SOCHA; GUERINOT, 2014). Under conditions with high Mn concentration, plant growth is reduced and responses are varied under these conditions (YAO et al., 2012). Mn stress can interfere with the plant metabolic process, reducing energy production and consequently resulting in an increase in ROS (GILL; TETEJA, 2010), chlorosis in young leaves and reduced growth (LI et al., 2019). Furthermore, there is a complex interaction between the elements available in the soil and the nutritional and toxicity status of the plants, where studies such as the one carried out by Carvalho et al. (2018) report that changes in Mn concentrations are associated with the magnitude of impacts caused by Cd in tomatoes. Mn hyperaccumulating species exhibit specific variability in the distribution of this element in leaf tissues (FERNANDO et al., 2012).

2.2 Phytoremediation and hiperaccumulator plants

Due to the increase in the disposal of pollutants in the environment and the consequent concern with the quality of the soil, there is a growing concern with the rehabilitation of contaminated areas (ASAD et al., 2019). A large part of the inorganic pollutants present in the soil is characterized by non-degradation, whether chemical or biological, thus maintaining high concentrations for long intervals of time (KUBIER et al., 2019), and there may be changes in bioavailability and chemical form (RASHID et al., 2018). Numerous technologies for remediation of contaminated areas have been consolidated, which can be classified into three groups: physical, which involves soil replacement and vitrification techniques, chemical, such as washing and stabilization techniques of elements, and biological, through absorption of the elements by microorganisms (PARK; SON, 2017; FAUZIAH et al., 2017) and with the use of plant species.

Among the current methods of soil restoration, phytoremediation is considered as an alternative with great potential due to the relatively low cost (WAN et al., 2016; ASHRAF et al., 2019), wide adaptability, and low alteration of ecological characteristics (PAN et al., 2018). This technique consists of using plants to remediate the soil, reducing the concentrations of potentially toxic elements through absorption, extraction and/or fixation, and later, enabling new uses for the soil (YU et al., 2019). There are now several phytoremediation techniques such as phytoextraction, phytostabilization, phytodegradation, phytovolatization, and rhizodegradation (CUNDY et al., 2016).

Phytoextraction is a highly efficient tool in the removal of potentially toxic elements from the soil and storage in plant tissues (ABBAS; ABDELHAFEZ, 2013). This technique consists of the use of plant species capable of absorbing soil elements and accumulating in tissues located aboveground tissue (SAWAR et al., 2013; JACOB et al., 2018). Phytostabilization is applied using tolerant species in order to reduce the bioavailability of elements in the soil, either by immobilization, precipitation or alteration of solubility and mobility (GERHARDT et al., 2017). Phytodegradation consists of the degradation of elements absorbed by plants in less phytotoxic forms or in the synthesis and release of exudates that are later secreted by plants and act directly on the soil (SIVARAMAKRISHNAN et al., 2018). Finally, phytovolatization is a strategy where elements are extracted from the soil by plant roots, translocated to the shoots and later released into the atmosphere with the transpiration flow, however, it is important to note that the elements are converted in less toxic forms (YAN et al., 2020).

One of the principles of phytoremediation, aiming at the successful application of the technique, is the selection and use of species tolerant to potentially toxic elements, essential or non-essential, in the environment and that they produce biomass, grow and develop under these conditions (GUTIÉRREZ et al. 2016; MINGORANCE et al., 2016). Plants differ in their ability to accumulate high concentrations of essential and potentially toxic elements (KACALKOVA et al., 2015) and species with phytoremediation potential have few limitations such as rapid growth, high biomass (SAWAR et al., 2017) and acclimatization to environmental conditions, especially nutritional deficiency (GERHARDT et al., 2017).

Kramer (2010) determined the limits of leaf concentration (dry weight) for plants hyperaccumulating elements as > 100 μg/g for cadmium (Cd); > 300 μg/g for cobalt (Co) and copper (Cu); > 1000 μg/g for nickel (Ni), arsenic (As) and lead (Pb); > 3000 μg/g for zinc (Zn); and > 10,000 μg/g for manganese (Mn). In this sense, plants have developed different strategies that give them the ability to tolerate high concentrations of these elements, however, maintaining growth and development. Some strategies involve restricting the absorption of elements by the roots and consequent translocation to the shoot (ARAUJO et al., 2020) or even absorbing and accumulating these ions in different compartments. Thus, the responses or strategies developed by plants happen intra and extracellularly, in order to reduce the deleterious effects of the elements in cells and tissues (RODRIGUES, 2016). In addition, it is important to highlight that the interaction between the plant and the pollutant is different between species, the level of toxicity of the element, concentrations, exposure time (SOUZA et al., 2011; RODRIGUES et al., 2016) and the multielemental occurrence.

Currently, more than 450 plant species have been identified as metal hyperaccumulators (SUMAN et al., 2018). In particular, under the condition of trace element stress, photosynthetic adjustments and the ability to allocate biomass in phytoremediation species are fundamental for the success of plant development and remediation of areas (LEI et al., 2019; WAN et al., 2017). Furthermore, it has been shown that an optimal biomass allocation strategy involves everything from achieving a balance of light energy to the absorption and use of water and nutrients (GIERTYCH et al., 2015). In addition to photosynthetic and nutritional parameters, the alteration in the activity of antioxidant enzymes and the adjustment of osmotic substances (SASMAZ; SASMAZ, 2009) can be considered key mechanisms in protecting against damage from stress by potentially toxic elements. The antioxidant enzyme system includes superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), which can remove excess ROS and reduce oxidative damage caused by trace element stress (Li et al., 2016). The adjustment of osmotic substances such as free proline, soluble proteins and sugar allows the maintenance of the water content and osmotic potential of the plant cell, thus protecting the biological macromolecules and reducing the damage caused by the osmotic loss of water (WANG et al., 2013).

Studies carried out with sunflower (*Helianthus annuus* L.) have shown that these plants have high tolerance to high concentrations of trace elements (RIZWAN et al., 2016, GOVARTHANANA et al., 2018). This species has characteristics such as high biomass, rapid growth rate and ability to extract and store varied trace elements from contaminated soils (FORTE; MUTITI, 2017). Promising results obtained by Alaboudi et al. (2018) showed that sunflower has the ability to accumulate Pb and Cd in the roots and shoots, an important fact based on the condition that soil contamination occurs in a multi-elemental way.

2.3 Sunflower (*Helianthus annuus* L.)

Sunflower (*Helianthus annuus* L.) is a dicot belonging to the Asteraceae family originated from the North American continent, however, due to the fact that it has the ability to acclimate to different soil and climate conditions, this species has been cultivated in different regions, including Brazil (SANTOS et al., 2016; SIMÕES et al., 2018). The cultivation of this species is considered a viable option for the crop rotation system because it has a short cycle, with wide acclimatization to variations in altitude and photoperiod, addition to being resistant to cold and high temperatures and promote improvement in the physicochemical and biological conditions of the soil (CARVALHO et al., 2015).

Regarding the phenological scale of sunflower, Schneiter and Miller (1981) divided the plant's development into vegetative and reproductive phases. The vegetative phase begins with the emergence of the seedling and the emergence of the first pair of leaves. This phase is subdivided in relation to the number of true leaves, presenting at least 4 cm in length, counted starting as V1, V2, V3, V4, etc. The number of days between the vegetative phases is variable and depends on genotypic and environmental factors. The sunflower's vegetative cycle varies between 90 and 130 days, while its flowering period varies between 10 and 15 days. The reproductive phase begins with the emergence of the floral bud and evolves until the physiological maturation of the plant.

Initially, the inflorescence, surrounded by the immature bract, becomes visible and presents several points, called the star stage. The process of formation of floral primordia begins with the establishment of (V8) 10 leaves and is the phase considered essential in determining the potential number of achenes. Subsequently, the internode located below the base of the flower bud elongates from 0.5 to 2.0 cm above the last leaf of the stem and then the inflorescence begins to open. The ligulate flowers are visible and this is considered the most critical period of development (R4). The next stage is described by the beginning of anthesis, with the ligulate flowers fully expanded and the floral disk becoming visible. Subsequently, all the tubular flowers open and the ligulate flowers lose turgidity (BLANCHET, 1994; SCHNEITER, MILLER, 1981; ROSSI, 1998). Finally, the development of achenes begins, where the back of the capitulum turns yellow and the bracts green and the leaf surface is reduced. The last phase is marked by the physiological maturation of the achenes.

Sunflower stands out among the oilseeds due to the high oil content in its seeds, with estimated amounts higher than 50%, with about 68% of these being linoleic acid, which is ideal for the production of biodiesel (WU et al., 2015). Seeds are also sources of proteins used in the production of bran and can be used for rations, fertilizers and forages (SANTOS et al., 2015). In addition to the various industrial uses of sunflower by-products, this species stands out as being considered moderately tolerant to drought and salinity (OLIVEIRA et al., 2014; NUNES JUNIOR et al., 2017) and also as efficient in the accumulation of trace elements (MARQUES, 2009).

High concentrations of potentially toxic trace elements can generate oxidative stress, directly or indirectly, through the generation of ROS (EHSAN et al., 2014), inactivation of PSSII through the inhibition of electron transfer (FAROOQ et al., 2016), alteration nutritional status, expression of defense mechanisms (XIE et al., 2018), and others. However, sunflowers are able to tolerate some trace elements by different mechanisms, varying according to the cultivar, the form of the elements, the type of soil, the concentration and the time of exposure of the plants to the stressful condition (DEBAEKE et al., 2021). These mechanisms are related to the allocation of biomass (BATISTA, 2013), to the accumulation in specific organs such as the roots and reduction of translocation to the shoot (JUNIO et al., 2014), the activity of the enzymatic antioxidant mechanisms (SAIDI et al., 2014) and adjustments in the synthesis and accumulation of protective osmolytes (WANG et al., 2013). Thus, considering the sunflower as a species with high phytoremediation potential for areas contaminated by trace elements, this work aims to understand how the joint disposition of the nonessential element Cd and the essential element Mn in high concentrations in the soil can affect the phytoremediation potential of this species, as well as understanding the response mechanisms related to this interaction and how they affect the synthesis of metabolites throughout different stages of plant development.

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Article 1: How the interaction of Cd and Mn jointly alters the phytoremediation potential and metabolism of sunflower plants: a soil-plant interface

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How the interaction of Cd and Mn jointly alters the phytoremediation potential and metabolism of sunflower plants: a soil-plant interface

ABSTRACT

Remediation of areas contaminated by potentially toxic elements and high essential elements concentrations are a current challenge, especially with regard to the interaction and factors that affect the bioavailability of these ions. *Helianthus annuus* L. is considered a phytoremediator, being tolerant to high concentrations of non-essential elements. Thus, this work aimed to evaluate how the jointly disposition of Cd and Mn in the soil can interfere in the phytoremediation potential and in the photosynthetic metabolism in sunflower plants. For this, the plants were cultivated during the entire biocycle under six conditions: control (T1); 1.3 mg. Kg^{-1} of Cd (T2); 5 mg. Kg^{-1} of Cd (T3); 400 mg.Kg^{-1} of Mn (T4); 1.3 mg.Kg^{-1} of Cd and 400 mg.Kg^{-1} of Mn (T5); 5 mg.Kg^{-1} of Cd and 400 mg.Kg^{-1} of Mn (T6). For this, the incident and absorbed irradiance data were evaluated throughout the experiment using a pyranometer. The photosynthetic parameters were evaluated in two moments of the vegetative and reproductive phases and, at the end of the experiment, the biomass, the oil content in the achenes, and the concentrations of Cd, Mn and P in the soil, root, leaf and achenes were quantified. Were calculated the bioaccumulation potential, translocation rate and element tolerance. In the end, it was concluded that the concentration of 400 Mn did not interfere in the phytoremediation potential of sunflower for Cd, and the bioaccumulation of this element was increased. However, Cd reduces Mn uptake and does not interfere with P uptake. Plants showed greater tolerance to Cd and to Cd and Mn together at both concentrations than at 400 mg Mn. Regarding photosynthetic metabolism, plants grown at 400mg Mn showed the lowest rates of *A*, Ci/Ca and g*s*, the other treatments showed adjustments in these parameters. For total dry matter and root/shoot ratio, the treatment containing 400 mg Mn also presented the lowest values, followed by treatments containing Cd and Mn at both concentrations. It was observed that plants grown in soil containing 400 mg of Mn and in the condition where there was joint availability of Mn and Cd at both concentrations showed a delay in development during the reproductive stage when , but all plants completed the biocycle. This fact was associated with the observed adjustments in photosynthetic metabolism of these treatments.

Keywords: phytoremediation; trace elements; bioaccumulation; metabolism; gas exchange; biocycle

1. INTRODUCTION

The soil contamination by trace elements is described as one of the biggest environmental concerns of the last decades, endangering human health through the food chain and reducing plant growth and development in these areas (Rizwan et al., 2016). Trace elements occur naturally in soil (Colin and Jaillais, 2020), however, considering the exponential increase in anthropic activities and the expansion of contaminated areas, the restoration of degraded ecosystems has become an urgent and complex challenge (Wang et al., 2017). The methods of rehabilitation of contaminated areas has been intensively discussed (Liu et al., 2018; Asad et al., 2019), and legal provisions have been created to regulate and manage the risks arising from the contamination of natural environments.

The CONAMA Resolution 420 of 2009 provides criteria for soil quality regarding the presence of chemical substances, among them trace elements, using reference values based on natural concentrations for prevention and investigation values. Most of the trace elements (TE) are also important nutrients for higher plants in various stages of metabolism, whether in gene regulation (Chen et al., 2019), cell signaling, CO_2 assimilation, stomatal conductance, and synthesis of specialized metabolites (Yadav et al., 2019). However, special attention has been directed to the elements described as essential which, at high concentrations, can cause disturbances in plant metabolism (Lai, 2015), i.e., reducing photosynthesis and nutrient uptake, causing an imbalance in ROS production, and oxidative damage (Jabeen et al., 2016; Rizwan et al., 2016).

Conventional methods used for revegetation of disturbed areas contaminated with trace elements have limitations such as high remediation price and damage to soil structure (Shu et al., 2015). In this sense, phytoremediation has stood out as an efficient, ecological, and economically viable technique (Wan et al., 2016; Nedjimi, 2020). The ideal species to perform phytoremediation present characteristics such as fast growth, high biomass production, and tolerance to edaphoclimatic variations (Liu et al., 2014). Sunflower (*Helianthus annus* L.) is one of the most studied species for remediating soils contaminated with non-essential trace elements (Niu et al., 2007; Mahar et al., 2016) such as cadmium. Furthermore, sunflower can be cultivated in different soil and climatic conditions, presenting rapid growth (Gouda et al., 2018), and with ability to acclimatize to a range of environments (Debaeke et al., 2021).

Since the contamination of the environment occurs in a complex way, evaluating the phytoremediation potential of the species regarding a single-element is not representative of the conditions found in the field (Ribeiro et al., 2020). Thus, since the essential and non-essential elements are arranged together in the soil, their uptake and accumulation in plants is dependent on the concentration and bioavailability of the ions to the roots (Bian et al., 2018). The uptake of potentially toxic elements occurs through mechanisms analogous to that of essential nutrients (Reuscher et al., 2016).

Reuscher et al., (2016) reported the interference of ions of the non-essential element cadmium (Cd) in relation to the accumulation and translocation of essential elements such as phosphorus (P). Nutrients can affect the bioavailability of Cd and the relationship of the element in the soil-plant continuum (Dheri et al., 2007). Since absorbed, Cd can cause nutritional disturbances, oxidative stress, and reduced growth in plants (Carvalho et al., 2020). On the other hand, high concentrations of the essential element manganese (Mn) can lead to iron (Fe) deficiency, also described as an essential element, which can cause significant changes in processes such as gas exchanges (Yamaguchi et al., 2017) negatively impacting plant growth and development.

The synergistic arrangement of elements in soil can trigger different strategies in plants, among them the reduction of the uptake of toxic elements and the mitigation of damage (Muszynska and Labudda, 2019). Thus, it was hypothesized that high concentrations of the micronutrient Mn in the soil can reduce the phytoremediation potential of sunflower for Cd through metabolic adjustments. In this way, this work aimed to evaluate how the jointly disposition of Cd and Mn in the soil can interfere in the phytoremediation potential, and in the photosynthetic metabolism of sunflower.

2. MATERIAL AND METHODS

2.1 Plant material, and experimental conditions

The experiment was conducted in a greenhouse at the Federal University of Lavras (UFLA) in Lavras/MG. According to Köppen, the climate classification is Cwa, considered warm and temperate (Alvares et al., 2013).

Seeds of the sunflower (*Helianthus annus* L.) simple hybrid (Helio 250) from commercial lot 04, commercially obtained. After harvest, the seeds were packed in multi-layered Kraft paper and polyethylene plastic bags and stored in a cold chamber with a constant temperature of 10ºC, and 9-10% water content.

The substrate used was composed of red oxisol (USDA 1999) of clayey texture, presenting moderate permeability and water retention, and sand in the proportion of 2:1. After homogenization, the substrate was placed in 20-liter capacity pots watered with solutions containing $CdCl_2$ (cadmium chloride) and/or $MnSO_4$ (manganese sulfate). The pots were reserved during 21 days for incubation. Then, the seeds were sown and the pots were kept in the greenhouse for 130 days with an average temperature of 25ºC, relative humidity (R.H.) of 60% and pH between 5.5 and 6.5. Fertilization was performed according to the recommendation of Malavolta (2006).

The concentrations of Cd and Mn were established using the values of prevention and investigation defined in Annex II of CONAMA Resolution no 420 of 2009. The experiment was performed in randomized blocks with six treatments, as follows: control (T1); 1.3 mg. Kg^{-1} of Cd (T2); 5 mg. Kg^{-1} of Cd (T3); 400 mg.Kg^{-1} of Mn (T4); 1.3 mg.$Kg^{-1}$of Cd and 400 mg.Kg^{-1} of Mn (T5); 5 mg.$Kg^{-1}$of Cd and 400 mg.Kg^{-1} of Mn (T6).

The data of incident and absorbed irradiance as well as gas exchange analyses were performed, using 4 plants per treatment, at V4 (4 leaves fully expanded), V8 (8 leaves fully expanded), R4 (first phase of flowering, characterized by presenting the first ligulate flowers), and R7 (beginning of the development of the achenes). At the end of the experiment, four plants from each treatment were collected to quantify biomass, oil content in the achenes, productivity analysis, and the concentrations of Cd, Mn and P in the soil, root, leaf and achenes. These data were used to calculate the bioaccumulation potential, the translocation rate and the tolerance of plants grown under these conditions.

2.2 Evaluation of cadmium, manganese and phosphorus content

The quantification of cadmium, manganese and phosphorus in the soil, roots, leaves and achenes was performed as described by Tedesco et al., (1995), using Atomic Absorption Spectrophotometer equipment. After the collection, the plant´s organs (root and leaves) were washed in running water, and in acid solution (HCl 1 %) to remove the metals adsorbed to the surface of the roots and finally in distilled water to remove the sediments associated with plant tissues. Subsequently, the roots, leaves and achenes were kept in an oven at 60°C for drying until they reached a constant weight. After the drying process, the samples were ground and subjected to the digestion step in concentrated nitric-perchloric acid. Finally, the elements were quantified by atomic absorption spectrometry.

2.3 Evaluation of bioconcentration, translocation and tolerance

The indicators of tolerance, bioaccumulation and translocation were calculated to evaluate the bioaccumulation potential and translocation efficiency of Cd and Mn by sunflower plants. The bioconcentration factor (BFC) indicates the efficiency of the plant to accumulate the target elements arranged in the soil in its tissues (Ladislas et al., 2012). It is calculated by the following equation (Zhuang et al., 2007).

$$Bioconcentration\ factor\ (BFC) = \frac{Metal\ concentratio\ in\ the\ plant}{Metal\ concentratoin\ in\ soil}$$

The translocation factor (TF) indicates the efficiency of the plant in translocating the analyzed element accumulated in the roots to the aerial part. The TF was calculated using the equation (Zacchini et al., 2009), and is presented as a percentage.

$$Translocation\ factor\ (TF) = \frac{C\ aerial}{C\ root}$$

The abiotic stress tolerance index (TI) was calculated as follows:

$$Tolerance\ index\ (TI) = \frac{BMT}{BMC}x100$$

BMT is the biomass accumulated in each treatment and BMC is the biomass accumulated in the control treatment (Rahman et al., 2013).

2.4 Gas exchange analysis

The measurements of gas exchange were performed between 8 and 10 hours, in the middle third of the third fully expanded leaf of each plant, using the infrared gas analyzer model LI- 6400XT, LI-COR, Lincoln, NE, USA. Measurements took place under a CO_2controlled system (6.400-01, Li-Cor Inc) at 400 µmol mol^{-1}, at a leaf temperature of 25°C and artificial light adjusted to 1,500 µmol photons $m^{-2}s^{-1}$ that was provided in a 2cm² area by a light emitting diode (LED) source (model 6400-02B Red-Blue, Li -Cor Inc). The following variables were analyzed: net photosynthetic rate (A, lmolm^{-2}s^{-1}), stomatal conductance (g_s, mol $m^{-2}s^{-1}$), transpiration (E, mmol $m^{-2}s^{-1}$) and the ratio between internal and external CO_2 concentration (Ci / Ca).

2.5 Biomass analysis

At the end of the experiment, the accumulated root, leaf, achene and total dry matter was quantified based on Cairo et al. (2008). The samples were placed in Kraft paper bags and taken to a forced circulation oven at 65ºC until reaching a constant weight. Dry mass values were obtained by weighing the plant material on digital scales.

2.6 Evaluation of achenes oil content

Ethereal extract determination was performed according to AOAC (2011) with modifications using a TE-044 Fat Determiner soxhlet extractor, using petroleum ether as solvent. Sunflower seeds grown in the 6 treatments were used for the analysis. Initially, the seed teguments were removed and then the samples were macerated and dried in a circulating-air oven at 105°C for 1 hour. After drying, 1 gram of the material was weighed into filter paper cartridges. The cartridges were placed inside reboilers plus 100 ml of petroleum ether. The samples remained immersed in the solvent for 3 hours at 70°C. After the end of the extraction by immersion, the samples were kept suspended for 30 minutes receiving the dripping of the condensed solvent. At the end, the cartridges containing the samples were removed and kept in an oven at 70ºC for 1 hour for complete evaporation of the solvent. Subsequently, the cartridges were transferred to the desiccator for 30 minutes and weighed for ethereal extract quantification using the reason:

$$EE\% = (Initial\ weight - final\ weight) * 100$$

2.7 Statistical analysis

Statistical analysis was performed in Rbio software (Bhering, 2017). Data were submitted to Analysis of Variance (ANOVA) and, in case of normal distribution, Tukey's test of means was performed at 5% significance level. Data without normal distribution were evaluated by GLM 48 analysis, assuming normality by observing the qq-plot graphs and then applying Tukey's test at 5% significance.

3. RESULTS

Plants show different strategies to respond to excess trace elements in the soil, resulting in distinct values of accumulation and toxicity. In this work, the bioconcentration, translocation and tolerance factors of sunflower plants submitted to the conditions under analysis were reported (Figure 1). It was observed that the treatment with the highest cadmium concentration also presented the highest bioconcentration of Mn, however, after exposure of the plants to Cd and Mn synergistically, a reduction in Mn accumulation was observed for both treatments. Interestingly, the treatment containing only Mn showed lower bioconcentration of the trace element compared to the control and the highest dose of cadmium used. Regarding the translocation of Mn from the root to the shoot, it was observed that T2 (1.3Cd) presented the highest rate for this parameter (Figura 1A). The control treatment and T3 (5.0 Cd) showed the highest concentrations bioaccumulated in the organs, but with lower rates of translocation (Figure 1B).

Concerning cadmium, the plants presented opposite responses to those observed for the Mn element. The treatments where Cd and Mn were disposed synergistically showed the highest values of bioconcentration of Cd (Figura 1D). The treatments with a concentration of 1.3 mg.kg^{-1} de Cd showed the highest accumulation of the element both when disposed alone and together with Mn. As for the translocation factor (Figure 1C), the treatment containing the highest concentration of Cd together with Mn showed the highest rate. However, in the presence of 1.3 mg.kg^{-1}of Cd the plants translocated higher concentrations of the element when compared to the treatment with 1.3 mg.kg^{-1}of Cd and 400 mg.kg^{-1}of Mn, even though they had the lowest bioconcentration rates for the element.

Besides that, when observed the dose of 5.0 mg.kg^{-1} of Cd, the responses were the opposite. For the phosphorus, the bioconcentration was statistically different only in treatment 1.3Cd + 400Mn, which was the lowest, value found. For the translocation factor, the highest rates were observed in the treatment 5.0Cd+500Mn followed by the treatments with the presence of cadmium (Figure 1 F). The translocation and bioconcentration factors directly interfered in the tolerance index of the plants.

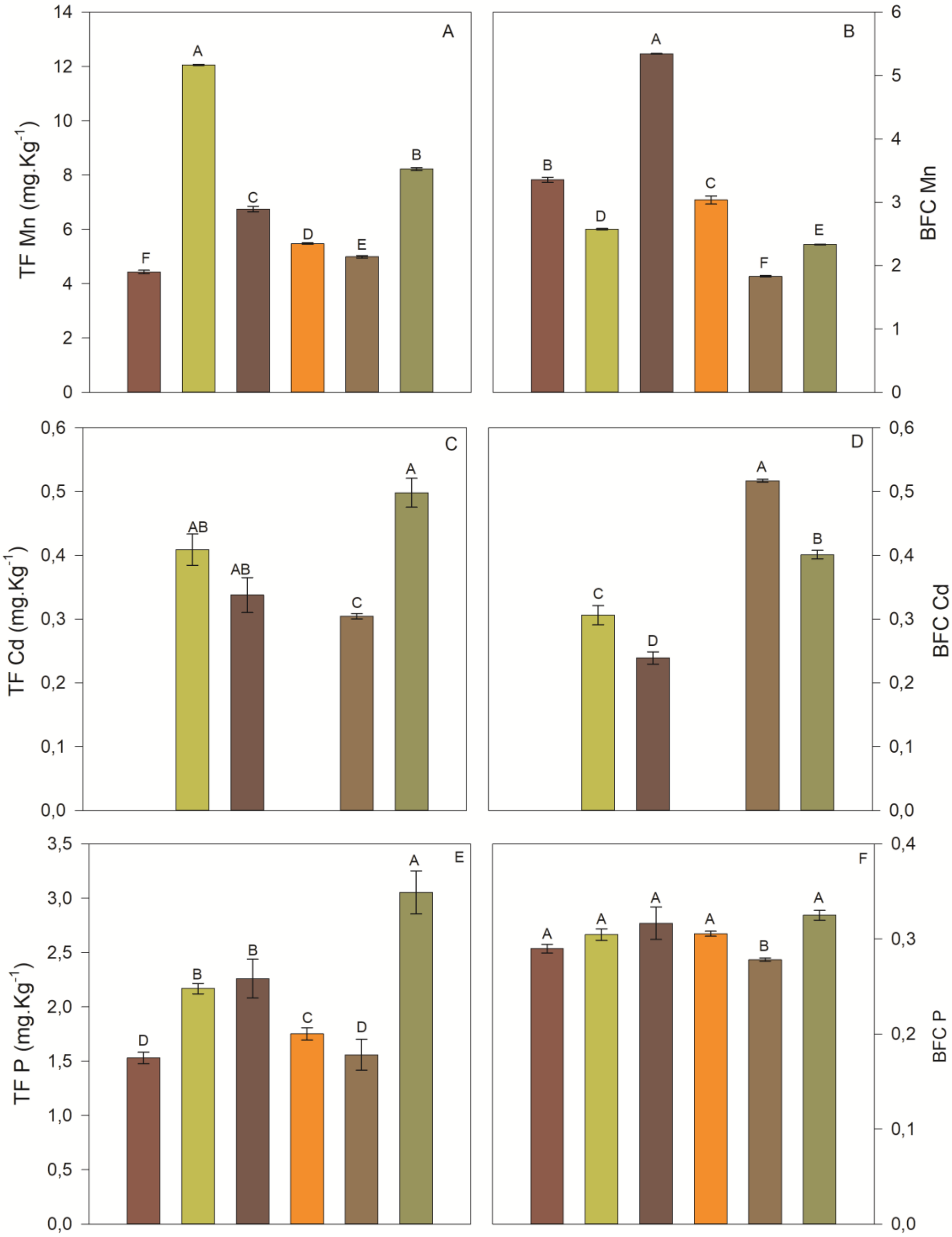

Figure1. Mn translocation factor (A), Mn bioconcentration factor (B), Cd translocation factor (C) Cd bioconcentration factor (D), P translocation factor (E), and P bioconcentration factor of sunflower (*Helianthus annuus* L.) plants grown in Cd and Mn contaminated soil. Letters compare the responses in each treatment. Equal letters show that there was no statistical difference between treatments by Tukey's test ($p < 0.05$). Bars represent means ± standard error (n = 4).

Observing Figure 2A, it is possible to verify that the treatment containing 400mg Mn, without the addition of cadmium, presented the lowest index of tolerance to the submitted condition. Regarding the treatments with Cd, it can be observed that the highest concentration of this element (5.0 mg Cd) the plants showed a tolerance index similar to the control, however, when disposed together with Mn (5.0 mg Cd + 400 mn Mn), plant tolerance was reduced. For the concentration of 1.3 Cd, when disposed alone or together with Mn (1.3 Cd + 400 mg Mn), its tolerance was not affected (Figure 2A).

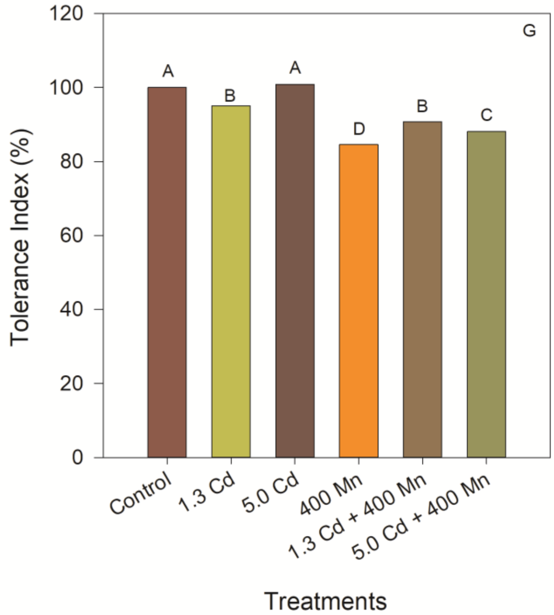

Figure 2. Tolerance index of sunflower (*Helianthus annuus* L.) plants grown in Cd and Mn contaminated soil. Letters compare the responses in each treatment. Equal letters show that there was no statistical difference between treatments by Tukey's test ($p < 0.05$). Bars represent means ± standard error ($n = 4$).

Different responses were observed regarding gas exchange in the cultivation conditions and stages of development analyzed. The CO2 assimilatory rate (A) (Figure 3A) and stomata conductance were lower in plants exposed to Mn during all periods analyzed. The treatment containing 1.3Cd + 400Mn presented high rates of CiCa, in comparison to the other treatments, while the rate of A was increased over time, being higher in the reproductive stage. The stomata conductance remained high until the end of the vegetative stage (V8) with a reduction in the reproductive rate, as well as the transpiration rate.

Interestingly, the treatment with 5Cd + 400 Mn showed high rates of *A*, *CiCa* and g*s* and, like the other treatments, such as the control, showed higher *A* and *E* in the vegetative stages compared to the reproductive period, with only the condition with 400Mn maintaining high transpiration rates over time. The highest g*s* values were observed at the V8 stage, followed by a decrease, except for the 1.3Cd+400Mn treatment.

The lowest ratio of internal and external CO2 concentration (Ci/Ca) (FIGURE 3D) for all treatments was observed at the V8 developmental stage, close to the transition between the vegetative and reproductive periods. During the reproductive stage, only the 5.0Cd treatment showed a significant reduction Ci/Ca. These results indicate that the greatest sensitivity of the photosynthetic machinery to the elements under analysis occurred during the early developmental period.

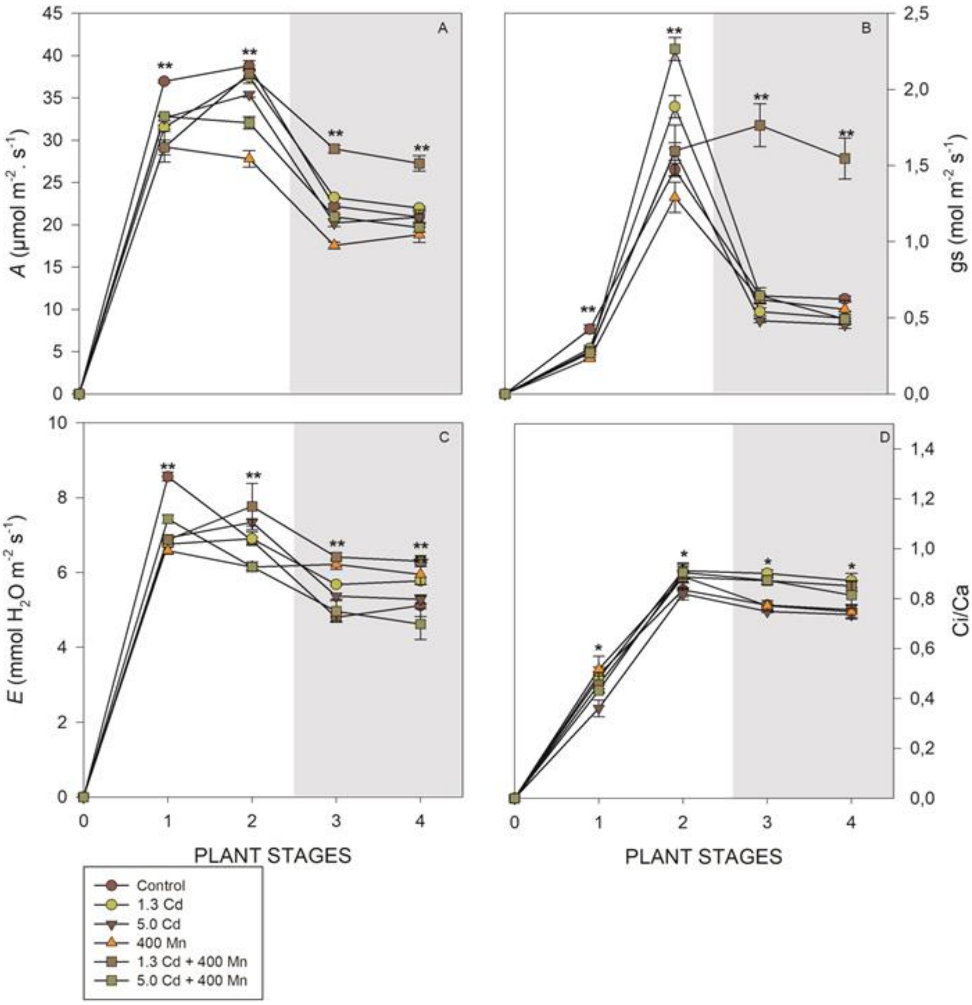

Figure 3 Photosynthesis (A), stomatal conductance (B), transpiration (C) and internal and atmospheric carbon ratio (D) in sunflower (*Helianthus annuus* L.) leaves grown in soil contaminated by Cd and Mn. Shading in gray indicates analysis performed during the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test ($p < 0.05$). Bars represent means ± standard error ($n = 4$). On the x axis: 1= V4; 2= V8; 3 = R4; 4 = R7.

Regarding the efficient conversion of photoassimilates into biomass, it was observed the proportionality of the results described by the tolerance index and the gas exchange data with the accumulated dry weight data, the ratio between root and aboveground part, and the weight of the achenes (Figure 4). For the total dry weight parameter (Figure 3A), the lowest conversion rate into biomass was found in plants grown in the conditions with 400mg Mn in the soil and 5.0Cd + 400mg Mn. Sunflower plants grown in soil containing only 1.3 and 5.0 Cd showed similar results to the control.

The root/shoot ratio was lower in the treatment containing 400 mg of Mn and in the treatment containing 5.0 mg Cd and 400 mg of Cd together. The lowest concentration of Cd (1.3 mg Cd) presented the same value of the ratio when disposed alone and together with Mn. Interestingly, at the concentration of 5.0 mg Cd, the root/shoot ratio was the same as that presented by the control treatment. When the organ of economic interest of the species was observed, the filling of the grains and consequently the weight of the achenes was lower when Mn was present. The treatments containing cadmium were similar to the control, but when Cd and Mn were used synergistically there was a significant reduction.

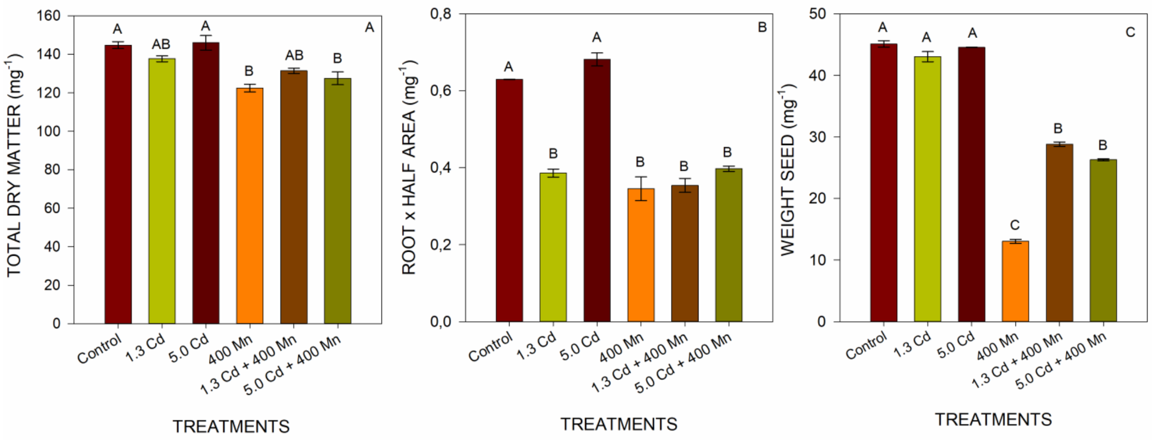

Figure 4 Total dry matter (A), root to aboveground ratio (B) and seed weight (C) of sunflower (*Helianthus annuus* L.) grown in Cd and Mn contaminated soil. Letters compare the responses in each treatment. Equal letters show that there was no statistical difference between treatments by Tukey's test ($p < 0.05$). Bars represent means ± standard error ($n = 4$).

As for the oil content in the achenes (Figure 5), the control treatment showed the highest values, with the other treatments not differing from each other. However, it is important to emphasize that was necessary larger number of achenes to reach the weight to quantify the oil.

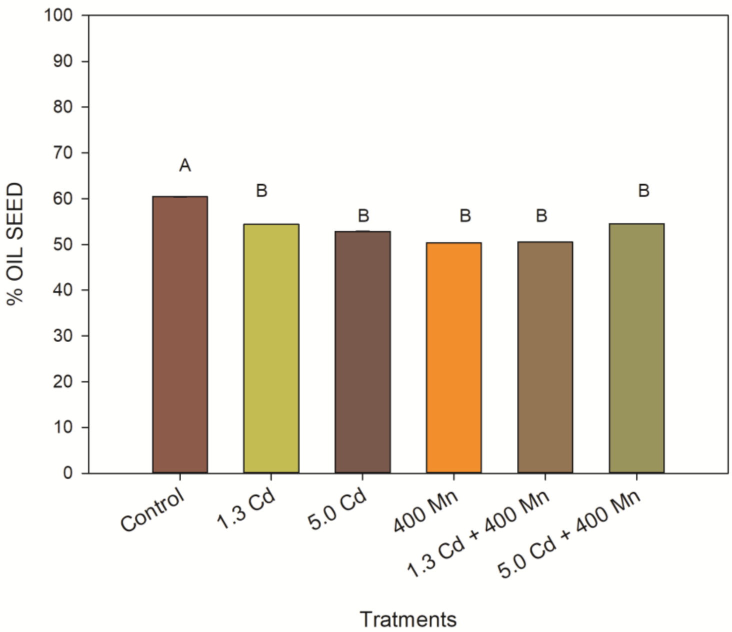

Figure 5. Percentage of oil (A) of sunflower (*Helianthus annuus L.*) seeds grown in soil contaminated by Cd and Mn. Letters compare the responses in each treatment. Equal letters show that there was no statistical difference between treatments by Tukey's test ($p < 0.05$). Bars represent means ± standard error ($n = 4$).

By monitoring the life cycle of sunflower plants and the responses presented over time, it was possible to observe the phenotypic changes that occurred in the developmental stages (Figure 6). It can be inferred that there was a developmental delay in sunflower plants grown in soil containing manganese at the concentration evaluated and in the condition where there was a synergistic availability of manganese and cadmium since the initial stages of development. However, in all conditions evaluated the plants completed the biocycle.

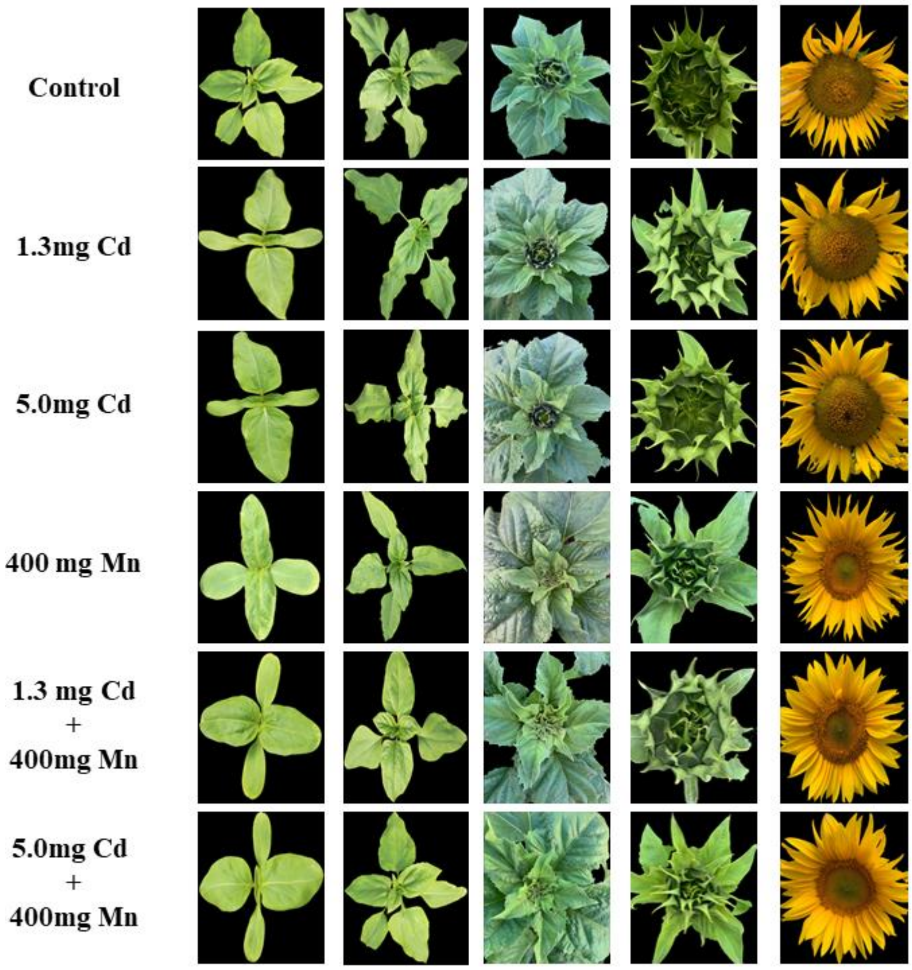

Figure 6 Characterization of the different developmental stages (V4, V8, R4 e R7) of sunflower (*Helianthus annuus* L.) plants grown in soil with different concentrations of Cd and Mn.

4. DISCUSSION

The results found in this study demonstrate that sunflower plants show different strategies to tolerate excess Cd and Mn in the soil, when arranged individually or synergistically. High amounts of Cd and Mn were taken up by the sunflower plants and translocated to the aerial part. The highest concentration of cadmium (T6) induced the highest translocation of cadmium to the aerial part when disposed together with Mn. The lowest concentration, on the other hand, induced the highest translocation of Mn to the aerial part, even though T6 (5.0 mg Cd) presented the highest accumulation. Curiously, Cd interfered negatively in the uptake of Mn when arranged together. These results corroborate those of Cutright et al., (2010) in which sunflower plants accumulated heavy metals simultaneously when cultivated in contaminated mixed soil. Thus, in this work, the plants presented adjustments in metabolism and in the accumulation and translocation of elements, which directly interfered in photosynthetic metabolism, production and biomass partitioning. The first strategy of plants when cultivated in areas with high concentrations of potentially toxic elements is the exclusion and/or complexation of these elements in the roots (Gratão et al., 2005). However, sunflower plants underwent the strategy of accumulation Cd and Mn in the tissues of the aerial part.

The lower uptake of Mn in plants when disposed together with the concentration of 1.3 mg Cd may be related to the responses induced by cadmium in the root structure, such as in the plasma membrane or by competition between the elements for the same transporter channels (Migocka and Klobus 2007; Wu et al., 2016). However, being required in small concentrations, the reduction of the absorption of Mn would not lead to the deficiency of this element. The translocation factor is an important factor to evaluate the phytoremediation potential of plant species, by assessing the efficiency of the plants in translocating the elements present in the soil to the aerial part (Mendez and Maier 2008). This evaluation indicates the potential of plants to accumulate the elements in the aerial part without significant damage to the metabolism that could affect the production of biomass

In non-stressful concentrations, cadmium can induce defense responses in plants (Poschenrieder et al., 2013). Moderate cadmium-induced stress involves a similar or better performance of plants in the presence of the element when compared to the control and to other potentially toxic elements, phenomenon called hormesis effect, as observed by Piotto et al., (2018) in tomato plants. Our results corroborate with those provided by Piotto et al., (2018) which allows inferring that cadmium tolerance depends on genotype-specific mechanisms, not depending exclusively on the lower concentration in plant tissues.

At physiological levels, the hormesis effect induced by Cd is associated to adjustments in photosynthetic activity and in the different forms of energy dissipation, in addition to the change in root length to provide a balance in the nutritional state of plants (Carvalho et al., 2020). In our results, adjustments were observed in the absorption and translocation rates of the elements under analysis, in the root/shoot ratio and in plant development. However, adjustments at the photosynthetic level may be more correlated with the stage of development, as seen in Figure 2.At higher concentrations trace elements can cause deficiency of essential nutrients in plants (Rizwan et al., 2016).

In this work, phosphorus uptake was not affected by the concentrations of Cd and Mn used. However, there was a higher translocation to the aerial part in the treatments with Cd, at both concentrations, and in the treatment with the highest dose of Cd together with Mn. The interactions between macro and micro elements can occur antagonistically or synergistically and this process is directly associated with the physiological processes of the plant and the environmental conditions (Kabata-Pendias 2011). Thus, it is possible to perceive that the mechanisms of response of plants to stress are multiple, varying mainly due to the intensity, exposure time and space where it occurs, being controlled by various signaling pathways.

Atkinson and Urwin (2012) elucidate in their work that plant responses to a combination of stresses do not simply represent the sum of the responses to each stressor condition. The conditions in the environment can interact in complex ways where a single stress can induce different answers and, on the other hand, different stresses can result in similar changes (Blum 2016) or cause favorable responses. In this way, observing the physiological adjustments presented by the plants regarding the extraction and absorption of the elements when arranged in an isolated and synergistic way, and also regarding the adjustments in photosynthetic metabolism under these conditions, it is possible affirm that, in the concentrations and conditions evaluated, the phytoremediation potential of sunflower is not affected when Cd and Mn are disposed together at the concentrations evaluated.

The concentrations of Cd alone, in this work, did not affect the photosynthesis, which was contrary to observations of Junior et al., (2016). However, 400 mg Mn when disposed in a singular manner and together with Cd caused changes in photosynthesis, especially in the initial stages of development evaluated. This fact can be associated with the reduction of biomass, the root to aerial part ratio, and of the achenes of the plants cultivated under these conditions. For the remediation of contaminated areas, it is necessary to select plants that are tolerant to the contaminants, have the ability to grow and reproduce in the adverse stressful conditions (Gutiérrez et al., 2016; Mingorance et al., 2016). Biomass production is associated with the conversion efficiency of photosynthetically active radiation absorbed by plants into photoassimilates from photosynthesis (Heinemann et al., 2006; Heldwein et al., 2007).

The increased bioaccumulation and translocation of Cd to the aerial part when disposed together with Mn in sunflower plants is intriguing. Cd-induced growth reduction may also be tied to reduced carbon fixation due to decreased photosynthesis and chlorophyll content (Hassanet al., 2005). When in a synergistic manner, Mn can assist in the mitigation of Cd damage by collaborating with the maintenance of membrane integrity and reduction of oxidative damage (Rahman et al., 2016), which explains the greater tolerance, biomass accumulation and mass of the achenes in this condition, where Mn bioaccumulation was lower.

In this work, the absence of a Mn-competitor element probably favored the absorption and translocation of Mn, which reduced plant tolerance and reflected in photosynthetic parameters, biomass, productivity and mass of the achenes. According to Ramos and collaborators (2002) the excess of Mn is stored in the aerial part mainly in the chloroplasts. In such case, the element can induce disorganization of chloroplast lamellae (Lavres Junior et al., 2010), resulting in damage to the photosynthetic apparatus and inducing the production of reactive oxygen species (ROS) (Rojas-Lillo et al., 2014). Moreover, the lower CO_2 assimilation rate in plants grown in the condition with excessive Mn indicates a negative effect of the metal on internal C concentration and carboxylation. Gururani et al., (2015) argue that the lower efficiency in carbon assimilation and, consequently, in the Calvin cycle, occurs due to the lower availability of NADP+ for the carbon fixation process and lower stability of photosystem II.

Interestingly, the Cd concentrations evaluated here only affected yield when synergistically disposed with Mn. The treatment with Mn drastically affected productivity and, although it did not show significant differences with respect to lipid content, a greater number of achenes were required to reach the mass necessary to perform the analysis. Thus, the reserve tissues were reduced in the Mn condition. This fact may be associated with reduced water uptake, which is also related to the reduction of gs, and nutrients, in addition to the translocation of these to the fruits (Hédiji et al., 2015; Kumar et al., 2015). In addition to plant biomass, stress conditions can modify progeny success by modifying fruit and seed filling (Herman and Sultan, 2011) and productivity, as observed in the results presented. Mn toxicity can result in the degradation of lipids, proteins, and carbohydrates, negatively altering cellular metabolism (Fernando et al., 2013). In addition, Cd can affect the allocation and partitioning of amino acids and sugars (Lima et al., 2019).

Plants grown in the presence of Mn and in the condition of both Mn and Cd available showed significant delays in the reproductive phase and in the formation of the floral receptacle, as well as in the filling and ripening of the grains, but they eventually completed the biocycle and produced offspring. These results suggest evidence of the occurrence of different strategies of tolerant plants to stressful condition, called slow syndrome (Sartori et al., 2019). Thus, it can be considered that sunflowers are tolerant to the concentrations of the studied elements and the arrangement of these, presenting different responses throughout the stage of development and in the different metabolic processes.

5. CONCLUSIONS

The potential of Cd uptake and bioaccumulation by sunflower plants was not affected when arranged together with the evaluated Mn concentration. On the contrary, in the presence of Mn, there was an increase in the rate of Cd bioaccumulation at both concentrations evaluated. At the concentration of 5.0 mg Cd, the rate of translocation to the shoot was even higher in the presence of Mn. For the treatment containing 400 mg Mn, Cd interfered with the absorption and bioaccumulation of the essential element. As for the element P, the treatments with Cd and Mn did not interfere in the bioaccumulation of the element. Sunflower plants showed greater tolerance to conditions with 1.3 and 5.0 mg Cd alone and together with 400mg Mn than the condition with 400 mg Mn single.

Regarding photosynthetic metabolism responses, it was observed that the treatment with 400mg Mn presented the greatest reductions in CO_2 assimilation rate, stomata conductance and *CiCa* ratio. The treatments containing Cd in both concentrations showed similar responses to the control and once the 400 mg Mn concentration was synergistically arranged, the treatment containing the highest concentration showed adjustments in photosynthetic parameters increasing the rates of g*s* and *A*.

These results directly affected the total dry matter, the root/shoot ratio and productivity, where the treatment containing 400 mg Mn also presented the lowest values, followed by the treatments containing Cd and Mn in both concentrations. When arranged in isolation, Cd did not affect these parameters. The filling of the grains was directly affected, it was possible to conclude that to obtain the oil contents a greater number of achenes was needed.

Sunflower plants showed a delay in plant development when grown in soil containing 400 mg Mn and in the condition where there was synergistic availability of Mn and Cd at both concentrations, especially in the reproductive period, but all plants completed the biocycle.

6. ACKNOWLEDGMENT

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Chapter 2: Improve to overcome: how sunflower plants perform to cadmium and manganese in soil over time

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IMPROVE TO OVERCOME: HOW SUNFLOWER PLANTS PERFORM TO CADMIUM AND MANGANESE IN SOIL OVER TIME

Abstract

Sunflower (*Helianthus annuus* L.) is a widely used species for remediation of contaminated areas due to the potential for accumulation of trace elements. Different mechanisms and metabolic adjustments are required for tolerance capacity, ranging from changes in enzymatic activity to osmolyte production. Thus, we aim to understand the adjustments promoted by the excess of Mn and Cd alone and together in the photosynthetic apparatus, in the enzymatic and non-enzymatic responses of the antioxidant system, in the synthesis of stress-related metabolites and in the accumulation of hydrogen peroxide and consequent oxidative damage. For this, the experimental design was randomized blocks (DBC), with 6 treatments: (T1) control, (T2) 1.3 mg.Kg^{-1} Cd, (T3) 5 mg.Kg^{-1} Cd, (T4) 400 mg. Kg^{-1} Mn, (T5) 1.3 mg.Kg^{-1} of Cd + 400 mg.Kg^{-1} Mn, and (T6) 5 mg.Kg^{-1} Cd + 400 mg.Kg^{-1}Mn and 4 collections along the biocycle. Biochemical analyzes, chlorophyll *a* fluorescence and quantification of Cd, Mn and P in soil, roots, leaves and achenes were performed. The greatest accumulation of Cd occurred in the root, compared to the shoot (phytostabilization) and the greatest accumulation of Mn occurred in the leaves. Unlike Cd, the absorption of Mn was reduced when combined with Cd. The photochemical adjustments not affected photosynthetic apparatus and and the quantum yield. Thus, it can be concluded that sunflower is tolerant to Cd in isolation and jointly with Mn, and the responses to the stressful condition are related to metabolic adjustments like metabolites synthesis os stresse-related, such as prolina, caronedoids and sugars, without significant damage to the photosynthetic apparatus.

Keywords: Trace elements · Tolerance · Photochemistry · Enzymatic metabolism · Biochemistry

Introduction

Contamination of soil and water by trace elements is one of the biggest environmental problems today (Rizwan et al. 2016). These elements can naturally occur in the environment, originating from volcanic eruptions and rock weathering (Ali et al. 2019). However, the intensification of anthropogenic activities such as mining, the phosphate fertilizers use, and untreated effluents discharge has contributed to an increase in their concentration in the environment, making them potentially harmful to plant species and the food chain (Alamgir et al. 2015; Bonanno et al. 2018).

Once exposed to high concentrations of essential and non- essential trace elements, plant species may have negative responses such as reduced gas exchange and activity of enzymes that act on stress-induced responses, such as SOD, CAT and others, as well as enzymes that act on carbon fixation, such as Rubisco (Dutta et al. 2018), structural changes in proteins, generation of ROS and oxidative damage to macromolecules (Chung et al. 2021). In addition to the direct metabolic effects, non-essential elements are also taken up by plants through competition for micro and macronutrients transport channels, enhancing the accumulation of potentially toxic ions (Han et al. 2019).

Plants have developed a precise regulatory system, including responses at transcriptional, post-transcriptional, post-translational and epigenetic levels (Chung et al. 2021). Thus, the tolerance of plant species to stressful conditions largely depends on the activity of enzymatic and non-enzymatic antioxidant mechanisms, the synthesis and, consequently, accumulation of macromolecules in cells (Maleva et al. 2017), such as proline (Wan et al. 2016). Some of these strategies are performed by plant species described as hyperaccumulators, which areable to tolerating high concentrations of non-essential and phytotoxic elements in the airline (Kramer 2010). These plants are best candidates in the remediation of contaminated soil in restoration programs.

Among plant species able to accumulate trace elements in high concentrations studies carried out by Rizwan et al (2015) and Govarthananan et al (2018) showed that sunflower (*Helianthus annuus* L.) has high tolerance to varying concentrations of these elements. It is reported that sunflower absorption and translocation of ions increases according to their concentration in the medium (Cornu et al. 2016). However, the sunflowers potentially toxic elements absorption is highly influenced by the properties of the soil (Kolbas et al. 2014), the mobility and availability of the elements, which are controlled to biogeochemical processes (He et al. 2015), the concentration and exposure time (Rizwan et al. 2016), soil pH (Oborn et al. 1995) and the presence of organic matter and other elements.

Several works were carried out considering monoelementary arrangement of trace elements in soils. However, environmental contamination, especially in mining areas and close to industrialization centers, occurs in a multi-elemental way (Ribeiro et al. 2020). Thus, it is essential that the studies are representative of the impacted areas and, in addition, that the responses and metabolic alterations are evaluated according to the conditions found in the field. Sunflowers are able to tolerate different concentrations and types of metals by different mechanisms (Rizwan et al. 2016). It is also reported that sunflower plants can hyperaccumulate several elements simultaneously (Cutright et al. 2010). Among Sewalem et al (2014) suggest that sunflower plants are capable of remediating soils contaminated by non-essential elements such as cadmium by phytostabilization.

Cd is a non-essential element that compromises plant growth and development even at low concentrations (Dias et al. 2013). Cd toxicity negatively affects gas exchange, promotes reduction of photosynthetic pigments and induces damage to chloroplasts (CI et al. 2009), promotes leaf chlorosis, reduced biomass (Rizwan et al. 2016) increasing of ROS formation (Farooq et al. 2019), consequently causing oxidative stress (Younis et al. 2016). In addition, high concentrations of Cd can reduce the absorption of essential elements by plants (Rochayati et al. 2011), harming plant metabolism and response mechanisms. However, it has been observed that manganese, an essential element for plant growth, can reduce Cd uptake, as demonstrated in rice plants (Wang et al. 2018).

Besidesan essential element for plants, Mn is widely distributed in nature, being the third most abundant transition metal in the world (Neculita and Rosa 2018). Being an essential element for plants, playing important roles in metabolic processes such as photosynthesis, ATP synthesis, fatty acids, amino acids and proteins (Millaleo et al. 2010), Mn transport occurs through active transport as a divalent cation , which can compete with bivalent Cd because they have common absorption and transport pathways (Pittman 2005). However, enhancing mining activity produces tons of toxic tailings, which leads to the release of Mn in potentially toxic concentrations in the environment (Huang et al. 2018; Neculita and Rosa 2018).

Therefore, the central question of this work arises, which seeks to clarify the response mechanisms related to the interaction between Cd and Mn and how this interaction affects the photosynthetic processes and consequently the synthesis of metabolites. Thus, this work aimed to study the adjustments promoted by the excess of Mn and Cd alone and together in the photosynthetic apparatus, in the enzymatic and non-enzymatic responses of the antioxidant system, in the synthesis of stress-related metabolites and in the accumulation of peroxide of hydrogen and consequent oxidative damage.

Material and Methods

Plant material and experimental conditions

The experiment was carried out in a greenhouse at the Plant Physiology Sector of the Federal University of Lavras (UFLA) in Lavras/MG, Brazil. Sunflower hybrid seeds (HELIO 250), were used as plant material, selected due to tolerance to high concentrations of trace elements in the soil (Mahar et al. 2016). After being collected, the seeds were stored in multilayer Kraft paper bags and polyethylene plastic bags and stored in a cold chamber with a constant temperature of 10ºC and water content of 9-10%.

There was used red oxisol with a clayey texture and sand (2x1). The substrate was homogenized and placed in 18L pots. The contamination of the homogenized soil was based on the prevention and investigation values for Cd and Mn established by the Resolution of the National Environmental Council (CONAMA) 420/2009, using solutions containing cadmium chloride ($CdCl_2$) and manganese sulfate (MnSO4).After soil contamination, the pots were incubated for 21 days. After incubation, soil fertilization was performed according to Malavolta (2006) recommendation, and the pH was between 5.5 and 6.5. The seeds were sown and the experiment was conducted in a greenhouse for 130 days with an average temperature of 25ºC, relative humidity (RH) of 60%.

The experimental design was randomized blocks (DBC), with 6 treatments: (T1) control, (T2) 1.3 $mg.Kg^{-1}$ Cd, (T3) 5 $mg.Kg^{-1}$ Cd, (T4) 400 $mg.Kg^{-1}$ Mn, (T5) 1.3 $mg.Kg^{-1}$ of Cd +400 $mg.Kg^{-1}$Mn, and (T6) 5 $mg.Kg^{-1}$ Cd +400 $mg.Kg^{-1}$Mn. Biochemical analyzes of chlorophyll a fluorescence and quantification of photosynthetic pigments in plant material were performed throughout the plant life cycle, in the vegetative and reproductive time: V4 (4 fully expanded leaves), V8 stage (8 fully expanded leaves), R4 (beginning of inflorescence opening, precedes anthesis) and R7 (on set of achene development).

The quantification of the metals accumulated in the soil, root, leaf and achenes was carried out at the end of the experiment, using 4 sampling for each treatment.

Analysis of cadmium, manganese and phosphorus content

The plants were collected and sectioned into root and leaf, washed in running water, acid solution (1% HCL) and again in running water for drying in an air circulation oven at 60ºC for 48 hours. The achenes were collected from the inflorescence, identified and kept in an oven at 60ºC until reaching constant weight. After drying, the samples were crushed and digested in concentrated nitric-perchloric acid and later the cadmium, manganese and phosphorus concentrations were quantified by the methodology described by Tedesco et al (1995), using the Atomic Absorption Spectrophotometer.

Quantification of photosynthetic pigments: chlorophyll *a*, *b* and carotenoids

The leaves were collected and stored in aluminum foil, properly identified and kept on ice. Subsequently, 0.1 g of fresh matter was grinded in 5mL of 80% acetone. After filtering the extract, the final volume was completed with 80% acetone to 10mL and then readings were taken in a spectrophotometer at 663.2 nm, 646.8 nm and 470 nm for chlorophyll *a*, *b* and carotenoids respectively, according to the described by Lichtenthaler and Buschmann (2001).

Chlorophyll *a* fluorescence

A portable PAM fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany) was used together with a leaf clip holder. The initial fluorescence (F0) and the maximum quantum yield of photosystem II (PSII, Fv / Fm) were determined in leaves acclimated to the dark for a period of 30 minutes (Genty et al. 1989). After the analyzes carried out in the dark, the leaves were exposed to a flow of photosynthetic photons of 1000 μmol m-2 s-1 for 60 seconds, followed by a saturation pulse in order to determine: the effective quantum yield of PSII (ϕPSII) (Genty et al. 1989), the photochemical quenching coefficient (q L) (Kramer et al. 2004) and the non-photochemical quenching of fluorescence (NPQ) (Bilger and Björkman 1990). To calculate the apparent electron transport rate (ETR), the equation was used: ETR = 0.5 x lA 9 /ϕ PSII x PPFD, where 0.5 is the assumed proportion of absorbed quanta used by the PSII reaction centers (Melis et al. 1987) and lA is leaf absorbance.

Biochemical analyzes - Quantification of hydrogen peroxide and lipid peroxidation

Lipid peroxidation was determined by quantification of malondialdehyde (MDA) as described by Dhindsa et al. (1981). Samples of 200 mg of root and leaf were grinded in liquid nitrogen and homogenized in 1.25 ml of trichloroacetic acid (TCA) (0.1%) and sodium duodecyl sulfate (SDS) (1%). The homogenate was centrifuged at 12,000 g for 15 min. For a 300 μL aliquot of the supernatant, 1mL of 20% trichloroacetic acid (TCA) was added to a vessel containing 0.5% thiobarbituric acid (TBA). The mixture was heated at 95°C for 30 minutes and then cooled in an ice bath. The estimate of lipid peroxidation was obtained from the absorbance reading at 532 nm and the MDA concentration was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} (Baryla et al. 2000).

The hydrogen peroxide (H_2O_2) levels were performed by the method of Velikova et al (2000). 100 mg of fresh leaves and roots were used, grinded in liquid nitrogen and homogenized with 1 mL of 0.1% trichloroacetic acid (TCA). The samples were centrifuged and the reaction was carried out with 10mM potassium phosphate (KH_2PO_4) buffer, pH 7.0 and 1M potassium iodide (KI). The samples were analyzed in a spectrophotometer at 390 nm and the H_2O_2 levels were quantified using a standard curve.

Antioxidant system enzymes

The activities of antioxidant metabolism enzymes (catalase – CAT, superoxide dismutase – SOD and ascorbate peroxidase – APX) were evaluated in leaf and root tissues. Enzyme extracts were obtained according to Biemelt et al (1998) in which 200 mg of leaf or root tissues were macerated in liquid nitrogen plus insoluble PVPP (Polyvinylpolypyrolidone) and 1.5 mL of extraction buffer composed of: Potassium phosphate 400 mM (pH 7.8), EDTA 10 mM and 200 mM ascorbic acid. The homogenate was centrifuged at 13,000 g for 10 minutes at4 °C and the supernatant collected.

SOD activity was estimated by the enzyme's ability to inhibit nitrotrazolium blue (NBT) photoreduction (Giannopolitis and Ries 1977). CAT activity was determined according to Havir and McHale (1987) and APX activity was determined according to Nakano and Asada (1981).

Quantification of compatible solute accumulation - Proline

The method described by Torello and Rice (1986) was usedto obtain the supernatant. Samples with 200 mg of fresh material (leaves) were homogenized with 10 ml of 3% sulfosalicylic acid and centrifuged at 5000 rpm for 20 minutes. In a test tube containing 2 ml of the supernatant, 2 ml of acid ninhydrin and 2 ml of glacial acetic acid were added (Bates et al. 1973). Then the samples were kept for 1 hour in a water bath at 100º C. After cooling, by immersion in an ice bath, the reading was performed in a spectrophotometer at 520 nm. The absorbance obtained wascompared with the standard curve for proline and the results obtained were expressed in micrograms of proline per g of fresh material.

Reducing sugars, total soluble sugars and amino acids content

Based on the methodology described by Zanandrea et al. (2010), the extraction of macroelements occurred through the homogenization of 0.2 g of dry matter (ms) of leaves and roots in 10mL of potassium phosphate buffer, 100 mM and pH 7.0, followed by a water bath for 30 minutes at 40ºC. Subsequently, the homogenate was centrifuged at 6,400 rpm for 10 minutes, collecting the supernatant. The supernatant aliquots were used for the quantification of reducing sugars using the Dinitrosalicylic Acid (DNS) method described by Miller (1959), total soluble sugars by the Anthrone method described by Yemm and Willis (1954) and total free amino acids by the ninhydrin assay described by Yemm and Cocking (1954).

Protein content

For protein extraction, approximately 100 mg of fresh matter from each experimental plot were macerated in liquid nitrogen and polyvinylpolypyrrolidone (PVPP) before homogenization, carried out in 10 mL of potassium phosphate buffer (KH_2PO_4) (100 mM, pH 8), added of 0.1 Mm ethylene diaminetetra acetic acid (EDTA) and 10 mM ascorbic acid ($C_6H_8O_6$). The resulting material was centrifuged at 13000 g for 10 min at 4°C. Using the collected supernatant, the determination of proteins was carried out according to the method of Bradford (1976), using the Comassie Blue G-250 dye, H_3PO_4 85% (v/v) and ethanol 95% (v/v).

Statistical analysis

The experiment was carried out in blocks in a 4x6 bidirectional factorial arrangement, with 4 collection periods and 6 treatments. Statistical analyzes were performed using the Rbio software (Bhering, 2017). The data were subjected to ANOVA and, when in normal distribution, the Tukey means test at 5% significance was performed.

Results

The highest absorption and accumulation of phosphorus in roots and leaves was observed at T3 and T6, with the highest concentration of cadmium evaluated in isolation and disposed together with Mn (Figure 1A). These same treatments showed a lower P translocation to the shoot, consequently the highest concentration of absorbed P was accumulated in the roots. For Cd, in all treatments, the greatest accumulation of the element was in the roots (Figure 1B). In the presence of Mn, there was a greater accumulation of Cd both in the roots and in the leaves and seeds. There was a considerable accumulation of Cd in the seeds in all Cd-treatments. Unlike Cd, the highest concentration of Mn was observed in leaves (Figure 1C). Interestingly, in theT4 (400mg Mn) there was a higher concentration of the element in the seeds than in the root. In the presence of Cd there was a lower Mn in both plant organs.

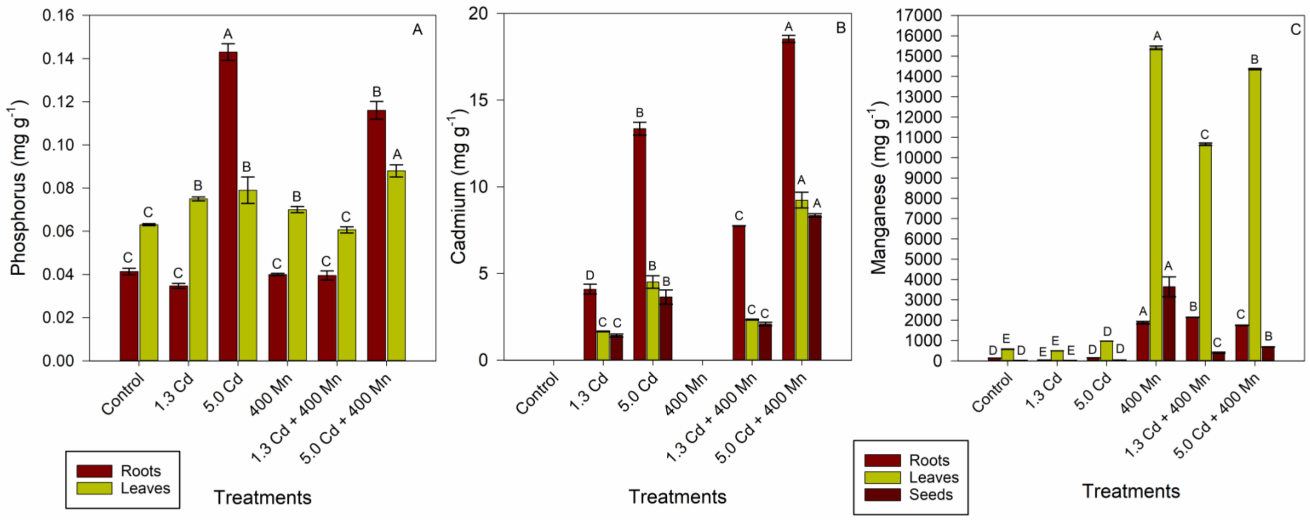

Fig. 0 P (A), Cd (B) and Mn (C) concentration in root, leaf and achenes of sunflower (*Helianthus annuus* L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The letters correspond to the responses presented in each treatment. Equal letters demonstrate that there was no statistical difference between treatments by the Tukey test (p <0.05).

There were significant changes in the levels of chlorophyll *a*, *b* and carotenoids (Figure 2), as well as differences in the content of these pigments between the treatments in each evaluation. Both treatments showed an increase in chlorophyll *a* and *b* content until the R4 development stage, with the exception of theT3 (5.0mg Cd). This treatment showed a reduction in the chlorophyll *a* content after the transition to the reproductive period and an increase in chlorophyll *b* from the V8 stage. The treatment with the concentration of T2 (1.3mg Cd) presented chlorophyll a content similar to the control treatment. Regarding carotenoids, the control and T2 (1.3mg Cd) showed the highest pigment concentrations over time. The other treatments showed a reduction in the carotenoid content in the V8 and R4 stages.

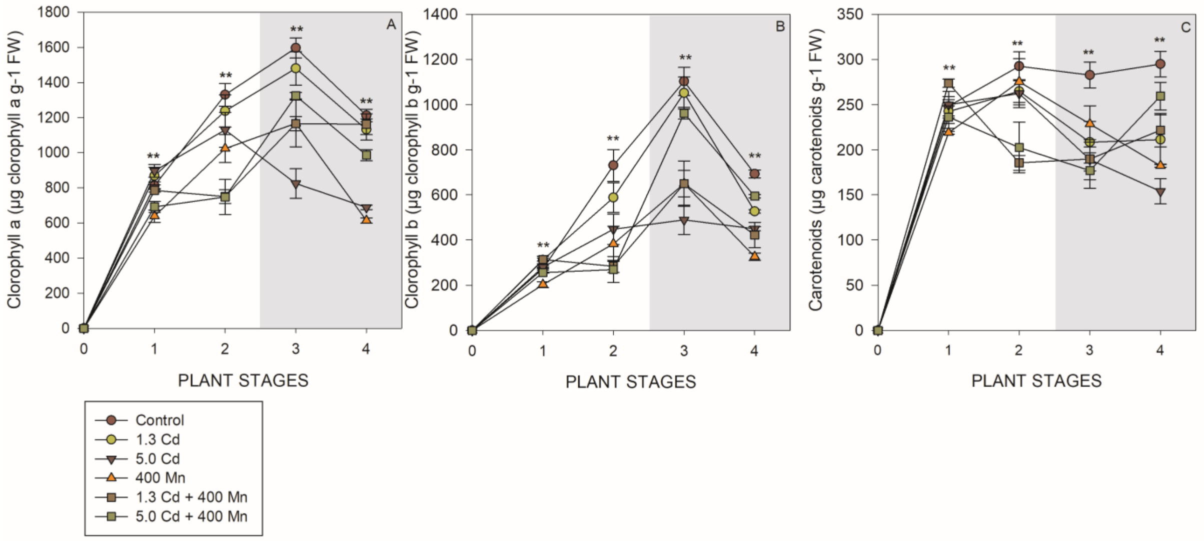

Fig. 2 Chlorophyll a (A), chlorophyll b (B) and carotenoids (C) content in sunflower leaves (*Helianthus annuus* L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The gray shading indicates the collections carried out in the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test (p <0.05).

The maximum quantum yield of PSII (*Fv/Fm*) did not show significant differences between treatments in each measurement (FIGURE 3A). The electron transport rate (ETR) (FIGURE 3B) and the effective quantum yield (Yield) (FIGURE 3C) showed interaction between time and treatment parameters. There was a significant increase over time in both until the R4 developmental stage, which were later reduced in the R7 stage. The treatments T5 (1.3mg Cd + 400mg Mn) presented the lowest ETRs in the initial stage of development, having later been equal to the other treatments and remaining until the R7 phase. The highest rates of ETR and Yield were identified in measurements 2 and 3, corresponding to phases V8 and R4, transition period between the vegetative and reproductive phases.

Regarding non-photochemical extinction (NPQ) (FIGURE 3D), the treatments showed a similar behavior over time, where in the vegetative and final reproductive stages (V8 and R7) the plants showed higher values in relation to the initial stages. It was observed that in the initial collections, the T4(400mg Mn), T5 (1.3mg Cd + 400mg Mn)e T6(5.0mg Cd + 400mg Mn)showed the highest rates of photochemical extinction, which was later reversed. The coefficients qL and qP (FIGURE 3E, F) showed similar responses, where the treatments showed variation over time with the lowest non-photochemical extinction rate in the initial V4 phase and the highest in the V8 stage, the period that precedes the transition from vegetative to reproductive phase. In the reproductive stage, there was a drop that was maintained over time. The T2 (1.3mg Cd) showed the lowest rates for these parameters in the first and last collection when compared to the other treatments.

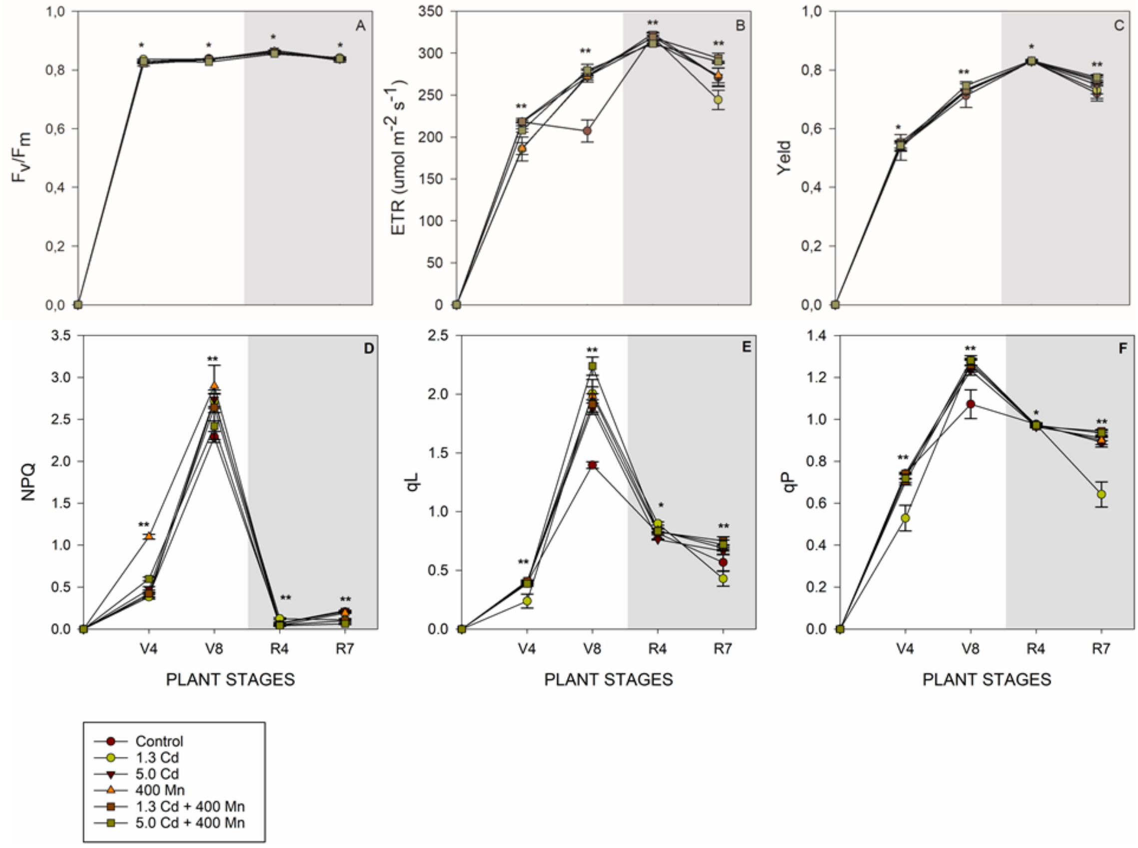

Fig. 3 Maximum quantum yield of PSII (*Fv/Fm*) (A), apparent electron transport rate (ETR) (B) and effective quantum yield of PSII (C), non-photochemical quenching (D), photochemical quenching coefficient (E) and photochemical extinction coefficient related to the interconnection of the PSII antenna complex (F). Analyzes performed on sunflower leaves (*Helianthus annuus* L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The gray shading indicates the collections carried out in the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test ($p < 0.05$).

The H_2O_2 content in the leaves (FIGURE 4A) was marked by an increase over time, with the highest values identified in the R7 phase, a period marked by the beginning of grain filling, leaf signaling and senescence. Interestingly, the control and T4 (400mg Mn) showed the highest concentrations of H_2O_2until the beginning of the reproductive period. However, MDA levels (FIGURE 4B) were low for all treatments until the reproductive period in R4, followed by a fall in the R7 stage. In the evaluation in R4, T4 (400mg Mn), T5 (1.3mg Cd + 400mg Mn) and T6 (5.0mg Cd + 400mg Mn) were lower than in the other treatments, inverse of the other evaluations.

In the roots, the T3 (5.0mg Cd) in the final phase evaluated showed a reduction of H_2O_2 (FIGURE 4C). In the initial phases of the vegetative and reproductive stages, the treatment with 400 Mn presented lower H_2O_2 contents regarding other treatments. All treatments showed the highest levels of MDA in the V8 and R4 stages (FIGURE 4D), considering the transition time of the initial stage of the reproductive period. A curious fact is that the T2 (1.3mg Cd) showed high rates of MDA until the R7 phase and, when combined with 400 Mn, the MDA content was higher since the first sampling in V4.

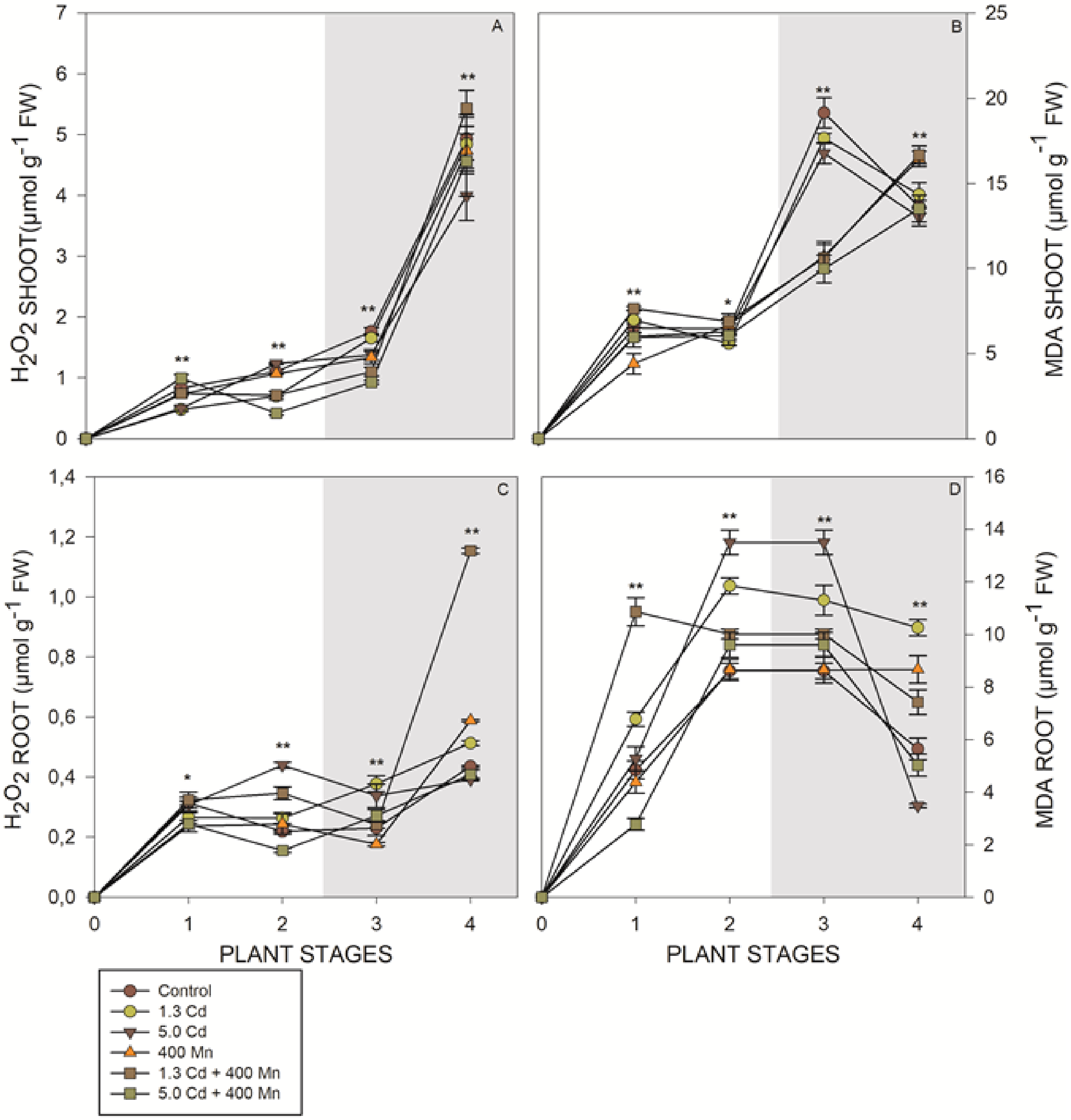

Fig. 4 Hydrogen peroxide (H_2O_2) in leaves (A) and roots (C); Malondialdehyde (MDA) in leaves (B) and roots (D) of sunflower (*Helianthus annuus* L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The shading indicates the collections carried out in the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test ($p < 0.05$).

The SOD activity in the leaves (FIGURE 5A) was variable according to the treatment and the stage of plant development. SOD activity in leaves was higher in the V4 phase for all treatments, but only in T3 (5.0 mg

Cd) and T5 (1.3 Cd +400 Mn) it was maintained, with the others reduced over time. The activity of the SOD enzyme (FIGURE 5D) showed a reduction throughout the development of the plants, with T6 showing an increase in the R4 (FIGURE 5B), followed by an increase in the V8 phase, decreasing again only in the R7 stage. The control and T2 (1.3 Cd) showed the highest CAT activity at the beginning of development. In the roots, the CAT activity (FIGURE 5E) increased over time for the treatments, with the exception of T5 (1.3 Cd +400 Mn), which showed high activity at the beginning and a reduction in the V8 stage. In the R4 stage, all treatments showed the highest activity for CAT.

The control treatment initially showed higher APX activity in leaf tissues (FIGURE 5C), since it showed low CAT activity at this stage. The R4 stage was marked by the reduction of APX activity in the leaves and roots (FIGURE 5F), due to the reduction in the activity of SOD and CAT activities, an increase in APX activity was observed in the R7 stage, the grain filling phase. At the end of the development stage, in this work the R7, onlyT4 (400mg Mn), T5 (1.3mg Cd + 400mg Mn) e T6(5.0mg Cd + 400mg Mn) showed a significant increase of this enzyme.

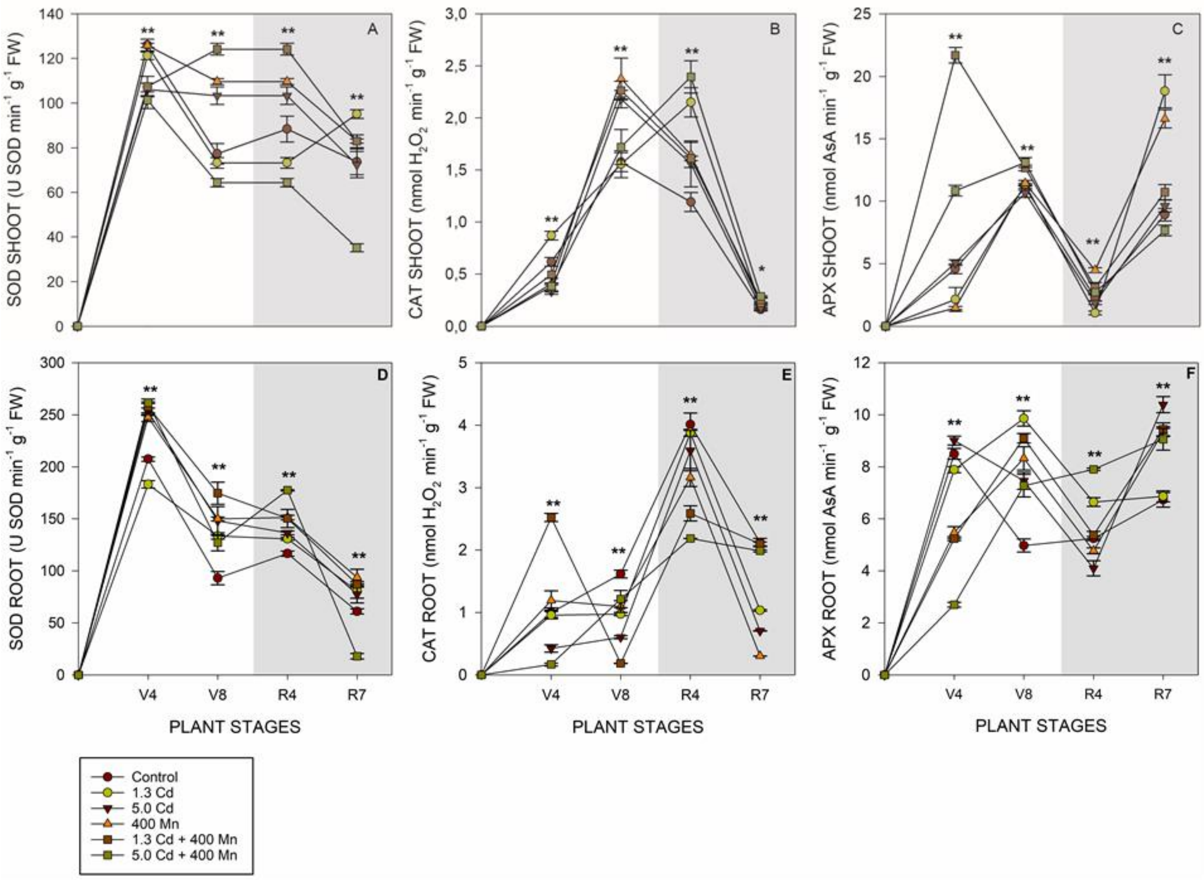

Fig. 5 Activity of superoxide dismutase enzymes in leaf (A) and root (D); leaf (B) and root (E) catalase; ascorbate peroxidase in leaves (C) and roots (F) in sunflower plants (*Helianthus annuus* L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The gray shading indicates the collections carried out in the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test (p <0.05).

The proline content was significantly altered as a result of time and treatments (FIGURE 6). In the initial phase of development (V4) only T5 (1.3mg Cd + 400 mg Mn) showed a considerable increase in this osmolyte, with T6 (5.0mg Cd + 400 mg Mn) having the lowest concentration. In the period that precedes the transition between the vegetative and reproductive stages, the control and T2 (1.3Cd) presented the highest proline content, both with a subsequent significant drop during the reproductive period. At the end of the reproductive stage (R7), only T6 (5.0mg Cd + 400 mg Mn) still had highproline content.

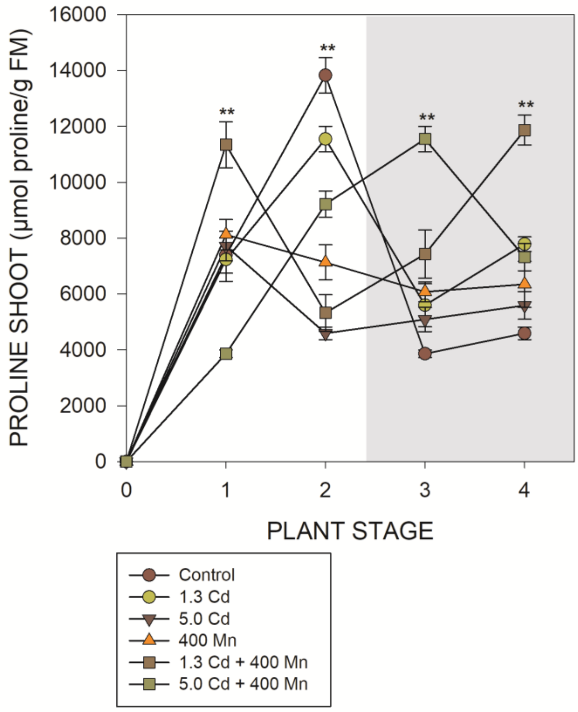

Fig. 6 Proline content in sunflower leaves (*Helianthus annuus* L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The gray shading indicates the collections carried out in the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test ($p < 0.05$).

Regarding total soluble and reducing sugars, there was an increase in their accumulation in both roots and leaf tissues over time until the reproductive stage of R4. In the roots, the highest concentrations of sugars were at T4 (400mg Mn) in the vegetative phases (FIGURE 7C, D), while in the reproductive phase the highest concentrations of reducing sugars were from the treatments with Cd only and for total soluble sugars the treatments containing Cd and Mn synergistically and just Mn. In leaf tissues (FIGURE 7A, B), described as source tissues, treatments containing Cd and Mn together presented the highest concentrations up to the R4 stage. In the R7 phase, period of grain filling, both for total sugars and for reducers, the control treatment presented the highest concentrations.

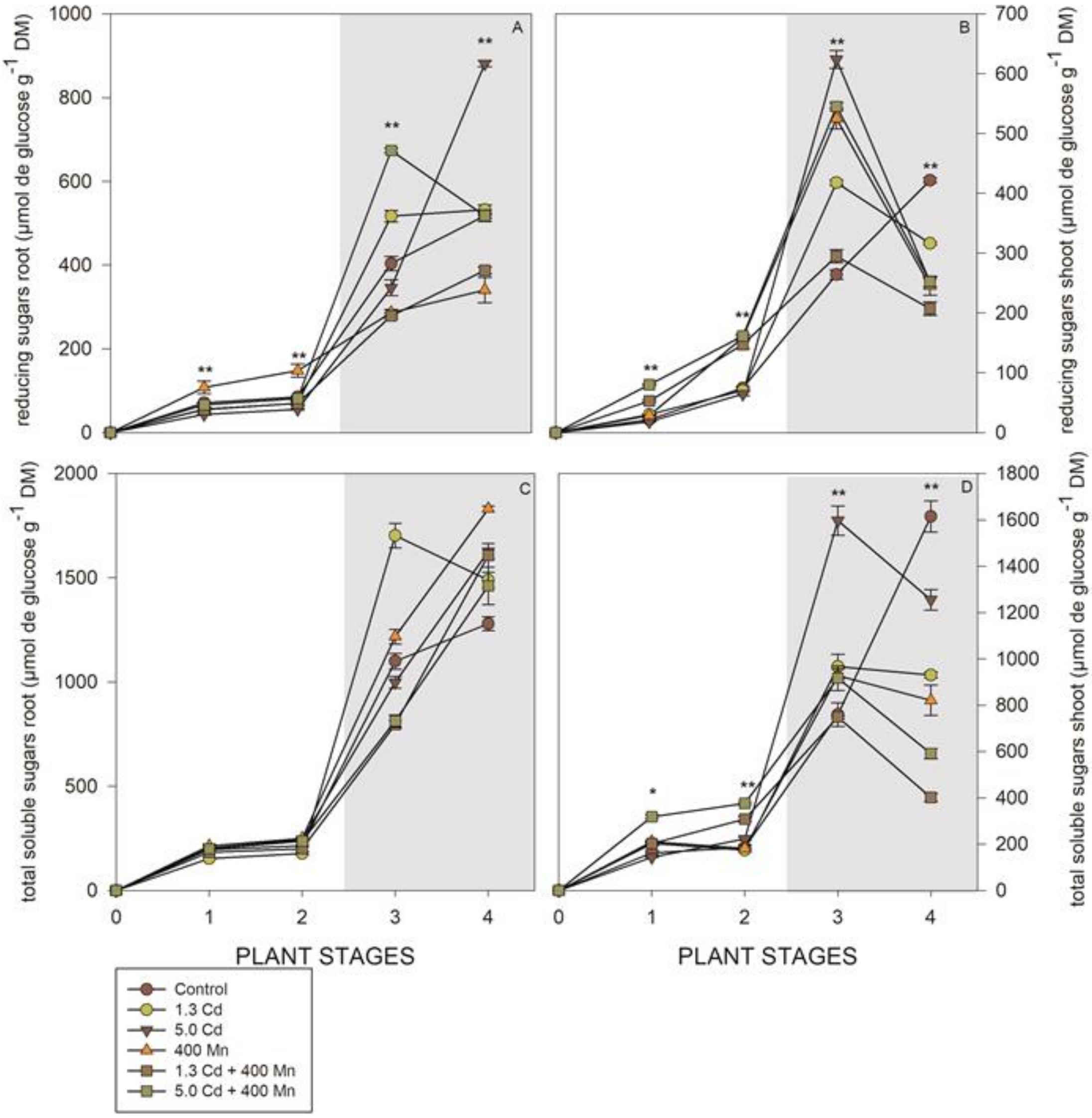

Fig. 7 Reducing sugar content in leaves and roots (A; C); Content of total soluble sugars in leaves and roots (B; D) of plants (*Helianthus annuus* L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The gray shading indicates the collections carried out in the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test ($p < 0.05$).

Regarding the variables amino acids and proteins, in the leaf tissues during the vegetative stage the highest contents of this organic compound were in treatments T5 (1.3mg Cd + 400mg Mn) and T6 (5.0 mg Cd + 400mg Mn), in the presence of Cd and Mn synergistically (FIGURE 8B). The lowest concentrations of amino acids in the roots were in the control and T2 (5.0mg Cd) (FIGURE 8A). There was a reduction in amino acid content in the reproductive period in both treatments in leaves and roots. However, in leaf tissues during the V8 stage, the control and T2 (5.0mg Cd)showed the highest concentrations of amino acids. These same treatments for the roots showed similar responses for the protein content where, during the V4 and R4 stages, there was an increase in this amino acid content and after a reduction at the end of the cycle (FIGURE 8C). In leaf tissues, the protein content significantly reduced in treatments during the reproductive period (FIGURE 8D). However, in the vegetative phase, the control treatment showed small variations in protein content.

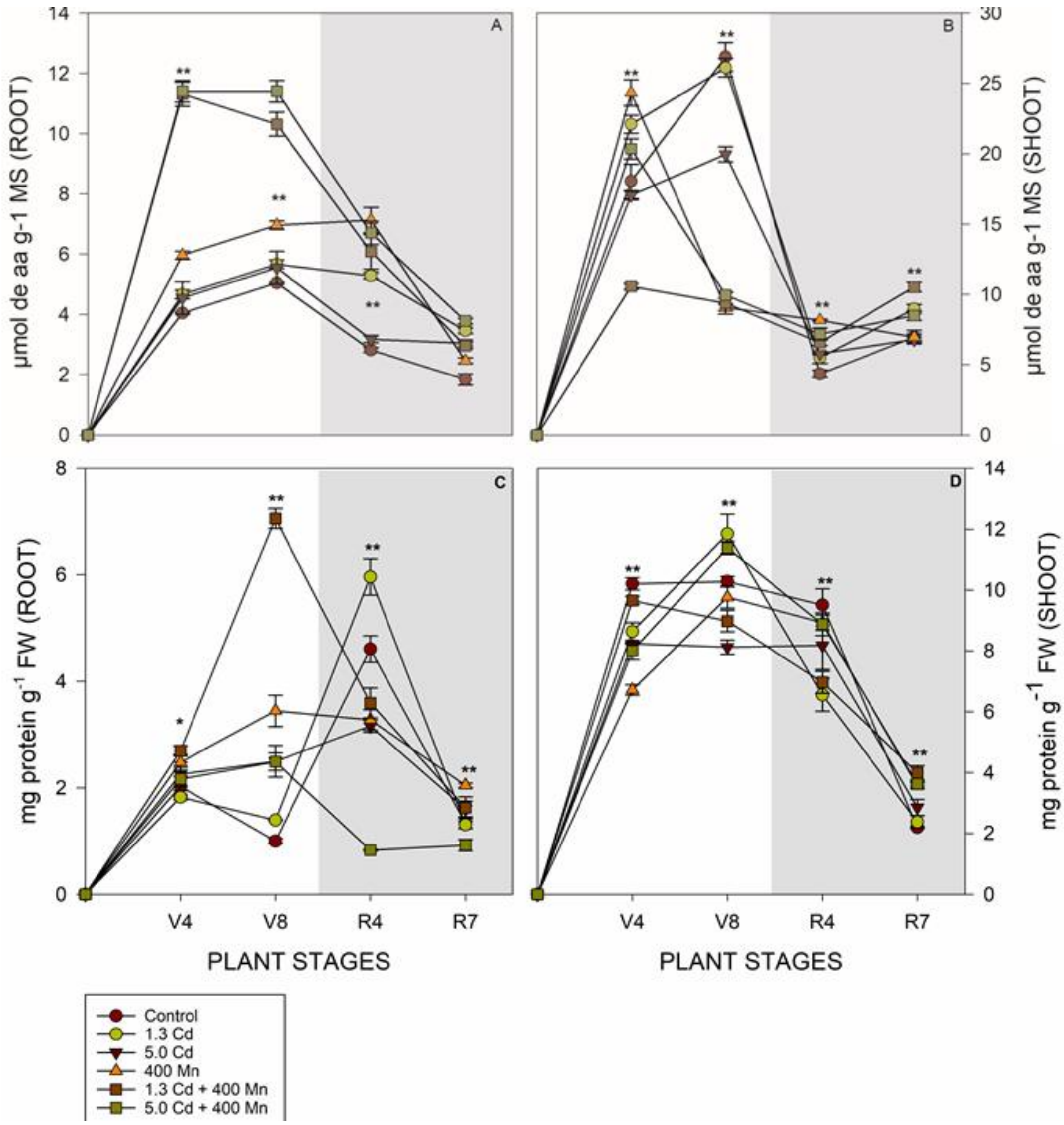

Fig. 8 Amino acid and protein content in root (A; C) and leaves (B; D) of plants (*Helianthus annuus*L.) cultivated in the different treatments under analysis. Values are means ± standard error (n = 4). The gray shading indicates the collections carried out in the reproductive period. One asterisk indicates significant difference between treatments and two asterisks indicate difference between treatments and interaction with the time factor by Tukey's test (p <0.05).

Discussion

Hyperaccumulator plants present opposite responses, allowing the absorption and translocation of high concentrations to the aerial part (Baker and Brooks 1989) as observed in the results found in this work. Sunflower belongs to a select group of plant species with the ability to tolerate and develop in areas contaminated with high concentrations of potentially toxic elements (Cutright et al. 2010; Govarthanana et al. 2018), including cadmium (Junior et al. 2014). As observed in our results, sunflowers showed the phytostabilization mechanism for cadmium, absorbing high concentrations and storing them in the roots, compared to the shoot, which are in agreement with Sewalen et al (2014). Furthermore, in the presence of Mn, the concentration of absorbed Cd was increased, contrary to the fact that the absorption of Mn was reduced in the presence of Cd in T4 (400mg Mn), T5 (1.3Cd + 400mg Mn) and T6 (5.0Cd + 400mg Mn). However, high Mn concentrations were translocated to the shoot.

The homeostasis of nutrients in cells is coordinated by several pathways of transport proteins, among them non-essential trace element tolerance proteins (Pinto and Ferreira 2015). Thus, many essential micro and macronutrients, such as Mn in this study, may have effects on the absorption and accumulation of Cd (Han et al.

2019; Xue et al. 2019). According to the results presented, Mn positively affected the absorption of Cd, corroborating the provisions of Liu et al (2017) where it is described that Mn is positively correlated with the Cd content in plants and rice grains.

In this work, the highest concentrations of Cd, even when combined with Mn, resulted in a reduction in P translocation rates from roots to shoots, contrary to all other treatments. It was shown that the absorption of trace elements in sunflowers increased with enhancing concentration in the medium (Lee et al. 2013) and it was also reported that different treatments with Cd did not affect the absorption of this element by the roots (Cornu et al. 2016). One of the mechanisms of Cd toxicity in plants is the structural similarity with essential nutrients such as P, which may result in competition for root uptake and translocation (Singh et al. 2016). Just as excess Mn can also prevent the uptake and translocation of this element and other essential ones (Millaleo et al. 2010). This fact corroborates the results found in this work where it can be observed (Figure 1A).Other treatments, however, all plants showed satisfactory levels of this element (Broadley et al. 2012).

The levels of photosynthetic pigments were significantly reduced at the beginning of the reproductive period only in T3 (5.0mg Cd) while in T5 (1.3mg Cd+400mg Mn) and T6 (5.0mg Cd+400mg Mn) did not cause representative damage. The tolerance mechanisms to trace elements can neutralize the damage caused by the presence of these in high concentrations (Yadav 2010). One of the immediate responses to Cd toxicity is the reduction of chloroplast pigments (Vassiley, Lidon, 2011) as also demonstrated by De Maria et al (2013) when evaluating chlorophyll levels in sunflower plants, chlorophyll levels decreased with increasing Cd concentration in leaves. The positive correlation between the synergistic disposition of Cd and Mn, even at the highest concentration of the element, may be linked to the role played by Mn in maintaining the structure of chloroplasts, corroborating plant metabolism, adjustments and prevention of oxidative damage (Liu et al. 2017) and pigment content.

Carotenoids were affected only at specific developmental stages, close to the transition between vegetative and reproductive phases and at the end of development. This pigment is essential in the process of reducing ROS, thus reducing various effects of free radicals (Watkins and Pogson 2020). Thus, it can be understood that the reduction of carotenoid content in the specific developmental stages may be linked to a higher generation of ROS linked to signaling. The maintenance of carotenoid levels in the other treatments may be associated with the dissipation of excess energy by the plants, favoring the maintenance of membrane integration (Krause et al. 2012; Lichtenthaler et al. 2013) and chlorophyll macromolecules, maintaining the photosynthetic rates (Guirao et al. 2013).

It is possible to associate the biochemical responses and the influence of Cd and Mn in high concentrations on energy metabolism, which directly affects plant growth and development. Marschner and Marschner (2012) and Sebastian and Prasad (2015) reported that phytotoxic concentrations of Mn and Cd can reduce Rubisco activity, photosystem II functioning and promote increased accumulation of these elements in the apoplast and/or associated with macromolecules in leaf tissues, besid that limiting stomatal conductance and reducing CO_2 absorption (Pan et al. 2018). In this work, the monitoring of changes over time in electron transport and in the quantum yield by plants in the different treatments, showed that the conversion of light energy into chemical remained efficient, protecting the photosynthetic system, as also reported by (Chang et al. 2020) for wheat. Small adjustments were presented mainly in the ETR, however, these did not negatively affect the quantum efficiency (FIGURE 3).

In our results, short changes in ETR and saturation related to PSII and consequently in Yeld in the final stages of the reproductive phase led to the release of light energy by carotenoid accessory pigment, promoting the reduction of damage to PSII and the oxidative balance by increasing the ROS concentrations, as reported by Sebastian and Prasad (2015). The *Fv/Fm* parameter is an important variable to evaluate the integrity of the photosynthetic mechanism and the selection of plants tolerant to the stressful condition evaluated. At the end, in the R7 stage, there is a reduction in both parameters that may be related to the period of remobilization of reserves and grain filling, culminating in the reduction of quantum yields.This positive relationship between the photochemical and non-photochemical parameters was observed, where with the increase of ETR and the effective yield promoted a reduction of NPQ and parameters related mainly to the photochemical extinction coefficient (qL and qP) as a protective mechanism also associated with the xanthophyll cycle (Demmig-Adams and Adams, 2006; Demmig-Adams et al. 2017).

The increase in H_2O_2 content occurred both in the roots and in the shoot in the reproductive stage, more precisely in the R7 phase, which is marked by the beginning of grain filling and decline in chlorophyll content, from photochemical quenching and non-photochemical and increased carotenoid content. The multiple metabolic variations that involve the signaling mechanisms in plant metabolism aim at the regulation of genes induced by the stressful condition that culminates in the encoding of proteins and enzymes, thus favoring acclimatization or alteration in metabolism (Casaretto et al. 2016). Energetic reactions and electron transfer lead to the occurrence of oxygen in more reduced forms (Choudhury et al. 2017), inducing the formation of ROS as part of cellular metabolism. However, the hyperaccumulation of these molecules can cause damage to macromolecules due to their reactive nature (Raja et al. 2017). As can be seen in Figure 4,

The increasing MDA content, in the shoot only in the reproductive stage andbetween the V8 and R4 phases in the roots, it can be inferred that the increase in oxidative damage occurred mostly due to metabolic changes during the biocycle and not necessarily due to the significant increase in ROS. ROS are molecules that can cause oxidative damage through redox imbalance (Xia et al. 2016), but they are also known as secondary messengers or signalers that carry signals through redox reactions in a variety of cellular mechanisms to increase tolerance against abiotic stresses (Singh et al, 2016).

The maintenance of the redox state and the consequent reduction of oxidative damage are correlated with the increase in the activity of enzymes of the antioxidant system, even at specific stages as in the results observed in Figure 5, and the accumulation of osmotic substances such as proline and sugars, being described as physiological mechanisms of tolerance to Mn (Li et al. 2016; Wan et al. 2016) and cadmium (Nogueirol et al. 2016). As can be seen in the results presented, the sunflower cultivar studied in this work showed tolerance to the cadmium concentrations analyzed, which can be attributed to tolerance and reduction of oxidative stress due to the elimination of ROS and the activities of these enzymes that make up the system antioxidant (Uraguchi et al. 2009; Zeng et al. 2017).

It is also proven that Mn plays a key role as a cofactor in Mn-SOD and Mn-CAT (Rahman et al. 2016) being an important contributor in the regulation of the antioxidant capacity of plant species. The increase in Mn can be used in the activation of antioxidant defense enzymes and in general metabolism (Rahman et al. 2016; Han et al. 2019) as observed throughout the development of sunflower plants (FIGURE 5). However, in this work, when synergistically disposed with Cd in the soil, thus favoring its absorption as demonstrated, the antioxidant enzymatic activity was reduced, culminating in a higher MDA content in the early stages of development.

In addition to antioxidant enzymes, non-enzymatic metabolites play important roles in stress tolerance. Stress factors, either alone or in combination, can cause changes in enzymatic activity, functional structure of proteins, accumulation of sugars and consequently the growth and development of plants (Gheyi et al. 2016). Throughout the development of sunflower seedlings, there was a significant increase in reducing sugars and total solubles in all treatments, with treatments containing Cd and Mn synergistically having the highest concentrations of these macroelements in leaf tissues.

A stress tolerance strategy is related to the maintenance of homeostasis of carbon and nitrogen metabolism, where interactions between these pathways are essential for plant growth and development (Nunes-Nesi et al. 2010). The carbohydrates accumulation collaborates with the maintenance of cellular turgor and guarantees the integrity of membranes and proteins (Verslues et al. 2006), in this way, plants produce proteins and soluble sugars to maintain osmotic regulation and reduce damage to plant cells (Yang et al. 2018).

The greatest accumulation of Cd occurred in the roots, as well as the amino acid content in the treatments containing Cd and Mn synergistically and in isolation. In the leaf tissues, during the vegetative stage, there was a lower content of amino acids in these same treatments. Cadmium accumulation and immobilization can also occur with the binding of these ions to amino acids such as proline and proteins as a common strategy in plants described as tolerant (Pal et al. 2018). As observed in the results obtained, the accumulation of trace elements can change the concentration of amino acids and antioxidant activity in plants (Islam et al. 2016). However, essential nutrients play key roles in protein and carbohydrate activation and synthesis, as well as signal transduction (Song et al. 2015).

Thus, being a tolerant species, the absorption of cadmium had little effect on the absorption of manganese and translocation of this element to the shoot, as well as phosphorus, according to the results presented. Thus, the enzymatic activity, antioxidant, amino acid and carbohydrate content, which in non-tolerant plants are affected by the accumulation of trace elements (Dave et al. 2013; Islam et al. 2016), were partially affected in sunflower plants even when synergistic of Cd and Mn. However, the adjustments along the development of the plants allowed the conclusion of the biocycle and the accumulation of biomass.

Regarding the visual symptoms observed, as a result of the toxicity of Mn (Marschner and Marschner 2012) and Cd (Jali et al. 2016) it deals with leaf chlorosis and brown spots, resulting in a reduction in biomass. As can be seen (FIGURE 9), the highest dose of Cd used and Mn disposed alone or together with Cd caused chlorosis, and, as observed in the data related to photosynthetic pigments, there was a reduction in the chlorophyll content. However, the maintenance of the maximum and effective quantum yield of the PSII was maintained.

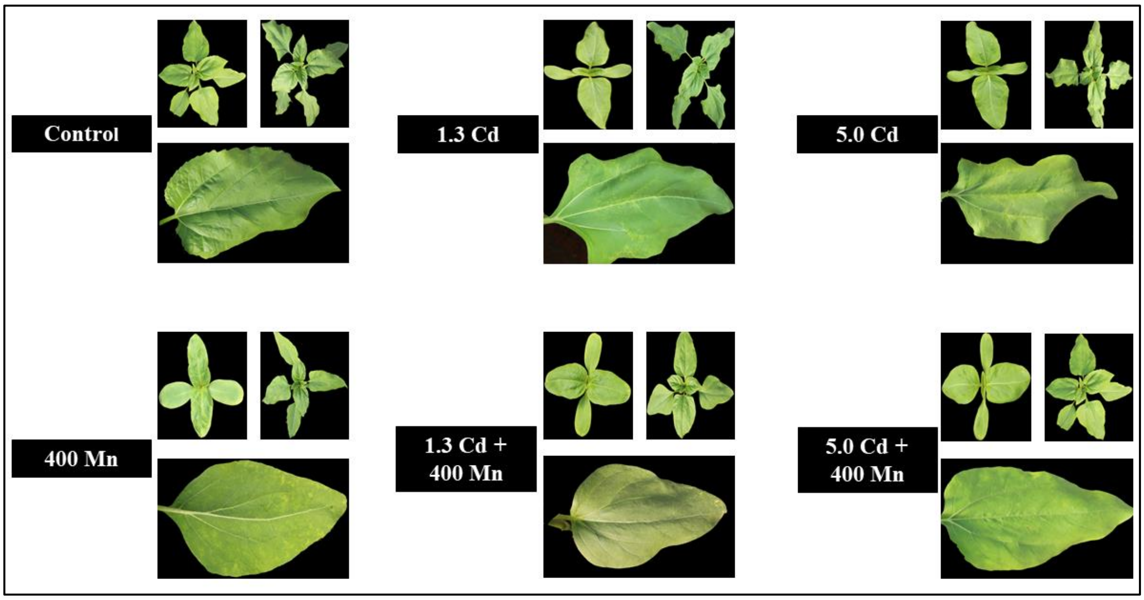

Fig. 9 Representation of leaf tissues of sunflower plants grown under different growing conditions.

Conclusion

It was concluded that Mn increases the absorption of Cd, which is accumulated in sunflowers in the roots (phytostabilization). On the other hand, Cd negatively affects the absorption of Mn, and this is accumulated in the leaves. Contrary to what happens in other species, it was concluded that Cd does not interfere with P absorption. Thus, it can be concluded that sunflower is tolerant to Cd and Mn in isolation and together, and the responses to the stressful condition are related to metabolic adjustments, without significant damage to the photosynthetic apparatus.

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3. FINAL CONSIDERATIONS

As final considerations, the phytoremediation potential of sunflower for Cd is not affected by the synergistic disposition of Mn at high concentrations. On the contrary, Mn enhances Cd absorption and Cd reduces Mn absorption. The mechanism related to the accumulation of Cd in plants was phytoextabilization, thus preventing this element from being translocated to the shoot, while the high concentration of Mn was translocated to the leaf tissues.

Regarding photosynthetic parameters, plants grown in soil containing Cd had similar responses to the control, while the addition of Mn significantly reduced stomatal conductance, CiCa ratio and Photosynthesis.

The relationship of adjustments in photosynthetic parameters had a negative impact on oil content, total dry mass and root/shoot ratio in plants grown in soil containing Mn and, to a lesser extent, in plants grown in soil containing Mn and Cd.

There was a delay in the development of plants in conditions of high concentration of Mn and Cd+Mn, however, the plants completed the biocycle presenting themselves as tolerant species. It was understood that sunflower is tolerant to Cd and Mn alone and together, and the responses to the stressful condition are related to metabolic adjustments, without significant damage to the photosynthetic device.