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BIANCA DE ALMEIDA MACHADO

X-ray spectroscopy unfolding the effects of glyphosate on soybean (*Glycine max* L.) manganese foliar uptake

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ABSTRACT

MACHADO, B. A. **X-ray spectroscopy unfolding the effects of glyphosate on soybean (*Glycine max* L.) manganese foliar uptake**. 2020. 125 p. Dissertação (Mestrado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

The spraying of tank mixtures with manganese (Mn) and glyphosate allows the nutrition of soybean crops resistant to glyphosate, while controlling weeds. However, complexation reactions can occur between these species, which has the potential to significantly decrease the efficiency of this operation. Thus, this study aimed to investigate the chemical reactions and the absorption of Mn by soybean supplied as $MnSO_4$, $MnCO_3$, Mn-ethylenediamino-tetraacetic (Mn-EDTA), Mn-phosphite and Mn-glycine. In tank mixtures, approximately 30% of the Mn supplied via $MnSO_4$ and Mn-glycine precipitated together with glyphosate molecules, in *ca*. 2:1 Mn:glyphosate molar ratio. X-ray absorption spectra (XAS) of the precipitates indicated that they presented the same chemical environment regardless of the employed source, this technique did not show evidence of soluble complexes. The use of Mn-EDTA, as well as maintaining the pH of the mixture below 2.5 prevents precipitation, while pH above 7 resulted in MnO(OH). $MnSO_4$, $MnCO_3$, Mn-EDTA and Mn-phosphite solutions/dispersions, with and without glyphosate, applied to soybean leaflets damaged the leaf cuticle, regardless of the glyphosate mixture. Except for $MnCO_3$, all sources increased the Mn content in the treated leaf petioles. The glyphosate mixture reduced the absorption and transport of Mn when supplied via $MnSO_4$ and Mn-phosphite, but XAS analysis showed no evidence of complexation of Mn by glyphosate inside the plants. The absorbed manganese is transported in a similar chemical environment when supplied as $MnSO_4$ and Mn-phosphite, but Mn-EDTA was found in its pristine form in the treated petioles. The foliar absorption of droplets of $MnSO_4$ and Mn-EDTA, with and without glyphosate, was monitored for 72 hours, following a sigmoid tendency of absorption. The glyphosate mixture decreased the leaf absorption of $MnSO_4$ due to the precipitation of complexes on the leaf surface, but no effect on the absorption of Mn-EDTA was observed. The absorption of $MnCO_3$ followed linear behavior, being absorbed very slowly. The application of glyphosate in conjunction with $MnSO_4$ increased the activity of the superoxide dismutase enzyme (SOD), probably due to the unavailability of Mn for leaf absorption. The results presented in this study show that the efficiency of foliar fertilization with Mn is reduced due to interactions that occur in solutions where free Mn^{2+} ions are available for complexation at pH above 2.5. No evidence of intracellular complexation was found. In this sense, the employment of chelated sources, such as Mn-EDTA, is an alternative to enable the application of tank mixtures in glyphosate-resistant soybean crops without losing the efficiency of the operation.

Keywords: Tank mixtures. Foliar fertilization. Micronutrients. Herbicide interaction.

RESUMO

MACHADO, B. A. **Espectroscopia de raios X revelando os efeitos do glifosato sobre a absorção foliar de manganês pela soja (*Glycine max* L.)**. 2020. 125 p. Dissertação (Mestrado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

A aplicação de misturas de tanque com manganês (Mn) e glifosato permite a nutrição de lavouras de soja resistentes ao glifosato, ao mesmo tempo em controla plantas daninhas. No entanto, reações de complexação podem ocorrer entre estas espécies, o que tem potencial para diminuir significativamente a eficiência desta operação. Desta forma, este estudo investigou as interações químicas e a eficiência de absorção do Mn pela soja quando fornecido via $MnSO_4$, $MnCO_3$, Mn-etilenodiamino tetra-acético (Mn-EDTA), Mn-fosfito e Mn-glicina. Em misturas de tanque, aproximadamente 30% do Mn fornecido via $MnSO_4$ e Mn-glicina precipitou junto com moléculas de glifosato, numa razão molar Mn:glifosato de cerca de 2:1. Espectros de absorção de raios X (XAS) dos precipitados indicaram que o ambiente químico do Mn independente da fonte utilizada, a técnica não mostrou evidências de formação de complexos solúveis. O uso de Mn-EDTA, bem como a manutenção do pH da mistura abaixo de 2,5 previne a precipitação, enquanto pH acima de 7 provoca a formação de MnO(OH). Soluções/dispersões de $MnSO_4$, $MnCO_3$, Mn-EDTA e Mn-fosfito, com e sem glifosato aplicadas em folíolos de soja causaram danos na cutícula foliar, independente da mistura de glifosato. Com exceção do $MnCO_3$, todas as fontes aumentaram o conteúdo de Mn nos pecíolos das folhas tratadas. A mistura de glifosato reduziu a absorção e transporte de Mn quando fornecido via $MnSO_4$ e Mn-fosfito, mas análises com XAS não mostraram evidências de complexação intracelular de Mn pelo glifosato. O manganês absorvido é transportado em um ambiente químico similar se fornecido via $MnSO_4$ e Mn-fosfito, mas o Mn-EDTA foi encontrado em sua forma original nos pecíolos tratados. A absorção de gotas de $MnSO_4$ e Mn-EDTA, com e sem glifosato, foi monitorada por 72 horas, revelando tendência sigmoide de absorção. A mistura de glifosato diminuiu a absorção foliar de $MnSO_4$ devido à precipitação de complexos sobre a superfície foliar, mas não apresentou efeitos sobre a absorção de Mn-EDTA. A absorção de $MnCO_3$ seguiu comportamento linear, sendo absorvido muito lentamente. A aplicação de glifosato em conjunto com o $MnSO_4$ aumentou a atividade da enzima superóxido dismutase (SOD), provavelmente devido à indisponibilização de Mn para absorção foliar. Os resultados apresentados neste estudo mostram que a eficiência da adubação foliar com Mn é reduzida devido a interações que ocorrem em soluções em que há a disponibilização de íons Mn^{2+} livres para complexação em pH acima de 2,5. Não foram encontradas evidências de complexação dentro da planta. Neste sentido, a escolha de fontes quelatadas, como o Mn-EDTA, é uma alternativa que pode viabilizar a aplicação de misturas de tanque em lavouras de soja resistentes ao glifosato sem perda eficiência na operação.

Palavras-chave: Misturas de tanque. Adubação foliar. Micronutrientes. Interação com herbicidas.

FIGURE LIST

TABLE LIST

SUMMARY

1 INTRODUCTION

Soybean (*Glycine max* L.) is the fourth most cultivated crop, covering an area of 122 million hectares worldwide. In the 2019/20 season, the world soybean grains production reached 337 million metric tons, which means an average yield of 2.75 tons per hectare. More than 68% of the cultivated area and 80% of the grain production comes from Brazil, United States of America (USA) and Argentina, which together own an average yield of 3.19 tons per hectare (USDA, 2020).

In the 2019/20 crop, Brazil is considered the biggest soybean producer worldwide (USDA, 2020), surpassing the USA in cultivated area (36.9 million hectares), production (126 million tons) and yield (3.49 tons per hectare) (USDA, 2020). Not surprisingly, this is the most cultivated crop in Brazil. Official data from the year of 2018 indicates that soybean represented more than 40% of the Brazilian agricultural exports market share (FAO, 2020).

The interest in the high protein content and quality within the grains are some of the drivers of soybean expansion, but many technologies had to work together to enable its cultivation worldwide. Soybean is native from a high latitude region in China, which is characterized by a temperate climate. It is thanks to breeding programs that its cultivation in the tropics had made possible, including Brazil and the Cerrado region (ALMEIDA et al., 1999), which, alone, is responsible for more than 48% of the Brazilian production (CONAB, 2020a).

In 1996 the genetically modified herbicide tolerant (GM HT) soybean was introduced in the USA. In the year of 2003, it arrived in Brazil. This technology conferred resistance to the herbicide glyphosate, simplifying the weed control in the fields. Hence, it had a very rapid adoption rate by soybean growers, which also contributed to boost its expansion (DILL, 2005).

Glyphosate is a post emergent broad-spectrum herbicide which inhibits the enzyme 5-enolpyruvyl-shikimate- 3-phosphate synthase (EPSPS) of the shikimate pathway (DUKE; POWLES, 2008). The EPSPS enzyme is required for the synthesis of essential aromatic amino acids such as tryptophan, tyrosine and phenylalanine (DUKE et al., 2012). Consequently, the EPSPS inhibition compromises the protein synthesis, leading plants to death.

The resistance to glyphosate was conferred by the insertion of the CP4 gene, from *Agrobacterium tumefaciens* strain CP4, into the soybean genome. This gene encodes the CP4 EPSP synthase enzyme, which allows the shikimate pathway to function in the presence of

glyphosate (DILL, 2005). Therefore, glyphosate can be applied over GM HT soybean cultivars without damaging them.

The release of glyphosate tolerant cultivars highly facilitated and reduced costs with weed management (DUKE, 2018). Thereupon, in 2017 more than 33 hectares were cultivated with GM HT soybean cultivars in Brazil, which represents an adoption rate of 97% (JAMES, 2017). Meanwhile, the USA and Argentina presented an adoption rate of 94 and 100%, respectively (JAMES, 2017). As a consequence, glyphosate holds the title of most successful herbicide in history (DUKE; POWLES, 2008).

In 2012, more than 1.2 billion kg of herbicide's active ingredient were employed worldwide. Only glyphosate represented 47% of the herbicides' market share, since more than 616 million kg was sold, of which 53% (333 million kg) were used on GM HT crops. Among GM HT crops, soybean is responsible for consuming 79% (265 million kg) of the whole glyphosate application (BENBROOK, 2016).

Higher yields derived from better weed control is also achieved due to the adoption of GM HT cultivars (BROOKES and BARFOOT, 2016). Since the release of the glyphosate resistant soybean until 2020, the yield had increased 40% in Brazil (CONAB, 2020b). However, such remarkable achievement cannot be attributed only to one technology.

The increasing use of mineral fertilizers played an important role in the development of agricultural yield. From 1991 to 2004, $N + P_2O_5 + K_2O$ (NPK) consumption per hectare increased almost 300% in Brazil (LOPES; GUILHERME, 2007), while the micronutrients employment increased more than 11-fold for the same period (MORAES; ABREU JUNIOR; LAVRES JUNIOR, 2010).

The use of micronutrients in soybean is widely practiced, and experimental responses have been usual at the Cerrado region. Broch and Fernandes (1999), based in results of 12 studies, showed an increase of 390 kg ha^{-1} on soybean yield trough seed treatment with micronutrients. Manganese has stood out from other micronutrients on soybean crop, due to the frequency of its deficiency in limed and high pH soils. Subtil and Dall Aglio (2000) observed Mn deficiency in 32.2% out of 268 soybean plant tissue samples, collected in an area of 200,000 hectares at the Cerrado region.

In plant metabolism, Mn is heavily involved in photosynthesis processes, where its changes on oxidation states mediate the water splitting into protons and oxygen (ANDRESEN et al., 2018). This nutrient is also involved in the catalysis and conversion of oxalate and O_2 into H_2O_2 and CO_2, as well as acting as an enzymatic activator of several enzymes, such as decarboxylases and dehydrogenases in the tricarboxylic acid cycle,

phenylalanine ammonia lyase in the shikimic acid pathway, and several glycosyltransferases in the Golgi apparatus (MARSCHNER, 2012). In summary, it is an essential element for photosynthesis processes, nitrogen metabolism, hormonal regulation and enzymes activation. Due to its fundamental role within photosynthesis, Mn deficiencies lead to a decreased oxygen evolution, which results in lower rates in the production of photo assimilates and decreased plant growth (NABLE et al., 1984).

According to Embrapa (2013), soybean requires 130 g of Mn to produce one ton of grains, of which 100 g remain in the system within the crop biomass, and 30 g are exported with the grains. This is usually supplied via soil or foliar. For soil fertilization, Sfredo et al. (2006) recommend from zero to 6 kg of Mn per hectare.

Foliar fertilization has been shown as an interesting alternative for delivering Mn. Embrapa (2013) recommends foliar application of 350 g of Mn per hectare in soybeans, which is a rate 11-fold lower than what it is suggested applied into soils with medium Mn content (4 kg ha^{-1}) (SFREDO et al., 2006).

There are several Mn sources available to be used on agricultural crops, such as manganese sulfate ($MnSO_4$), manganese oxide (MnO), manganese cabonate ($MnCO_3$) and Mn-ethylenediamine tetraacetic acid (Mn-EDTA). Among these sources, the solubility, reactivity and Mn content varies. Ferradon and Chamel (1998) observed that Mn-EDTA is absorbed in a lower quantity by the leaves than the $MnSO_4$, but it is less withheld at the leaf cuticle and is more redistributed to other plant parts than the sulfate salt. Migliavacca (2018) found that foliar application $MnSO_4$ was able to circumvent visual symptoms of Mn deficiency in soybean plants, while $MnCO_3$ was not.

It has been observed that Mn deficiency usually coincided with post emergency herbicides application, thus, tank mixtures with Mn sources and glyphosate became a common practice among soybean growers (BERNARDS et al., 2005). On the one hand, this is a suitable alternative for saving costs, on the other hand, glyphosate molecules can act as chelating agent and form stable complexes with di and trivalent metal cations such as manganese (GLASS, 1984). Such interactions can reduce the Mn and glyphosate efficiency on accomplish their roles.

Bernards et al. (2005), observed that manganese sulfate + lignin sulfonate chelate (Mn-LS), manganese sulfate + ethylaminoacetate chelate (Mn- EAA) and manganese sulfate monohydrate ($MnSO_4$), reduced glyphosate efficacy over velvetleaf (*Abutilon theophrasti*), while Mn ethylenediamine-tetraacetate (Mn-EDTA) did not. Likewise Bernards, Thelen and Penner (2005) found that tank mixtures with Mn-EDTA and glyphosate did not reduce

glyphosate's efficiency, on the contrary, an increasing herbicidal action was reported due to this mixture. These results suggest that the employed Mn source matters in tank mixtures.

In addition to interactions within tank mixtures, some studies raised the hypothesis that glyphosate can also bind with the intracellular Mn in GM HT soybeans. Bernards et al. (2005), observed modifications the electron paramagnetic resonance (EPR) spectra of $MnSO_4$ + glyphosate solutions in a physiological pH range, suggesting that such interactions can extend to the cytoplasmatic environment.

The complexation by glyphosate would unavailable Mn for accomplish its role on plant metabolism, which could explain the yellowing of the leaves which are often reported followed glyphosate application in soybean fields (DUKE et al., 2012). This yellowing is similar to the symptoms of Mn deficiency, and is popularly called as *yellowflash*, since it is a temporary effect which usually disappear within 21 days (DUKE et al., 2012). However, some studies have attributed the *yellowflash* symptoms to the toxicity of AMPA (amino-methylphosphonic acid), which is a metabolite formed through glyphosate's degradation, rather than to the intracellular complexation of Mn by glyphosate (DUKE et al., 2012; REDDY; RIMANDO; DUKE, 2004).

1.1 Hypothesis

This study raised the following hypothesis:

i) Glyphosate molecules can complex Mn^{2+} in aqueous solutions.

ii) The use of chelated sources and pH manipulation of the solution can avoid undesirable interactions between manganese and glyphosate in tank mixtures.

iii) Tank mixtures with manganese and glyphosate can decrease the efficiency of the Mn foliar uptake.

iv) Glyphosate can complex with manganese inside the plant tissue or cells.

1.2 Objectives

The general objective of this master's dissertation was to assess how tank mixtures with manganese ($MnSO_4$, Mn-EDTA, $MnCO_3$, and $MnHPO_3$) and glyphosate affect the manganese foliar fertilization of soybean (*Glycine max* L.). Thus, a series of experiments were designed to test the above mentioned hypothesis.

To achieve the objectives, we investigated the tank mixtures between Mn ($MnSO_4$, Mn-EDTA, $MnCO_3$, Mn-glycine, and $MnHPO_3$) and glyphosate by monitoring the electric conductivity (EC) and pH of the mixtures were monitored over 72 hours. X-ray fluorescence spectroscopy (XRF) quantified the amount of glyphosate and Mn found in the solution and in the precipitates. Chemical speciation analysis using X-ray absorption near edge structure (XANES) determined the Mn chemical environment for both supernatants and precipitates. The chemistry of $MnSO_4$ + glyphosate supernatants at different pH was evaluated through XANES. Thermogravimetric analysis revealed the composition of precipitates at pH 7, and zeta potential and dynamic light scattering (DLS) analysis showed the charge and diameter of the precipitates' particles in a varying pH.

To compare the foliar absorption and transport of Mn in soybean leaves exposed to four Mn sources ($MnSO_4$, Mn-EDTA, $MnCO_3$, and $MnHPO_3$) with and without the addition of glyphosate, *in vivo* transport kinetics was measured by XRF whistle X-ray absorption near edge spectroscopy (XANES) revealed the chemical environment of the Mn during its transport from leaves (source) to other plant parts (sink). Finally, scanning electron microscopy (SEM) demonstrated the effects of the spray mix on the anatomy of the leaf surface.

To investigate the *in situ* foliar absorption of fertilizers droplets ($MnSO_4$, Mn-EDTA, and $MnCO_3$), with and without glyphosate, we employed time resolved XRF line scans over the treated area. To evaluate the efficiency of tank mixtures on providing Mn for soybean fertilization, we determined the Mn concentration in the shoot of soybean (plants at the R6 phenological stage) using XRF. Finally, the activity of enzymes of the antioxidant system allowed to investigate the consequences of tank mixtures application on attenuate Mn deficiency symptoms.

1.3 Structure of the dissertation

This dissertation contains this general introductory text followed by three chapters. First chapter comprises a manuscript accepted and currently under revision to the journal *SN Applied Sciences*.

The second chapter comprises a manuscript entitled “X-ray spectroscopy fostering the understanding of foliar uptake and transport of Mn by soybean (*Glycine max* L. Merril): kinetics, chemical speciation and effects of glyphosate” published by the *Journal of Agricultural and Food Chemistry* v. 67, n. 47, p. 13010–13020, 2019.

The third chapter will be submitted to the journal Experimental and Environmental Botany, with the title “Effects of glyphosate on foliar absorption and metabolism of manganese by soybean plants (*Glycine max* L.)”. The original texts were adapted to comply with institutional format requirements.

2 UNDERSTANDING THE CHEMISTRY OF MIXTURES OF MANGANESE FERTILIZERS AND GLYPHOSATE USING SYNCHROTRON X-RAY SPECTROMETRY

Abstract

The spraying of tank mixtures with manganese (Mn) and glyphosate is a practical alternative to alleviate nutritional deficiency while controlling weeds. Thereby, this study investigates the chemical interactions between glyphosate and commercial sources of Mn, such as $MnSO_4$, Mn-phosphite, Mn-EDTA, Mn-glycine, and $MnCO_3$. Nearly 30% of the Mn supplied as $MnSO_4$ and Mn-glycine precipitated with glyphosate, yielding a Mn:glyphosate solid complex with molar ratio of nearly 2:1, both presenting similar chemical environment. XANES analysis of the supernatants indicate no formation of Mn-glyphosate soluble complexes. The use of Mn-EDTA as well as the maintenance of the mixture pH below 2.5 prevented precipitation, while pH above 7 caused the formation of MnO(OH). In conclusion, the Mn source and the pH of the mixtures matter. The absence of Mn-glyphosate soluble complexes suggests that dissolved Mn and glyphosate are still able to accomplish their functions, however, the precipitation significantly decreases their active availability.

Keywords: tank mixture, Mn-glyphosate complex, soybean, weed control, XAS.

2.1 Introduction

In the 2019/2020 crop season, Brazil cultivated 36 million of hectares of soybean, harvesting more than 125 million tons of grains. Such achievement made Brazil the world's largest soybean producer, a title previously belonged to the USA (USDA, 2020). The increasing cultivation under no-tillage systems has caused the widespread use of lime applications in Brazil. This practice raise the topsoil pH, decreasing availability and consequent root uptake of transition metal micronutrients, such as manganese (Mn) (CAIRES; FONSECA, 2000). Manganese is required by many plant enzymes, and plays an important role in photosynthesis, nitrogen metabolism, nodulation, and respiration. It is also a component of aromatic amino acids, auxins, phenols and lignin (GRAHAM; HANNAM; UREN, 1988). The importance of Mn for plant nutrition and the

current evidence of Mn deficiency in soybean trials increased the usage of Mn by soybean growers all over Brazil.

Among many factors, the introduction and rapid adoption of transgenic glyphosate-resistant soybean are some of the drivers of its expansion in Brazil, since it highly facilitates the weed management. Glyphosate ([*N*-(phosphonomethyl)glycine]) is a non-selective herbicide which mechanism of action consists of competitive inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is essential for the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan (GROSSBARD; ATKINSON, 1985). Besides the introduction of transgenic crops, which caused the increased use of glyphosate worldwide, there are several other reasons for glyphosate's success, such as its highly effective broad spectrum and low toxicity for animals (DUKE; POWLES, 2008), being also a very cheap herbicide (DUKE, 2018). Glyphosate is considered a once in a century herbicide (DUKE; POWLES, 2008), being the best-selling active ingredient for herbicides worldwide (BELBIN et al., 2019). In 2018, more than 190 thousands of tons of glyphosate's active ingredient and its salts were sold within the Brazilian territory, four times more than the second best-selling herbicide, the 2,4-D (ALCÁNTARA-DE LA CRUZ et al., 2020; IBAMA, 2018).

Manganese can be supplied through soil or foliar fertilization (SFREDO, 2008). In Brazilian soybean fields, Mn foliar fertilization is often performed in conjunction with glyphosate at least twice during the crop cycle, generally around the V3 and V7 phenological stages. The tank mixing is as an alternative to reduce the number of sprayings in the field, farmers costs, soil compaction, and mechanical damage to the crop (SOLTANI; SHROPSHIRE; SIKKEMA, 2011), however, this practice can compromise not only the efficacy of weed control by glyphosate but also the nutrition potential of the dissolved ions. Bernards et al. (2005) demonstrated that glyphosate interactions with Mn increased at higher pH values of the mixture similar to those found in plant symplast. In soybean plants, foliar applications of glyphosate significantly decreased leaf Mn content (CAKMAK et al., 2009).

Given the wide adoption of tank mixtures with Mn and glyphosate, and considering their antagonist potential on both weed control and plant nutrition, this study aimed to provide a better understanding on the chemical reactions between this herbicide and five of the most common Mn sources ($MnSO_4$, Mn-EDTA, Mn-phosphite, Mn-glycine, and $MnCO_3$ nanopowder). The effect of the pH on $MnSO_4$ + glyphosate interactions was also evaluated. Therefore, the electric conductivity (EC) and pH of the mixtures were monitored over 72 hours, while X-ray fluorescence spectroscopy (XRF) quantified the amount of

glyphosate and Mn found in the solution and in the precipitates. Chemical speciation analysis using X-ray absorption near edge structure (XANES) determined the Mn chemical environment for both supernatants and precipitates. The chemistry of $MnSO_4$ + glyphosate supernatants at different pH was evaluated through XANES. Thermogravimetric analysis revealed the composition of precipitates at pH 7, and zeta potential and dynamic light scattering (DLS) analysis showed the charge and diameter of the precipitates' particles in a varying pH.

2.2 Materials and Methods

2.2.1 Reagents and experimental design

The following Mn sources were employed: $MnSO_4 \cdot H_2O$ (31.85 wt% Mn, Synth, Brazil), $MnCO_3$ nanopowder 80-100 nm (47.78 wt% Mn, Nanoshel LLC, USA), Mn-ethylenediamine tetraacetic acid (Mn-EDTA) (13 wt% Mn, Stoller, USA), Mn-phosphite ($MnHPO_3$) (8 wt% Mn, Agrivalle, Brazil), and Mn-Glycine (22 wt%, Stoller, USA). The employed Mn-EDTA, Mn-phosphite and Mn-glycine are commercial products, while the $MnSO_4$ and $MnCO_3$ are lab reagents. The glyphosate employed in this study was also a commercial product ($C_3H_8NO_5P$) (48 wt% active ingredient, 36 wt% acid equivalent, and 68.5 wt% of inert ingredients), obtained from Ameribrás (Brazil). It worth mentioning that the real composition regarding the inert ingredients from the glyphosate source is not known. Generally, it is composed by surfactants, diluents or adjuvants which stabilize the formulation and improve the product penetration within the plants, but the real composition is considered confidential business information (DEFARGE; SPIROUX DE VENDÔMOIS; SÉRALINI, 2018). The experiments were carried out in triplicate using five Mn sources, with and without glyphosate.

2.2.2 Solution and dispersion preparation

The solutions and dispersions containing Mn, with and without glyphosate, were prepared based on the recommended field doses of 350 g ha^{-1} of Mn (EMBRAPA, 2013) and 1.5 L ha^{-1} of glyphosate. Considering the application of 50 L ha^{-1} of the spray mix, the employed concentrations were 7 g L^{-1} of Mn and 30 mL L^{-1} of glyphosate (10.8 g L^{-1} acid equivalent). It corresponds to a Mn:glyphosate molar ratio of nearly 2:1.

All measurements (pH, electric conductivity, Mn fractionation, thermal analysis, pH assay, and XAS) were carried out in three repetitions.

2.2.3 pH and electric conductivity measurements

The Mn solutions/dispersion (25 mL) were transferred to 50 mL plastic vials, and the pH and electric conductivity (EC) were measured using InLab® Expert Pro-ISM and InLab® 731-ISM sensors, respectively, coupled to the SG-23 SevenGo DuoTM equipment (Mettler-Toledo, Switzerland). Subsequently, glyphosate (750 μL) was added in the solution/dispersion and fully homogenized by manually shaking. Immediately after, the pH and EC were measured. From this time on, the plastic vials were closed and left undisturbed, under room temperature and dark. Later, the pH and EC measurements were carried out every 4 hours in the first 24 hours, with an extra measurement made around 72 hours after the glyphosate addition (Figure 2.1). This time range was employed because in field conditions spraying solutions hardly exceeds this time limit, being the first 24 hours the more critic period.

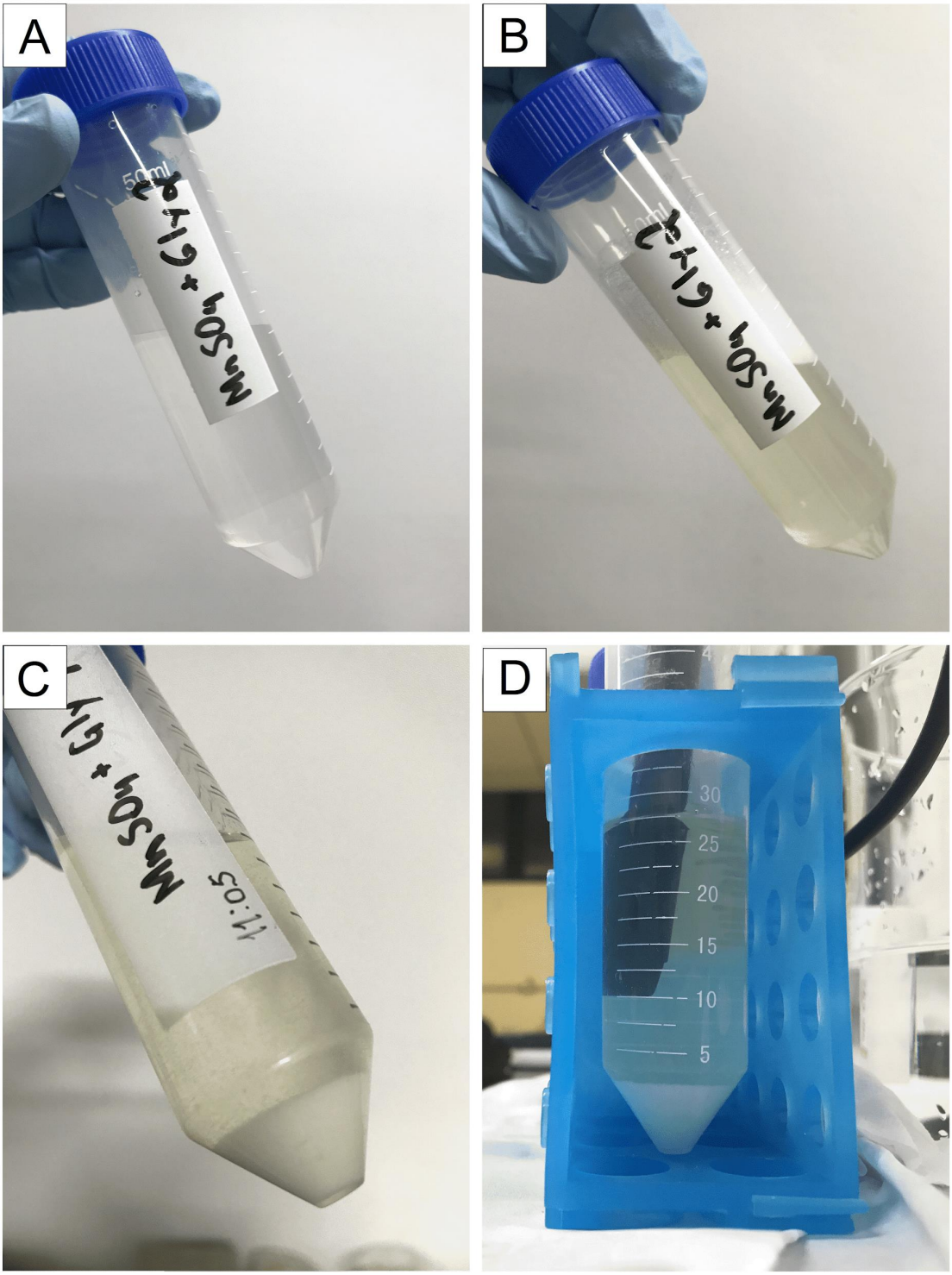

Figure 2.1. Preparation of the Mn + Glyphosate solutions and the pH and EC measurements. MnSO4 solution without glyphosate (A); MnSO4 solution right after the mixture of glyphosate (B); Formation of precipitates 10 hours after the mixture of glyphosate in a MnSO4 solution (C); pH and EC measurements carried out at the supernatants (D)

2.2.4 Mn fractionation

The Mn fractionation was determined based on Almeida et al. (DE ALMEIDA et al., 2019), with modifications. The fertilizers solutions and dispersions, with and without glyphosate, were prepared following the sample preparation described above (section 2.2). Aliquots (7 μL) of the solutions/dispersions were sampled right after the mixtures. Then, the samples were centrifuged (1,464 g) for 2 hours, in order to separate any precipitate from supernatants, being the supernatants also sampled (7 μL).

The 7 μL aliquots were transferred into 1 mL plastic vials, then, the surfactant Triton X-100 solution at 5% (v/v) (5 μL), purified water (938 μL), and 1,003 mg Ga L^{-1} internal standard (50 μL) were subsequently added.

For the treatments that formed precipitate, the insoluble phase was collected and dried in a laboratory oven (60°C) until reach constant mass. The precipitates were weighed, ground manually using an agate mortar, and microwave-assisted acid digested. For the latter procedure, the precipitates (20 mg) were transferred into pre-cleaned Teflon TFM tubes, then HNO_3 20% (v/v) (5 mL) and H_2O_2 30% (v/v) (2 mL) were added. So, the samples were placed in the microwave oven (DGT 100 Plus, Provecto Analítica, Brazil), and heating program was: 7 minutes at 400 W, 15 minutes at 850 W and 7 minutes at 320 W. The final solution was transferred to 50 mL plastic vials and diluted up to 40 mL with purified water. Finally, an aliquot of the solutions (330 μL) was transferred to 1 mL plastic vial, and the surfactant Triton X-100 solution at 5% (v/v) (5 μL), purified water (615 μL), and 1,003 mg Ga L^{-1} internal standard (50 μL) were subsequently added.

The concentration of Mn, P and S in the pristine solutions and dispersions containing Mn, with and without glyphosate, supernatants and in the precipitate (after microwave-assisted digestion) were determined by energy dispersive X-ray fluorescence spectrometer (EDXRF, Shimadzu, EDX-720, Japan). For that, 10 μL of each prepared sample were pipetted on the external side of the 31 mm diameter poly(ethylene) XRF cuvette (cat. no. 1530, Chemplex, USA) covered with 6 μm thick polypropylene film (VHG Labs, USA). Then, the samples were dried in laboratory oven (60°C).

The EDXRF operating condition was Rh X-ray tube at 50 kV and auto-tunable current to a 30 % maximum dead time. The measurement time was 200 s, and the analysis was carried out under vacuum and using a 10-mm diameter collimator (Shimadzu, Japan). The X-ray spectrum was acquired by a Si(Li) detector. The quantification was carried out using external calibration (Figure 2.2).

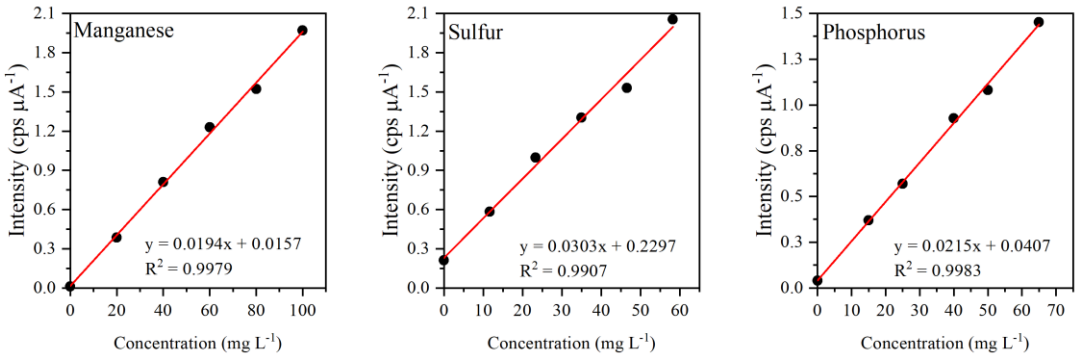

Figure 2.2. Calibration curves used for obtaining the Mn, P and S concentrations at the EDXRF analysis

Limits of detection (LOD) and quantification (LOQ) were determined as stated by Almeida et al. (2019) and Kadachi and Al-Eshaikh (2012), respectively, following the equations (1) and (2):

$$LOD = \frac{3}{I_{Ga} * S} \sqrt{\frac{BG}{t}} \quad (1)$$

$$LOQ = LOD \times 3.33 \quad (2)$$

where LOD, LOQ, S, I_{Ga}, BG and t are the limit of detection (mg L^{-1}), limit of quantification (mg L^{-1}) relative elemental sensitivity (mg^{-1} L), Ga intensity (cps), background intensity (cps) of the analyte, and the acquisition time (s).

2.2.5 X-ray absorption (XAS) characterization

The x-ray absorption near edge structure (XANES) measurements were carried out in the liquid and solid phases formed before and after the glyphosate mixture into the Mn solutions/dispersion. To simulate field conditions, glyphosate was added to the liquid samples *ca.* 2.5 hours prior to the analysis. Solid samples preparation was done according to Gomes et al. (2019) with modifications. Therefore, solid samples (precipitates and $MnCO_3$) were previously dried in a laboratory oven at 60° C and diluted in cellulose at 1.5 Mn wt.%. Then, 100 mg pellets were prepared by using a 13 mm stainless steel die set pressed at 500 kg for 1 min (Carver 3912, USA).

The liquid samples were placed in acrylic 1 mm thick sample holders covered with Kapton tape (Figure 2.3a). Solid ones were fixed in sample holders using Kapton tape (Figure 2.3b).

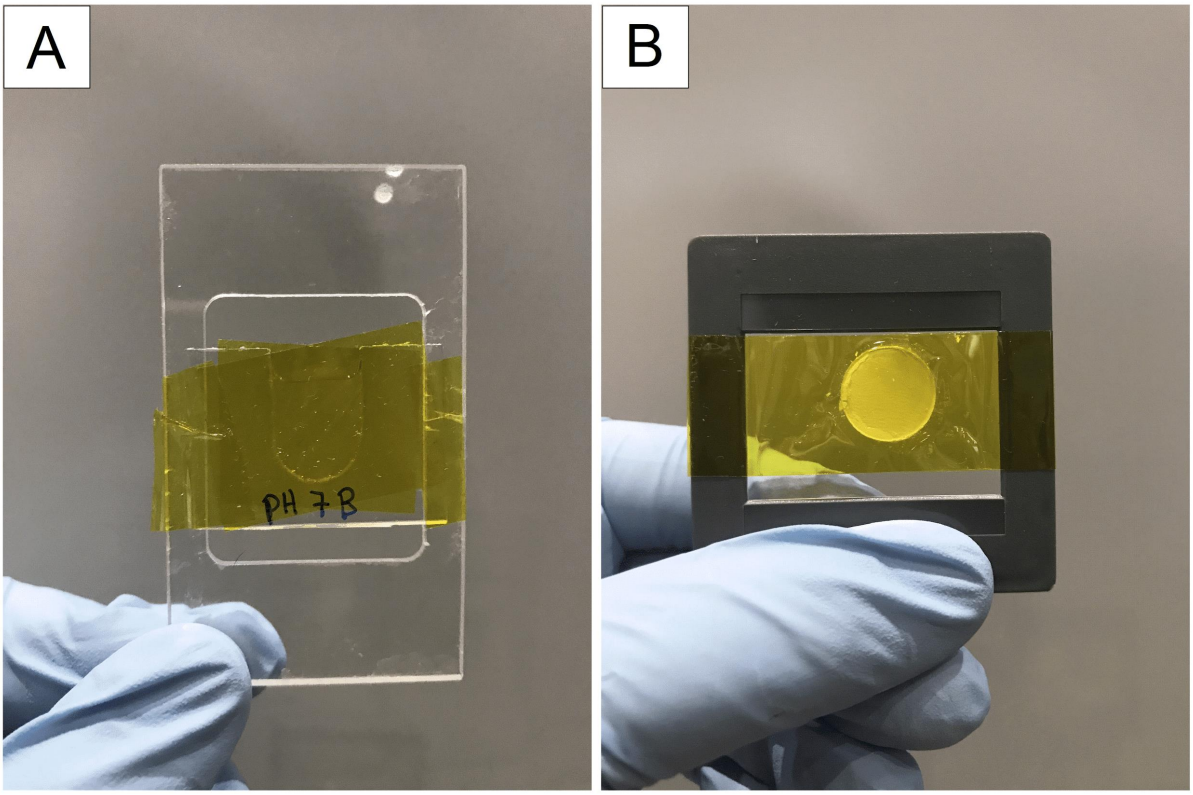

Figure 2.3. Samples measured at the LNLS. Liquid sample placed in an acrylic cell (A); 100 mg pellet were prepared from solid sample at 1.5 % Mn (w/w) and fixed with Kapton tape in sample holders (B)

The measurements were carried out in transmission mode at the XRF beamline of the Brazilian Synchrotron Light Laboratory (LNLS). The XRF beamline is equipped with a bending-magnet, Si (111) double crystal monochromator, KB mirror system resulting in a 20 μm diameter spot size, the detection was made using ionization chambers before and after the sample. The 20 μm beam was positioned at the center of the samples, recording the XANES spectra between -100 and 200 eV across the Mn-K edge. Three scans per sample were collected and merged to improve the signal-to-noise ratio. The measurements were carried out in three replicates.

The spectra were merged, energy-calibrated using a Mn foil, and then normalized using Athena software within the IFEFFIT package (RAVEL; NEWVILLE, 2005).

2.2.6 pH assay: chemical speciation of $MnSO_4$ + glyphosate solutions at different pH

$MnSO_4$ solutions (7 g L^{-1} of Mn) were mixed with glyphosate (18 g L^{-1} of acid equivalent) and had their pH adjusted to 1, 3, 5 and 7, by adding H_2SO_4 or NaOH. The pH was measured using the pH meter Tec-2 (Tecnal, Brazil). Subsequently, these solutions were centrifuged (1,464 g) for 2 hours, and the supernatants were separated from the precipitates using pasteur pipettes.

Chemical speciation analysis of the supernatants was carried out in transmission mode at the XRF beamline of the Brazilian Synchrotron Light Laboratory (LNLS), just as above the mentioned XAS characterization for liquid samples.

2.2.7 Thermal analysis (Gravimetric – TGA and Differential – DrTGA)

Thermal analysis was made in the precipitates formed after increasing the pH of $MnSO_4$ solutions with and without glyphosate. To prepare the samples, $MnSO_4 \cdot H_2O$ was dissolved in purified water at the concentration of 7 g L^{-1} of Mn. The pH, which initially was nearly 4.4, was adjusted to 7 using NaOH solution. The titration was made under stirring by a magnetic mixer. The pH increasing to nearly 7 caused the formation of a brownish compound that precipitated.

For $MnSO_4$ + glyphosate samples, glyphosate (18 g L^{-1} of equivalent acid) was added to the $MnSO_4$ solution at pH 7. The glyphosate addition caused the pH decreasing, thus, NaOH was employed to adjust the pH to 7 again. In this case, a lighter brownish compound was formed and precipitated.

The supernatants were properly discarded after centrifuging the samples at 1,464 g for 1h. The precipitates were placed into the laboratory oven (50ºC) until reach constant mass. After drying, the precipitates were ground manually using an agate mortar, weighted, and followed to the thermal analysis. Each treatment relied on three repetitions.

The thermal analysis was performed by a Shimadzu DTG-60H equipment - simultaneous TG-DrTGA, which operated in heating and cooling cycle, from room temperature to 1100°C, under N_2 atmosphere and heating rate of 10°C min^{-1}.

2.2.8 Dynamic light scattering analysis (DLS) and microelectrophoresis (zeta potential) analysis

The DLS and zeta potential analysis were performed in $MnSO_4$ + glyphosate solutions under several pH values. A $MnSO_4$ stock solution was prepared at the concentration of 7 g L^{-1} of Mn, which presented an initial pH of *ca.* 4.4, then 10 mL aliquots of the stock solutions were separated for the pH adjustments to 5 or 7 using NaOH solution. Later, 300 µL of glyphosate were added to the aliquots, aiming a final glyphosate concentration of 30 mL L^{-1}. Then, the pH was adjusted to 5 or 7 again. A treatment with no pH adjustment was also evaluated, which presented a pH of 2.5 after the glyphosate addition. The Mn and glyphosate concentrations were the same ones used in field applications (EMBRAPA, 2013).

The DLS and zeta potential analysis were performed using a ZetaSizer Nano ZS90 particle analyzer (Malvern Instruments). Samples at pH 2.5 and 5 had to be diluted 10 and 20-fold, respectively, due to their light scattering properties, while samples at pH 7 did not require any dilution. Three repetitions of each treatment were evaluated.

2.3 Results and Discussion

2.3.1 Monitoring the pH and electric conductivity

The pH and electric conductivity (EC) of the tank mixtures were monitored for *ca.* 72 hours. Glyphosate addition promoted changes in pH and EC of solution/dispersion for all Mn sources, especially during the first 5 hours after its addition (Figure 2.4). After 5 hours, the EC remained constant for all mixtures; however, for $MnSO_4$ and Mn-glycine, the pH continued to decrease until the last measurement, while it increased from 24 to 72 hours after the glyphosate mixture for $MnCO_3$ (Figure 2.4a and 2.4b). As a general trend, the electrical conductivity and pH were inversely proportional. It is worth mentioning that both $MnSO_4$ and Mn-glycine presented a white precipitate after mixing with glyphosate, while $MnCO_3$ precipitated regardless glyphosate addition, since it is a low solubility source.

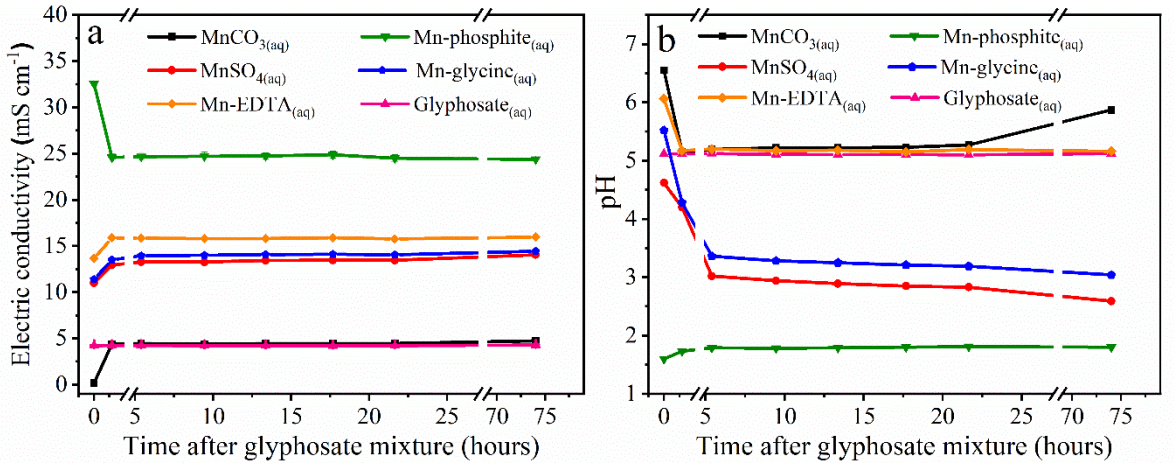

Figure 2.4. Electric conductivity (A) and pH (B) of Mn aqueous solutions/dispersion as function of time after glyphosate mixture. The data shows that glyphosate increased the electric conductivity (EC) of the solutions/dispersion, except for Mn-phosphite. On the other hand, the dispersion/solutions' pH decreased with the glyphosate addition, except for Mn-phosphite

Following dissolution in water, $MnSO_{4(aq)}$ forms an outer-sphere complex $[Mn(H_2O)_6]^{2+}SO_4^{2-}$ (BERNARDS et al., 2005), while the solubility product constant (K_{sp}) for Mn-glyphosate solution buffered at pH 7 is $0.955x10^{-6}$ (SUNDARAM; SUNDARAM, 1997). The reduction in pH followed by the EC increasing is likely related to the correlation between conductivity and availability of H^+ ions in solution. The pH of solution decreased more intensively for $MnSO_4$ (from 4.6 to 2.6) and Mn-glycine (from 5.5 to 3.0), probably due to the deprotonation of glyphosate molecules. The glyphosate deprotonation provides H^+ ions to the solution, which also releases adsorption sites for Mn^{2+} complexation.

Chahal et al. (2012) also investigated the influence of glyphosate addition to a Mn solution using a commercial source based on $MnSO_4$ (Nutrisol 8% Mn, Coastal AgroBusiness). The concentrations were *ca.* 0.8 g L^{-1} of Mn and 6.7 g L^{-1} of glyphosate. Differently from our data, they found an initial pH of 3 for the Mn solution, which is more acid than ours, and glyphosate raised the pH to 4.2 right after its addition, showing no more changes in this parameter until elapsed 72 hours. Conversely to our observations, these authors did not observe precipitate formation.

The pH of the tank mixtures is considered an important parameter to assure the efficiency of the involved compounds. Machado et al. (2019) reported severe epidermal cell shrinking due to the application of very acid Mn solutions (pH 1.4) on soybeans' leaves, but further studies are still necessary to unravel the effects of the solution pH on foliar fertilization efficiency. On the other hand, the influence of the pH on glyphosate's efficiency was better investigated. Previous studies had demonstrated that glyphosate's activity is

higher in acidic pH than that in alkaline (BUHLER; BURNSIDE, 1983; SHEA; TUPY, 1984; SHILLING; HALLER, 1989). Shea and Tupy (SHEA; TUPY, 1984) observed that the increase of the glyphosate solution pH from 4 to 5.5 was sufficient to reduce its efficiency on weed control, but no significant difference was resultant due to the adjustment of the pH from 5.5 to 6 or 10.

2.3.2 Mn fractionation

Values above the LOQ ($4.6\ 10^{-7}$, $5.8\ 10^{-7}$, and $4.2\ 10^{-7}$ mol for Mn, P and S, respectively) were considered to determine the Mn, P and S content in the obtained fractions (solutions, dispersions, supernatants, and precipitates). For $MnSO_{4(aq)}$ (Figure 2.5a) and Mn-glycine$_{(aq)}$ (Figure 2.5c) without glyphosate, no significant changes were observed for pH, EC, Mn and S content before and after the centrifugation process.

Most of the Mn and P provided by the mixture of $MnSO_{4(aq)}$ and glyphosate remained in solution (Table 2.1), however, nearly 29% of the Mn precipitated along with *ca.* 28% of the P. A *ca.* 2:1 Mn:P molar ratio was found in the $MnSO_4$ precipitate (Table 2.2). Regarding the Mn-glycine, one should expect that the Mn complexation by glycine could prevent some interactions with glyphosate; however, the addition of glyphosate to the Mn-glycine solution caused the precipitation of *ca.* 43% of the employed Mn, forming a precipitate with a Mn:P molar ratio of *ca.* 3:1 (Table 2.2), indicating that the stability constant of glyphosate is higher than that of glycine.

Table 2.1. Remaining percentage of Mn and P in liquid and precipitated phases after the centrifugation of the Mn sources mixed with glyphosate

Mn source	**Liquid phase**		**Precipitate**	
	Mn (%)	**P (%)**	**Mn (%)**	**P (%)**
	Mean value ± standard error (n=3)			
$MnSO_4$	68 ± 5	62 ± 10	29 ± 3	28 ± 9
Mn-glycine	61 ± 3	28 ± 9	43 ± 2	39 ± 2
$MnCO_3$	< LOQ	56 ± 6	81 ± 9	23 ± 8
Mn-EDTA	96 ± 6	90 ± 30	none	none
Mn-phosphite	101 ± 3	104 ± 6	none	none

Values obtained by the comparison with the Mn and P contents in the initial solution (before centrifugation).

Table 2.2. Mn:P molar ratio in the phases obtained after the interaction between glyphosate and the Mn sources

Mn source	**Mn:P molar ratio**		
	Solution	**Liquid phase**	**Precipitate**
	Mean value ± standard error (n=3)		
$MnSO_4$	2.2 ± 0.3	2.4 ± 0.4	2.4 ± 0.9
Mn-glycine	2.52 ± 0.06	6 ± 2	3.0 ± 0.9
$MnCO_3$	2.19 ± 0.16	< LOQ	8 ± 3
Mn-EDTA	2.7 ± 0.6	2.84 ± 0.04	none
Mn-phosphite	0.22 ± 0.01	0.214 ± 0.003	none

The addition of glyphosate in both sulfur-containing sources ($MnSO_4$ and Mn-glycine) did not change the S content in the solution, revealing that the SO_4^{2-} does not precipitate along with glyphosate molecules (Figures 2.5b and 2.5d). It is worth noting that glycine ($C_2H_5NO_2$) does not contain sulfur, however this element was detected in the mixture. It suggests that the Mn-glycine fertilizer was prepared through an attempt to complex Mn^{2+} with glycine displacing the SO_4^{2-}. However, it was not completely purified.

The precipitation suggests that both $MnSO_4$ and Mn-glycine can lose efficiency on foliar fertilization. On this subject, little research is available about Mn-glycine as fertilizer, but the $MnSO_4$ performance showed to be negatively affected by tank mixtures with glyphosate (MACHADO et al., 2019). Likewise, the herbicidal action of glyphosate may also be affected due to precipitation. Bernards et al. (2005), by tracing the glyphosate movement within *Abutilon theophrasti* leaves (velvetleaf) using ^{14}C radiolabeled glyphosate, verified that the absorption of glyphosate by the plant was reduced in the presence of Mn-ethylaminoacetate (Mn:glyphosate *ca.* 5:1), Mn-lignin sulfonate (Mn:glyphosate *ca.* 6:1), and $MnSO_4$ (Mn:glyphosate *ca.* 25:1), while Mn-EDTA (Mn:glyphosate *ca.* 7:1) was the least likely to diminish glyphosate absorption, translocation and efficacy.

Mn-$EDTA_{(aq)}$ presented stable Mn and P contents before and after the centrifugation, regardless of the glyphosate mixture (Figures 2.5g and 2.5h). Although the glyphosate changed the pH and EC of the Mn-EDTA solution, no precipitate was formed. The stability constant of glyphosate, log K = 5.53, is much lower than that of Mn^{2+} stability constant of EDTA, log K = 13.81 (MADSEN et al., 1978). In this context, the higher stability for EDTA prevents Mn complexing with glyphosate (Figure 2.5h).

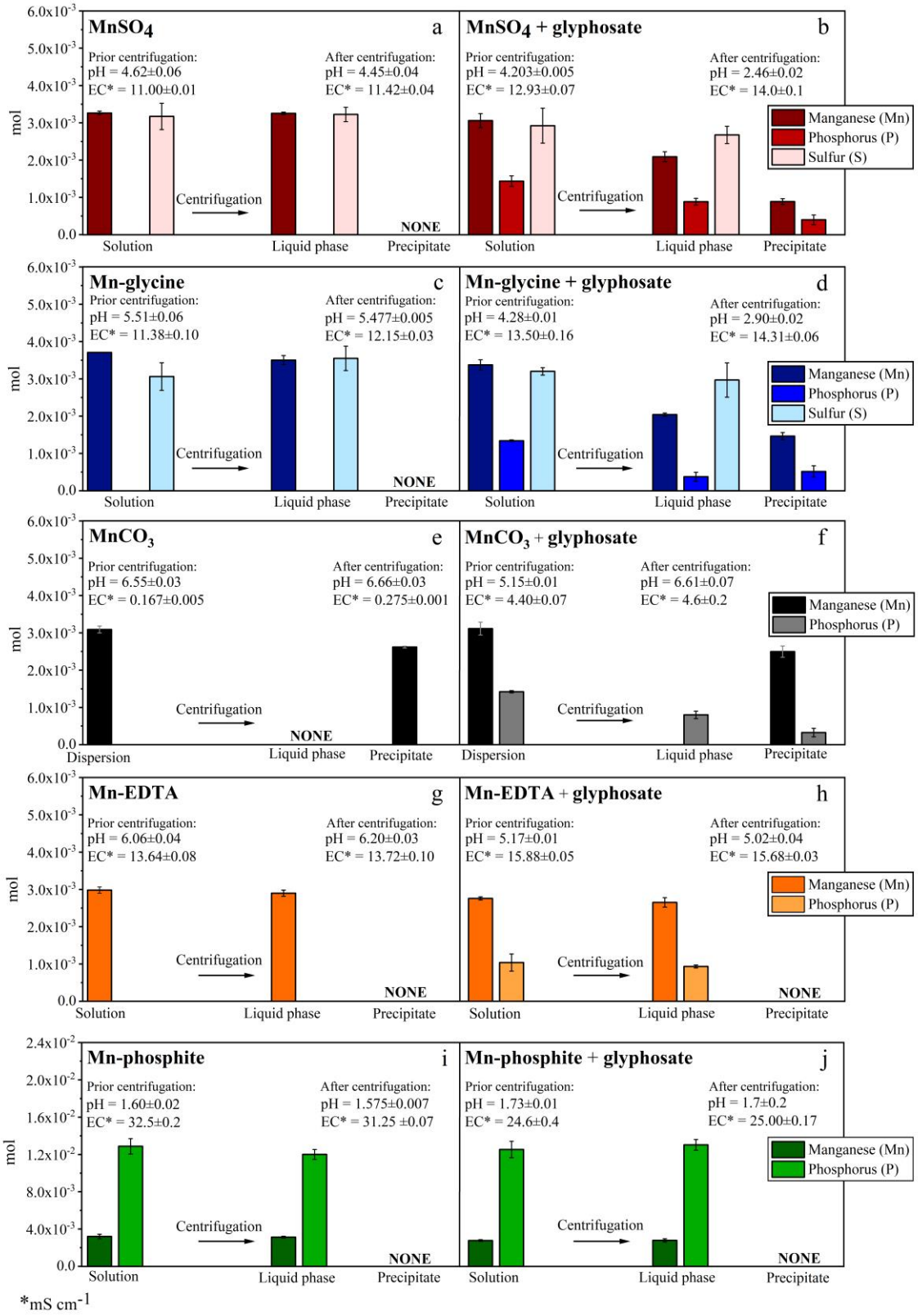

Figure 2.5. Fractions formed by Mn-containing fertilizers before and after mixing with glyphosate, with their respective Mn, P and S content (when present). $MnSO_4$ (A); $MnSO_4$ + glyphosate (B); Mn-glycine (C); Mn-glycine + glyphosate (D); $MnCO_3$ (E); $MnCO_3$ + glyphosate (F); Mn-EDTA (G); Mn-EDTA + glyphosate (H); Mn-phosphite (I); Mn-phosphite + glyphosate (J). Only values above LOQ ($4.6\ 10^{-7}$, $5.8\ 10^{-7}$, and $4.2\ 10^{-7}$ mol for Mn, P and S, respectively) were considered

Several studies showed that Mn-EDTA did not antagonize glyphosate efficacy (BERNARDS et al., 2005; BERNARDS; THELEN; PENNER, 2005; SOLTANI; SHROPSHIRE; SIKKEMA, 2011), on the contrary, it can even increase its herbicidal action (BERNARDS et al., 2005; BERNARDS; THELEN; PENNER, 2005). It happens most likely due to EDTA potential to chelate with cations when using tap water for the spray mixture preparation, preventing the formation of metal-glyphosate complexes. On the plant nutrition point of view, using *in vivo* X-ray spectrometry, it was previously showed that Mn foliar absorption and transport kinetics was not affected by the mixture of Mn-EDTA with glyphosate as well (MACHADO et al., 2019).

The $MnCO_3$ (80-100 nm) precipitated regardless of glyphosate addition (Figures 2.5e and 2.5f). The Mn content that remained in the supernatant was lower than the established limit of quantification. When glyphosate was added *ca.* 23% of the total P precipitated with 81% of the total Mn applied (Table 2.2). EC and pH increased after the centrifugation of the $MnCO_3$ dispersion (Figure 2.5e). Then, when glyphosate was added, the pH decreased while the EC increased. After centrifugation, the pH was raised, reaching a value near to that observed in the absence of glyphosate, while the EC increased only 0.2 mS cm^{-1} (Figure 2.5f).

Based on the behavior of the $MnCO_3$ dispersion, one can notice that the $MnCO_3$ source employed in this study showed itself as an inadequate source for foliar fertilization, regardless of glyphosate mixture. Since it precipitated in its entirely, no Mn remained available in solution for foliar uptake. Accordingly to this statement, Migliavacca (2018) found that foliar application of $MnCO_3$ did not circumvent Mn deficiency in soybean plants. Likewise, Machado et al. (2019) found that $MnCO_3$ was not able increase the Mn content in soybean petioles 48 h after leaf spraying, regardless of glyphosate mixture.

Regarding to the Mn-phosphite$_{(aq)}$ solution, the addition of glyphosate slightly increased the pH and decreased the EC. This is related to the acidic behavior of phosphite compounds. Likewise, Mn-phosphite also presents high P content (P concentration *ca.* 4-fold higher than Mn). Therefore, P content was not significantly changed by the glyphosate mixture (Figures 2.5i and 2.5j). It was not observed any formation of precipitates after the addition of glyphosate to the Mn-phosphite$_{(aq)}$ solution, since at such low pH (*ca.* 1.7), the glyphosate molecules remain protonated that ultimately providing few Mn^{+2} adsorption sites.

Considering the absence of visual interactions, such as precipitate formation, one should expect no negative consequences on the mixture between glyphosate and Mn-phosphite$_{(aq)}$. However, attention must be paid regarding the pH of the solution.

On the one hand, such acid pH should not disturb glyphosate efficacy on weed control, once its activity increases as pH decreases (SHEA; TUPY, 1984). On the other hand, it can be harmful to the main crop. Machado et al. (2019) showed that Mn-phosphite solutions, with and without glyphosate (pH 1.6 and 1.8, respectively), caused severe epidermal cell shrinking on soybean leaves. Additionally, it was also shown that glyphosate reduces the Mn leaf uptake from Mn-phosphate.

Among other factors, impairment of micronutrient root to shoot transport was also reported due to glyphosate, although the mechanisms behind were not elucidated. However, it has been demonstrated that foliar applied glyphosate can be translocated and further exudate by roots (NEUMANN et al., 2006), potentially precipitating Mn in soil or nutrient solution. On the other hand, Duke et al. (2012) listed several studies showing no restriction of micronutrients availability for GR crops treated with glyphosate.

2.3.3 Chemical speciation

Figure 2.6a presents the spectra for pristine Mn fertilizers and nanopowder without glyphosate, while Figure 2.6b shows the spectra for the supernatants obtained after glyphosate addition and centrifugation, and Figure 2.6c shows the spectra for recovered precipitates. The pristine solutions of Mn-glycine, $MnSO_4$ and Mn-phosphite present similar spectra, while Mn-EDTA and $MnCO_3$ show features that clearly differentiate them from the others.

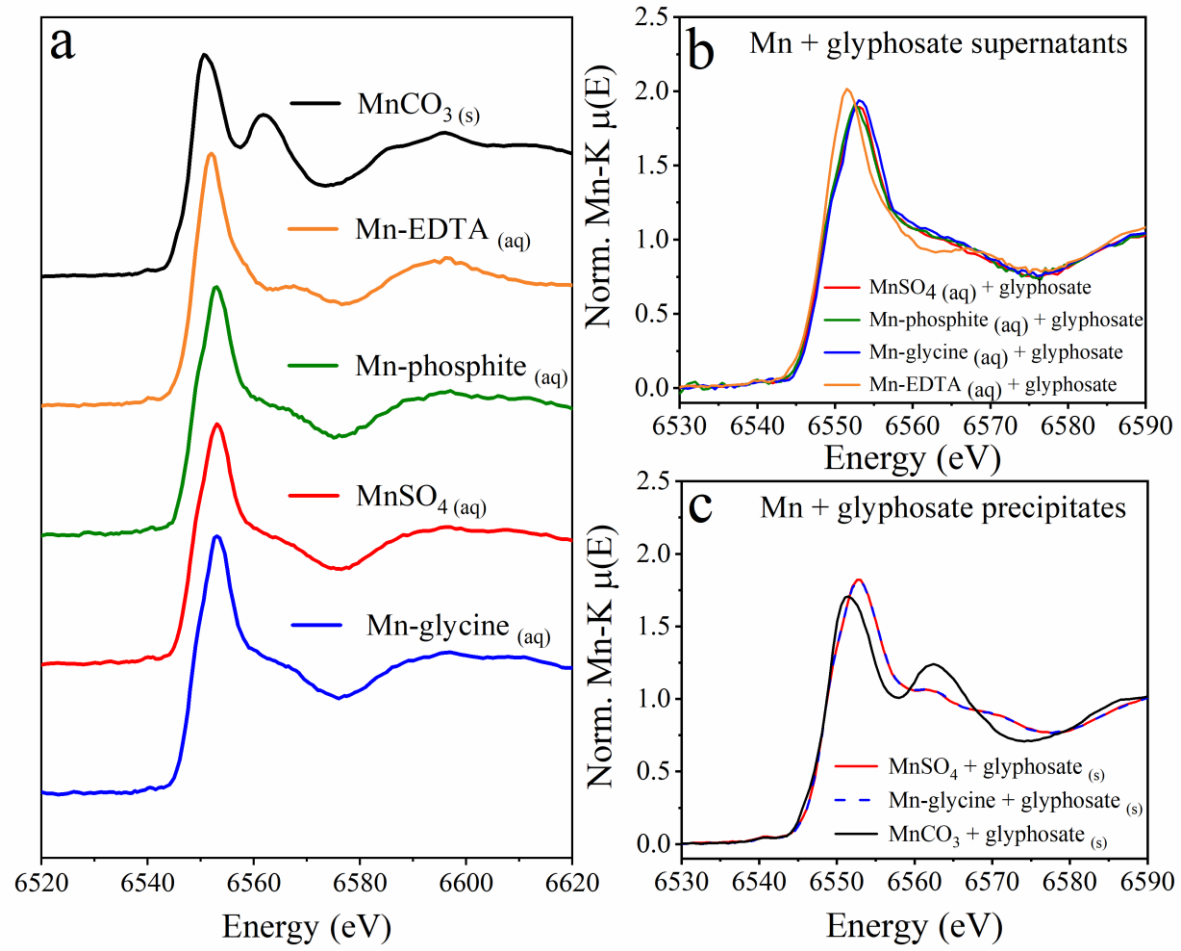

Figure 2.6. XANES spectra for the Mn sources in aqueous solution and solid $MnCO_3$ (a), liquid phase (supernatant) (b) and solid phase (precipitated) (c) obtained after mixing glyphosate to the Mn sources. Data shows that the Mn chemical environment of the liquid phase after glyphosate mixture are different, however, it is the same for the precipitates coming from Mn-glycine and $MnSO_4$

Although the stoichiometry shows that part of the glyphosate remained in liquid phase, the spectra for the supernatants did not present remarkable spectral changes to support a glyphosate reaction with Mn^{2+} that would yield an inner-sphere Mn-glyphosate complex (Figure 2.7). This would be consistent with electronic paramagnetic resonance (EPR) measurements of Mn^{2+} + glyphosate performed by Bernards et al. (2005) in a similar pH condition. On the other hand, likewise in Figure 2.6b, these overlapped spectra clearly show similarities between the chemical environment of the suspended Mn-glycine, $MnSO_4$ and Mn-phosphite.

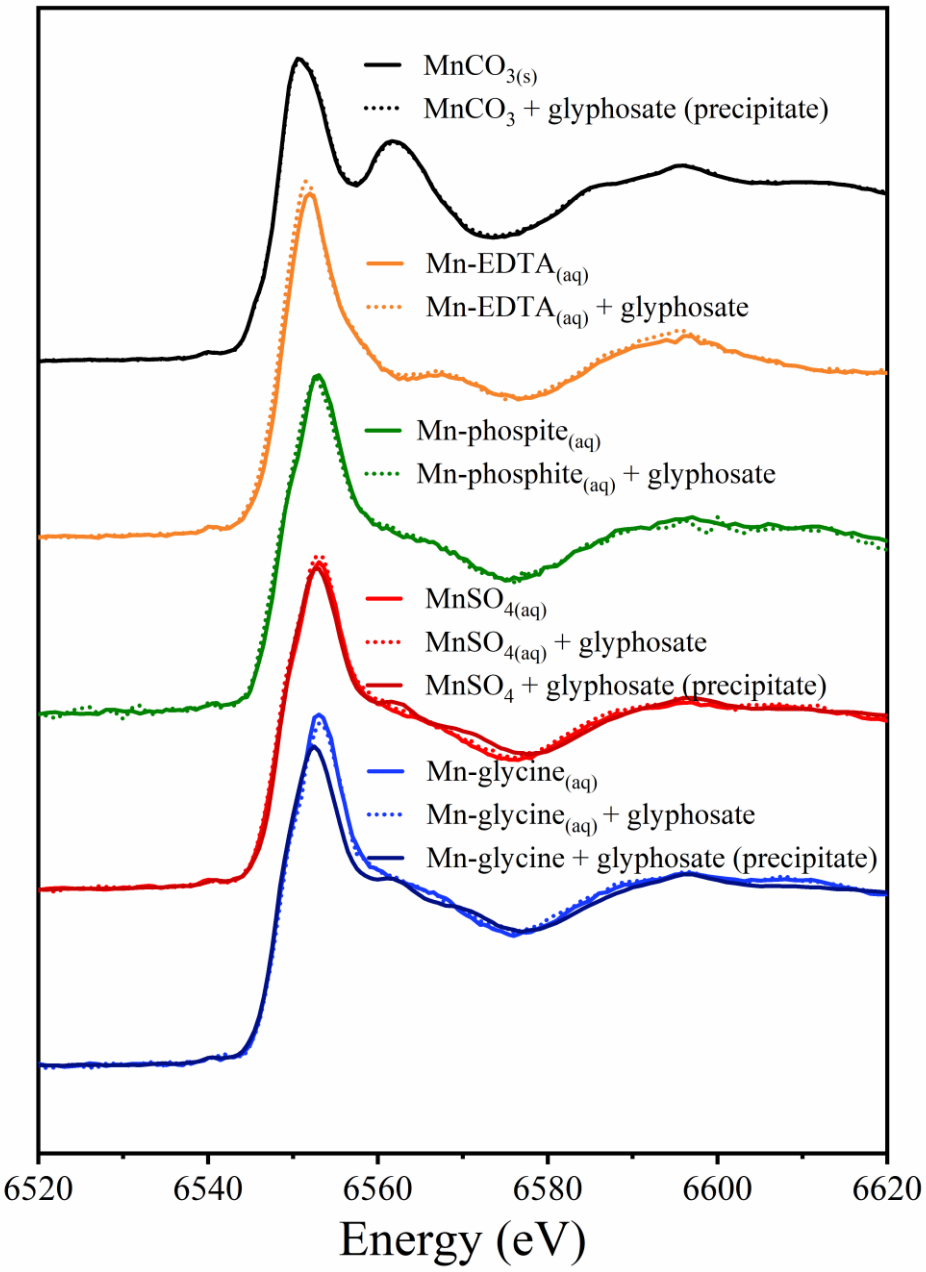

Figure 2.7. XANES spectra for the specimens obtained after the mixture between Mn sources and glyphosate. Glyphosate altered the Mn chemical environment coming from Mn-glycine and $MnSO_4$, which suggests that glyphosate molecules complex Mn when it is available as an ion (Mn^{2+}) in solution

Machado et al. (2019) obtained the Mn chemical speciation in the petioles of soybean plants which were foliar fertilized with $MnSO_4$, Mn-EDTA and Mn-phosphite, mixed or not with glyphosate. The acquired Mn XANES spectra found no Mn-glyphosate complexes inside the plants regardless of the applied Mn source. While $MnSO_4$ and Mn-phosphite provided Mn for plants in a similar chemical environment, the Mn-EDTA was still found in its pristine form, showing that the bonding force between Mn and EDTA is powerful enough to keep the Mn chelated all over the way from the leaf to the petiole.

Despite the reactions between aqueous Mn^{2+} and glyphosate within tank mixtures, the above-mentioned results (MACHADO et al., 2019) show that Mn remaining in solution is being absorbed and transported in a non-complexed form. These data, together with Bernards et al. (2005), also support the rejection of the soluble complexes' formation hypothesis, since they indicate that glyphosate does not interfere with the Mn chemical environment inside the plants, and that both Mn and glyphosate remaining in solution can accomplish their physiological impacts. It seems that the major cause for the loss of efficiency observed when Mn^{2+} and glyphosate are mixed, is due to the relative amount of both species available in solution.

The $MnSO_4$ and Mn-glycine formed similar precipitated products with glyphosate (Figure 2.6c). Their spectra presented a slightly reduction of whiteline intensity compared to the pristine and supernatant forms (Figure 2.7). The reduction of whiteline intensity is associated to the increase of electron density on p unoccupied states of Mn^{2+} ions, while edge shifts towards lower energies can be caused by increase of Mn-ligand bond length (TRAULSEN et al., 2017). This corroborates the chemical affinity between Mn-glyphosate which yielded a more stable molecule than $MnSO_4$ and Mn-glycine.

As previously reported that glyphosate can form low soluble complexes with Ca^{2+} which cannot be absorbed by the plants' leaves (HALL; HART; JONES, 2000), the $MnSO_4$ + glyphosate precipitate shows clear spectral differences from soluble Mn (Figure 2.7), which support that such changes in the Mn chemical environment may also make the Mn-glyphosate complexes unavailable for plant uptake. Furthermore, the precipitates' formation also consists of a serious practical problem, since the formed particles can clog the sprayer nozzles during field application, which compromises the operation's efficiency.

Figure 2.8 shows the XANES spectra recorded for Mn-glyphosate precipitate, $Mn_3(PO_4)_{2(s)}$, Mn-malate$_{(aq)}$ and Mn-citrate$_{(aq)}$. The XANES spectra are highly affected by symmetry and oxidation state. Although the precipitate remains Mn^{2+} its structure is closer to carboxyl complexed Mn^{2+} (Mn-malate and Mn-citrate) than $Mn_3(PO_4)_{2(s)}$. Subramaniam and Hoggard (1988) prepared non-soluble glyphosate complexes with Cu, Ni, and Fe. The crystalline nature of the formed compounds was solved by X-ray diffraction. Infrared Fourier transform recorded for bulk glyphosate and solid complexes evidenced changes on several bands which, in principle, would suggest that amine, carboxyl and phosphonate groups participate of Mn^{2+} binding.

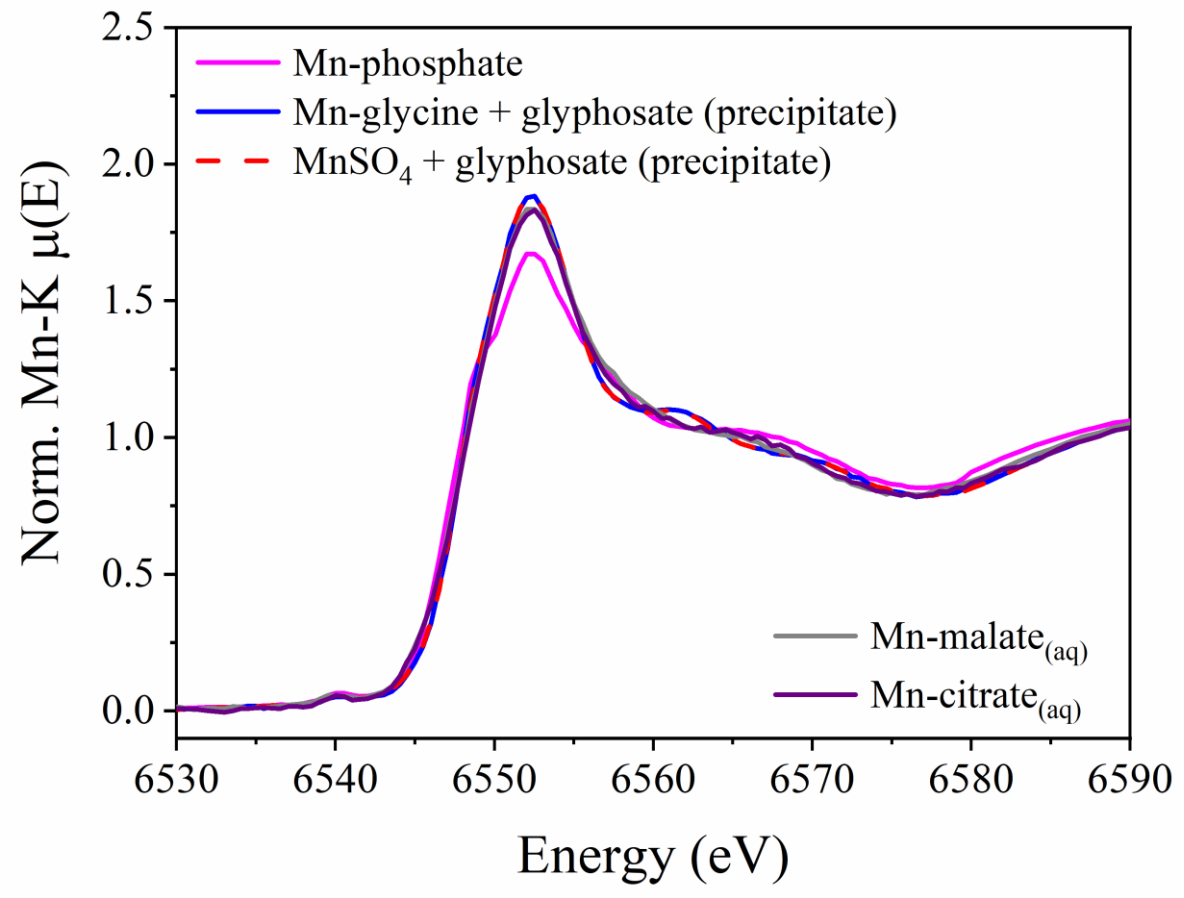

Figure 2.8. XANES spectra recorded for Mn-phosphate(s), Mn-malate$_{(aq)}$, Mn-citrate$_{(aq)}$ and two Mn-glyphosate precipitates, one obtained from $MnSO_4$ + glyphosate and the other from Mn-glycine + Glyphosate. Both Mn-glyphosate precipitates formed the same compound, which is different from Mn-phosphate, Mn-malate$_{(aq)}$ and Mn-citrate$_{(aq)}$. In aqueous solution, Mn-malate and Mn-citrate present a similar spectrum between them, while Mn-phosphate differs from all the overlapped spectra in this image

Nowack and Stone (2000) reported that Mn^{2+} quickly decomposed nitrilotris(methylene)phosphonic acid releasing orthophosphate ions, imino(dimethylene)phosphonic acid, and *N*-formylimino- (dimethylene)phosphonic acid. Conversely, Barrett and McBride (2005) reported that Mn^{3+} cleavaged glyphosate producing glycine and orthophosphate, while this did not happen for Mn^{2+} at 1:1 Mn:glyphosate ratio. However, increasing Mn^{2+}:glyphosate proportion to 4:1 lead to breakdown of glyphosate molecules. In the present study, it was employed a 2:1 Mn:glyphosate molar ratio. Although the orthophosphate had not been determined in solution, the XANES spectra recorded for the precipitates (Figure 2.8) do not suggest formation of $Mn_3(PO_4)_2$ which would have happened if glyphosate was decomposed by Mn^{2+}.

Barrett and McBride (2005) reported that less than 1% of Mn^{2+} should be complexed to glyphosate at pH 5 and it should increase to *ca.* 10 % at pH 7, they calculate these figures based on Motekaitis and Martel (1985). On the hand, our experiments showed that *ca.* 29% of Mn^{2+} precipitate at pH 2.5. A possible explanation for the deviation from theory observed in our study might be related to the concentration of ligands and Mn^{2+}. Since our study mimicked what happens in a tank mixture, the concentration of reactive chemical species, ie. Mn^{2+} and glyphosate, was nearly 60-fold higher than that employed by Motekaitis and Martel (1985).

2.3.4 Mn-glyphosate chemistry as function of pH

Among the fertilizers tested in this study, manganese sulphate is the most common, cheap and glyphosate-reactive source of Mn. Therefore, the chemistry of $MnSO_4$ + glyphosate solutions was evaluated as function of pH.

Glyphosate has four pKa values: < 2, 2.6, 5.6, and 10.6 (SPRANKLE; MEGGITT; PENNER, 1975). From pH 3-5 both carboxyl and phosphite groups are ionized. In such condition, an equilibrium between aqueous and precipitate Mn was observed. Precipitation immediately occurred for samples at pH 3 and 5, and to a larger extent at pH 5. At pH 7, glyphosate net charge is 2- and negligible brownish precipitate was found. Zeta potential values for samples at pH 2.5, 5 and 7 were close to neutrality, which explains that the suspended particles were within the micrometric size range (Table 2.3). The hydrodynamic diameter and zeta potential decreased as the pH increased.

Table 2.3. Zeta potential, polydispersive index and hydronaymic diameter for suspended precipitates' particles as function of pH

pH	**Zeta potential** **mV**		**Polydispersive index**		**Hydrodynamic diameter** **nm**	
	Mean value + standard error (n=6)					
2.5	1.9	± 1.6	0.25	± 0.06	4000	± 600
5.0	-2.4	± 0.5	0.40	± 0.18	3600	± 1100
7.0	-7.8	± 1.1	0.5	± 0.2	2200	± 700

Figure 2.9 shows the XANES spectra recorded for liquid phase recovered after centrifugation of $MnSO_4$-glyphosate mixture as function of pH. Under pH 1-5 the Mn^{2+} is in the same structure; the only difference was observed for pH 7 that shows a slight witheline intensity reduction. These findings are in agreement with Bernards et al. (2005) that showed that EPR spectra for $MnSO_{4(aq)}$ and $MnSO_{4(aq)}$ + glyphosate were identical in pH ranging from 2.8 to 4.5. Under pH 6-7.5 they reported reduction on EPR signal amplitude which would be associated with the displacement of water and coordination by the glyphosate.

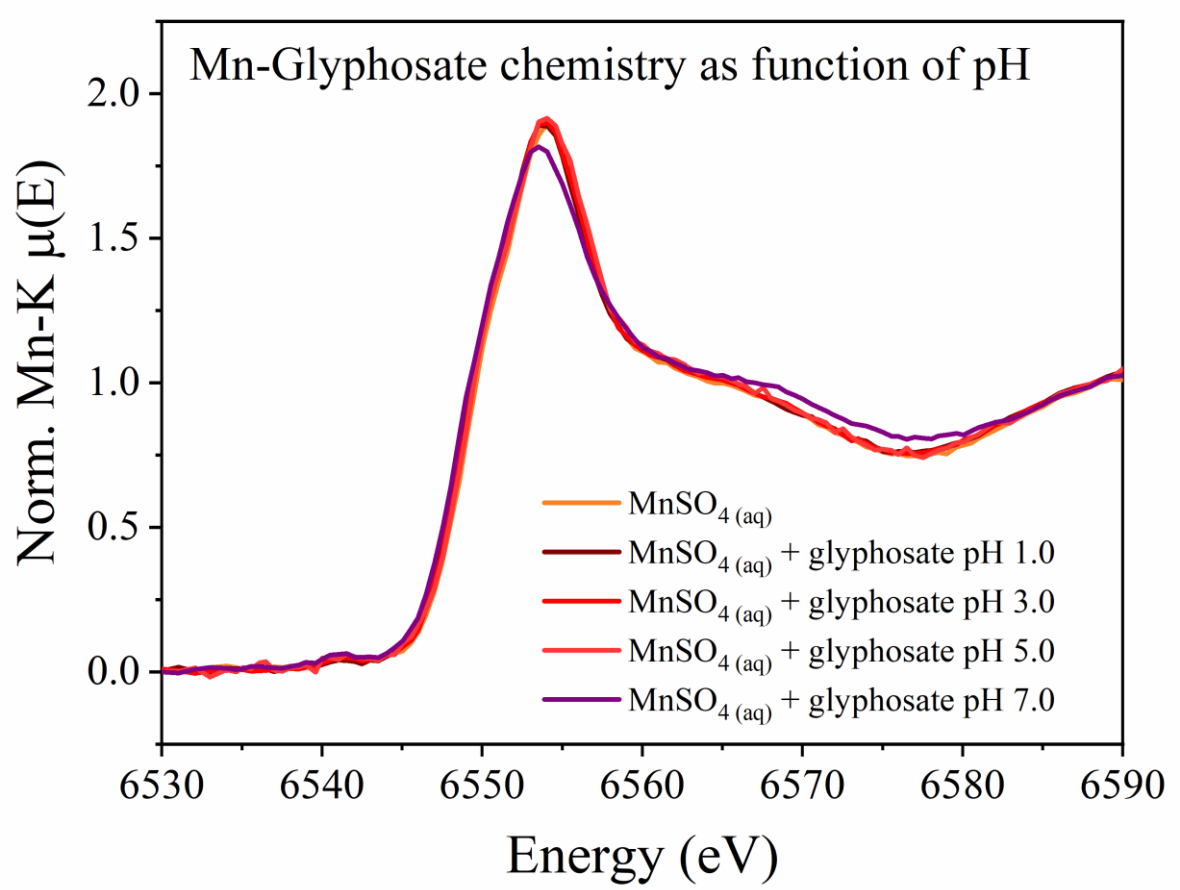

Figure 2.9. XANES spectra recorded from $MnSO_{4(aq)}$ + glyphosate supernatants at pH ranging from 1 to 7. Data show different spectral features only at the pH 7, while the other pH conditions offer the same one Mn chemical environment

The values of attenuation coefficient found for the non-normalized XANES spectra ofe $MnSO_4$ + glyphosate solutions in ranging pH (Figure 2.10) reflects the concentration of Mn in solution, are consistent with the observed precipitation yield in Figure 2.11, ie. most of the Mn-glyphosate precipitated at pH =5. The brownish suspended particles were not detected during by XANES since they were precipitated during the centrifugation.

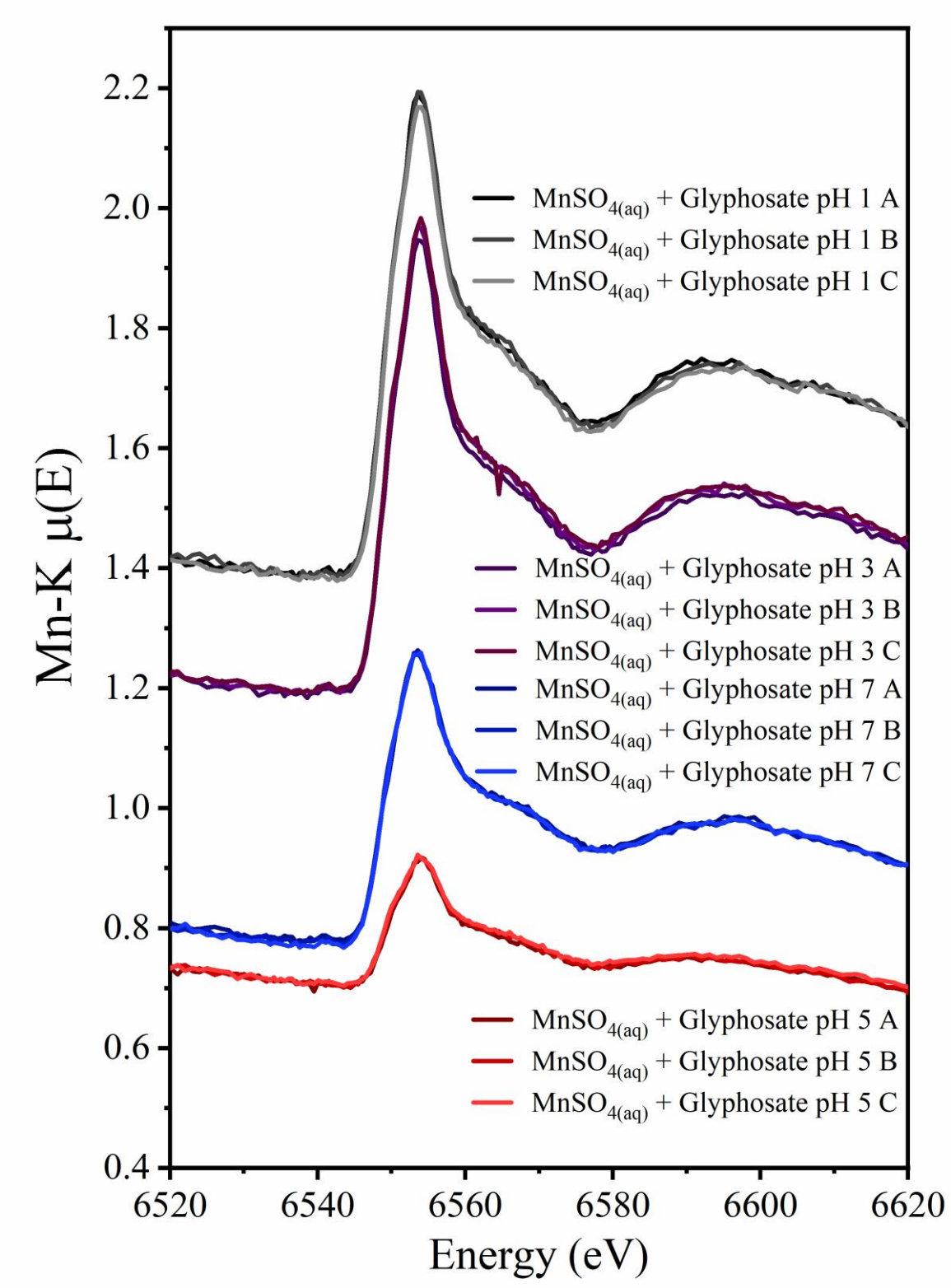

Figure 2.10. Non-normalized XANES spectra recorded for the MnSO4(aq) + Glyphosate solutions in pH ranging from 1 to 7

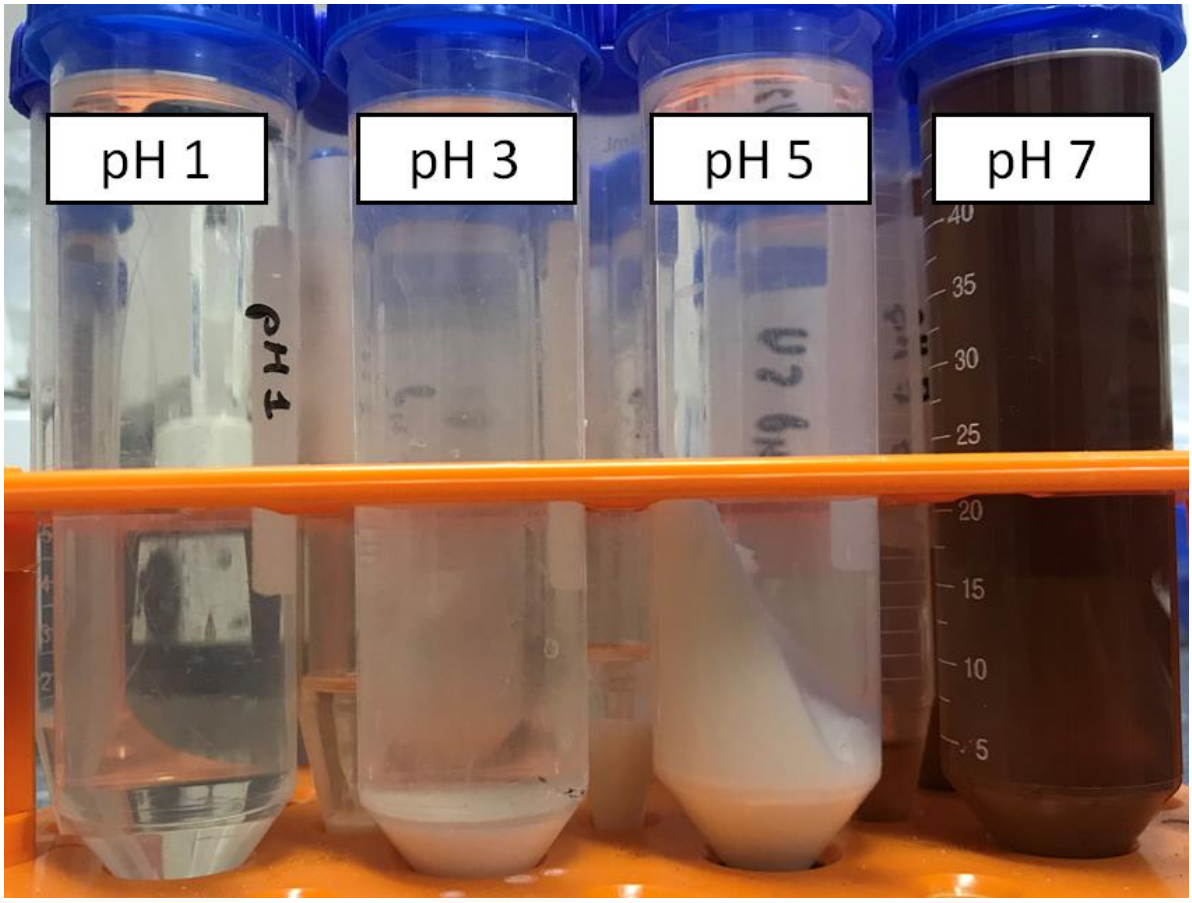

Figure 2.11. MnSO4(aq) + Glyphosate solutions in pH ranging from 1 to 7. Precipitation occurred for samples at pH 3 and 5, at larger extent for pH 5. At pH 7, the mixture turned brownish

The XANES spectrum recorded for the supernatant in pH 7 did not show change of Mn^{2+} redox state. However, the thermogravimentric analysis (Figure 2.12) shows a weight loss between 350-400 ºC, which indicates the occurrence of dihydroxylation and the loss of SO_4 between *ca*. 730-1060 ºC. This is consistent with Figueira, Angélica and Scheller (2008) that reported 10 wt.% weight loss between 350-400 ºC for manganite (γ-MnOOH), with an endothermic peak around 370 ºC related to the oxidation of γ-MnOOH and β-MnO_2 (pyrolusite) formation. Hem (1963) also obtained brown or black precipitates when Mn solutions were adjusted to pH higher than 8, which is due to the formation of oxides, since such pH and oxidation-reduction potential (Eh) conditions comprehend the oxide-stability region. Hem (1963) also states that oxidation by air may occur at pH conditions above 8, which decreases the concentration of dissolved Mn in solution. The thermogravimetric curves obtained from the precipitate at pH 7 (Figure 2.13) is not conclusive about the structure of the Mn-glyphosate solid complex.

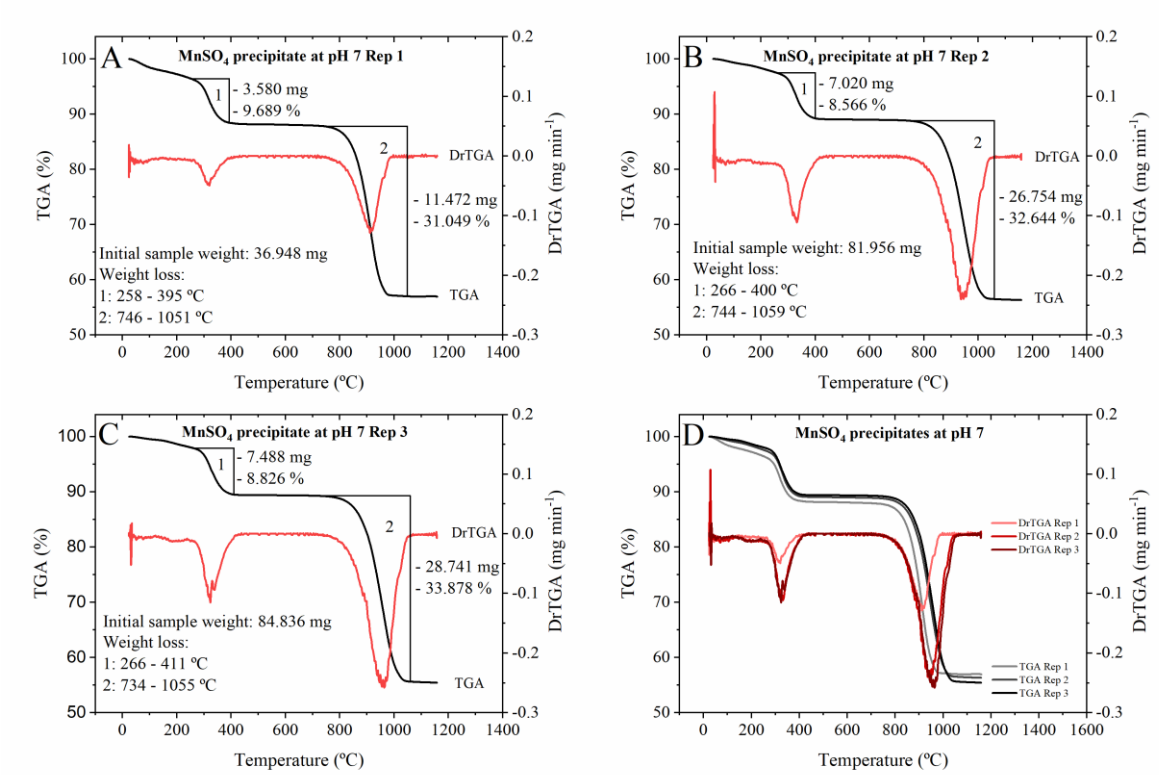

Figure 2.12. Thermal analysis (Gravimetric – TGA and Differential – DrTGA) for MnSO4 precipitates at pH 7. (A), (B) and (C) correspond to repetitions 1, 2 and 3, respectively, while (D) shows the merged thermal behavior of these three repetitions. Data show that the precipitate is composed by a mixture of MnO(OH) and SO42-. The weight loss observed between 300 and 400ºC indicates dehydroxylation, while the weight loss between 950 and 1000ºC indicates the loss of SO42-

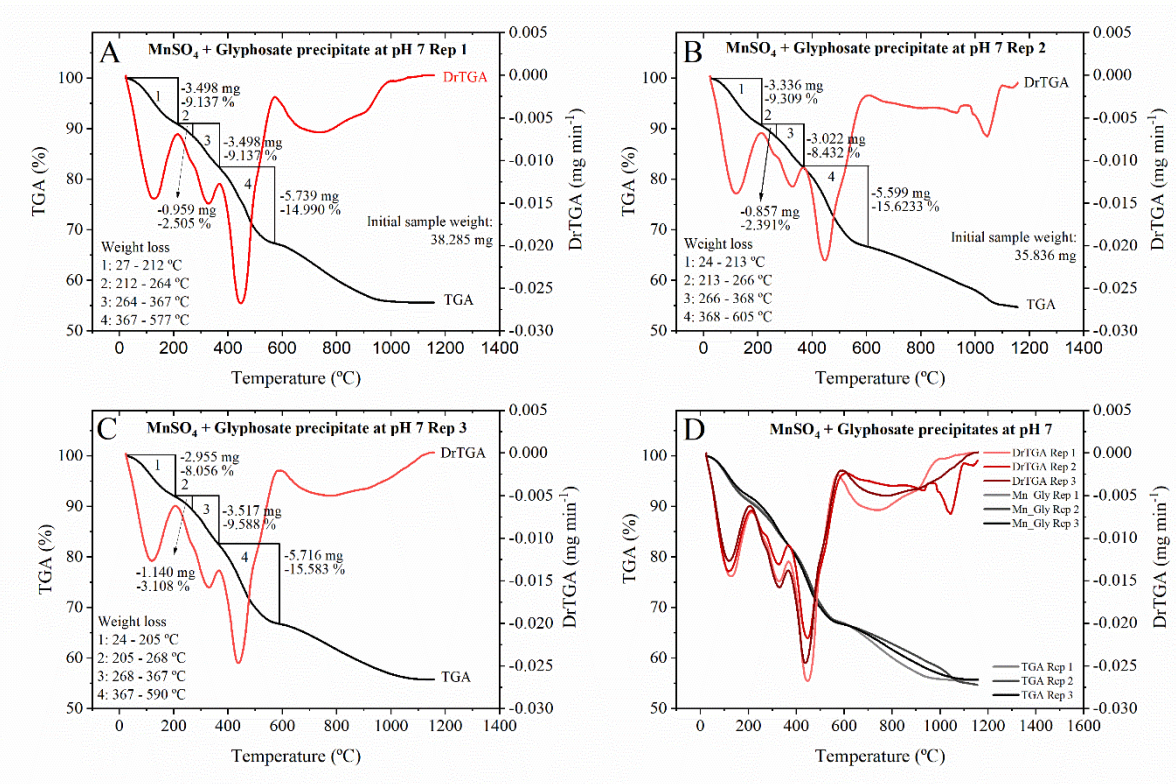

Figure 2.13. Thermal analysis (Gravimetric – TGA and Differential – DrTGA) for MnSO4 + glyphosate precipitates at pH 7. (A), (B) and (C) correspond to repetitions 1, 2 and 3, respectively, while (D) shows the merged thermal behavior of those three repetitions. Data show that the precipitate is composed by a mixture of MnO(OH) and Mn-glyphosate complexes. The weight loss observed between 300 and 400ºC indicates dehydroxylation, while the weight loss observed under other temperatures may be due to the volatilization of glyphosate compounds

Before ending the discussion about the results on glyphosate and Mn interactions, it is worth mentioning that there are several kinds of glyphosate formulations available. Some of them present distinct active ingredients, like isopropylamine salt of glyphosate (glyphosate-IPA) or potassium salt of glyphosate (glyphosate-K), for example. Others present the same active ingredient, but with distinct formulants employed for the product stabilization. The formulants are often composed by surfactants, diluents or adjuvants, but companies do not need to declare their composition, because this information is considered confidential business (DEFARGE; SPIROUX DE VENDÔMOIS; SÉRALINI, 2018). The glyphosate formulation employed in this study is a glyphosate-IPA based, with 48 wt% of active ingredient, 36 wt% of acid equivalent and 68.5 wt% of formulants. The formulants are considered inert ingredients according to the product's label. Recent studies found that some non-declared formulants of glyphosate based herbicides can be even more toxic than glyphosate itself (DEFARGE; SPIROUX DE VENDÔMOIS; SÉRALINI, 2018;

VANLAEYS et al., 2018). Given the unknown chemistry of the formulants, attention must be paid to the fact that different glyphosate brands may present distinct performances in tank mixtures.

2.4 Conclusions

This study showed that the Mn source and the solutions' pH matter when preparing spray mixtures with glyphosate. Glyphosate can form low solubility complexes with free Mn^{2+} ions in a pH ranging from 2.5 to 5. The complexes were found with a Mn:glyphosate molar ratio of nearly 2:1.

Features found in the XANES spectra showed same chemical environment for the precipitates, regardless of the employed Mn source, and suggest the non-formation of soluble Mn-glyphosate complexes. Further studies using the X-ray diffraction and extended absorption fine structure shall be conducted aiming at unraveling the structure of Mn-glyphosate solids.

Our results did not support the hypothesis of a soluble inner sphere Mn-glyphosate complex under pH 1-7. For sake of clarity, it is important mentioning that due to intrinsic errors introduced during the normalization of the spectra, it would be unlikely that the XANES spectra detect a fraction of soluble complex below 5 wt.% of the total manganese. Hence, if present, Mn-glyphosate soluble complexes in tank mixtures would be below *ca.* 5 wt.% concentration range.

The use of chelated fertilizers, such as Mn-EDTA, or the decreasing of the pH of the tank mixture to values < 2.5 are alternatives to circumvent the formation of precipitates.

3 X-RAY SPECTROSCOPY FOSTERING THE UNDERSTANDING OF FOLIAR UPTAKE AND TRANSPORT OF MN BY SOYBEAN (*Glycine max* L. Merril): KINETICS, CHEMICAL SPECIATION AND EFFECTS OF GLYPHOSATE

Abstract

Increasing soybean yield is a humankind challenge dependent on several management practices, such as fertilizing and weed control. While glyphosate contributes to controlling weeds, it can interfere with spray mixtures stability and, supposedly, complexing with micronutrients within the plant tissue. This study investigated the effects of glyphosate on soybean foliar uptake and transport of Mn supplied as MnSO4, MnHPO3, Mn-EDTA, and MnCO3. Those fertilizers induced ultrastructural changes on the leaf cuticle, regardless of glyphosate mixture. Except for MnCO3, all tested sources increased Mn content in the petiole. The mixture of glyphosate impaired Mn transport from MnSO4 and MnHPO3, but no evidence of Mn-glyphosate complexation within the plant was found. Manganese is rather transported in a similar chemical environment regardless of the source, except for Mn-EDTA, which was absorbed and transported in its pristine form. Interferences of glyphosate seem related to complexations in the tank mixture rather than affecting nutrients' metabolism.

Keywords: *Glycine max*, manganese, glyphosate, foliar uptake, XRF, XANES, SEM

3.1 Introduction

Foliar fertilization is a target-oriented way to supply micronutrients to crops. It can even be more environmentally friendly and cost-effective than soil broadcasting since small amounts of nutrients are directly applied to the plant. Thereby, foliar fertilization reduces fertilizer losses and the environmental impact caused by nutrient leaching or adsorption to soil colloids (FERNANDEZ; EICHERT, 2009). Additionally, the spraying of foliar fertilizers is usually carried out together with fungicides, herbicides, insecticides, and overhead irrigation that allow to split the doses and further facilitate the application of nutrients and reduce their cost (FAGERIA et al., 2009). For instance, optimal soybean yield was obtained by Mn foliar application at a rate of 0.1 kg Mn ha^{-1}, which is more advantageous than soil fertilization that reached optimal yield by applying 14 kg Mn ha^{-1} (MASCAGNI; COX, 1985).

Manganese (Mn) is available to plants as Mn2+, this is the more abundant form in most acidic, neutral and moderate alkaline soils. At higher pH soils, however, Mn is more prevalent in other forms, such as Mn (III) and Mn (IV), which plants cannot absorb and transport. Manganese is essential for plant metabolism, development and occurs in approximately 35 enzymes of a plant cell, serving as catalytically active metal or playing a role in activation of enzymes (SOCHA; GUERINOT, 2014) Foliar application of Mn increased the grain yield of soybean (ALT et al., 2018), wheat (KARIM et al., 2012), and cotton (SAWAN et al., 1993). It also increased the Fe content in wheat grain (STEPIEN; WOJTKOWIAK, 2016), mitigated the detrimental effects caused by salinity in green gram (SHAHI; SRIVASTAVA, 2018) and improved the chlorophyll content in cotton (DORDAS, 2009).

Despite the positive effects of foliar fertilization, Mn mobility within leaf tissues can vary according to the source, being the major limitation for fertilizer effectiveness in crops. The most common Mn sources are water soluble sources such as sulfates (MnSO4), chelate (Mn-EDTA), nitrate (Mn(NO3)2) and chloride (MnCl2) (FAGERIA et al., 2009; 2012). Less soluble sources such as oxides (MnO) and carbonate (MnCO3) are also marketed.

Glyphosate [N-(phosphonomethyl)glycine] is the most used herbicide worldwide. Its mechanism of action involves the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), whose absence is responsible for reducing the synthesis of aromatic amino acids, auxins, vitamins, as well as a number of key metabolites that causes growth inhibition in plants and some microorganisms, but not in animals (STEINRÜCKEN; AMRHEIN, 1980). Glyphosate has also the ability to interfere with the plant nutrient status by increasing or decreasing the uptake and translocation of nutrients (CAKMAK et al., 2009).

Although glyphosate is an herbicide, it does not kill the Roundup ReadyTM transgenic soybean. This plant is glyphosate-resistant thanks to its insensitive 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme which was transferred from a strain of *Agrobacterium* (HINCHEE et al., 1988). The technology was first introduced in North America in 1996. Mixtures of glyphosate with Mn and/or other micronutrients are common practices to control weed species while correcting plant nutritional deficiencies. Although tank mixtures are not ideal because of the interaction between the products, they decrease farmers' costs since multiple pulverizations can be combined in a single one.

Based on electron paramagnetic resonance recorded in solution (pH ranging from 2.8 to 7.5), a study suggested that glyphosate-Mn complexation could be responsible for nutritional deficiency (BERNARDS et al., 2005). Some marked attenuations in the spectral

amplitude of the solutions were found at pH 6 and 7.5, which was attributed to the formation of a glyphosate-Mn complex in tank mixtures. Once these pH values comprise the same pH range found in plant cells, it was proposed that these complexes may not just be formed in tank mixtures, but it could extend to the cytoplasm, where glyphosate could complex Mn^{2+} ions as well.

The combination of X-ray fluorescence spectroscopy (XRF) and X-ray absorption spectroscopy (XAS) arises as an alternative to investigate whether glyphosate can complex or not the Mn within the plant tissue. Once they are non-destructive analytical tools that determine the concentration, distribution and chemical environment of elements, they provide the possibility of real time assays in hydrated tissues, at room temperature and pressure (KOPITTKE et al., 2018). Since these techniques require minimum or none sample preparation, they allow studying the plants under in vivo conditions. Ultimately it grants the possibility of monitoring biochemical processes while they are happening.

Therefore, this study aimed at comparing the foliar absorption and transport of Mn in soybean leaves exposed to four Mn sources (MnSO4, Mn-EDTA, $MnCO_3$, and $MnHPO_3$) with and without the addition of glyphosate. In vivo transport kinetics were measured by XRF whistle X-ray absorption near edge spectroscopy (XANES) revealed the chemical environment of the Mn during its transport from leaves. Finally, scanning electron microscopy (SEM) demonstrated the effects of the spray mix on the anatomy of the leaf surface.

3.2 Material and Methods

3.2.1 Plant Growth and Treatments

Soybean plants (*Glycine max* (L). Merril cv M7739 IPRO, Monsoy) were grown in pots with sand in a growth room, where the temperature was kept at ± 27ºC, with air relative humidity at 80% and photoperiod of 12 h at photon flux of 250 µmol m^{-2} s^{-1}, provided by led lamps (6500 K).

The plants were daily irrigated with deionized water, and a Hoagland's nutritive solution was applied once a week to provide nutrients for appropriate development. The treatments were foliar applied when the plants reached the V3 phenological stage (with two trifoliate leaves completely expanded).

The treatments consisted of foliar application of aqueous solutions/dispersion of the following Mn sources: $MnSO_4.H_2O$ (31.85 wt% Mn, Synth), $MnCO_3$ nanopowder 80-100 nm (47.78 wt% Mn, Nanoshel), Mn-ethylenediamine tetraacetic acid (Mn-EDTA) (13 wt% Mn, StollerTM) and Mn-phosphite ($MnHPO_3$) (8 wt% Mn, AgrivalleTM), mixed or not with glyphosate (48 wt% active ingredient and 36 wt% acid equivalent, AmeribrásTM).

3.2.2 Aqueous solutions/dispersion preparation

Resende (2004) recommends 350 g ha^{-1} of Mn, while using 1.5 L ha^{-1} of glyphosate is the supplier recommendation for several types of weeds. Considering the application of 50 L ha^{-1} of spray mix ha^{-1} (commonly adopted by soybean growers), we prepared a solution employing 7 g Mn L^{-1} and 10.8 g of glyphosate (acid equivalent) L^{-1}, which corresponded to a molar ratio of 2:1 Mn/glyphosate.

For Scanning electron microscopy (SEM) analysis we employed the above-mentioned concentrations for Mn and glyphosate. For the in vivo transport kinetics and chemical speciation analysis, considering the employed methodology and limits of detection of such techniques, we employed solutions 1.6-fold more concentrated for both Mn (11.5 g L^{-1} of Mn) and glyphosate (18 g L^{-1} of acid equivalent), keeping the same Mn/glyphosate molar ratio. One can see in Table 3.1 the pH and electric conductivity of the treatments.

Table 3.1. pH and electric conductivity (EC) of the Mn sources, in the XRF/XAS experiments and in the SEM experiments

Treatment	XRF and XAS trials		SEM trials	
	pH	**EC (mS cm^{-1})**	**pH**	**EC (mS cm^{-1})**
$MnSO_4$	4.27	15.32	4.62	11.00
$MnSO_4$ + Glyphosate	2.68	18.62	2.59	14.04
Mn-EDTA	6.07	18.96	6.06	13.64
Mn-EDTA + Glyphosate	5.01	20.05	5.16	15.96
$MnHPO_3$	1.23	37.98	1.6	32.53
$MnHPO_3$ + Glyphosate	1.46	29.40	1.8	24.37
$MnCO_3$	6.53	0.37	6.55	0.17
$MnCO_3$ + Glyphosate	4.95	1.12	5.87	4.73

3.2.3 *In vivo* transport kinetics

At the V3 phenological stage (two fully expanded trefoils), soybean plants were settled in sample holders specifically designed to fit within the XRF equipment. The plants were positioned with the abaxial surface of the second trifoliate leaf upwards, then 0.07 mL of the spray mix was spread on the upper half of the abaxial face of the central leaflet using a cotton swab. The volume was adjusted by mass measurement.

The transport kinetics was monitored in vivo for 48 h, by measuring the Mn X-ray fluorescence emitted by the petiole of the treated leaflet. Measurements were made in the petiole to avoid interferences of the solid particles deposited over the leaflet and also to monitor the transport of nutrient which occurs through the phloem. XRF spectra were recorded 2 mm far from the leaf edge before and elapsed 1, 6, 12, 24, 36 and 48 h of the treatment application. The measurements were carried out in three biological replicates.

To perform the measurements, the plants were loaded within the Orbis PC X-ray spectrometer (ORBIS PC, EDAX, USA). X-ray was provided by a rhodium anode operating at 36 W. To improve the signal to noise ratio, a 250 µm thick primary Al filter was employed. A 1 mm wide X-ray beam shaped by a collimator was centered in the petiole of the treated leaflet, with a dwell time of 360 s (three shots of 120 s each) and a dead time smaller than 2%. The X-ray fluorescence photons were detected by a 30 mm^2 silicon drift detector.

The Mn-Kα intensity was normalized by the Rh-Kα intensity to determine the relative Mn content. Aiming at avoiding scattering of Mn photons from the deposited fertilizers, the leaves were covered with a 1 mm thick Pb foil. The number of Mn photon counts was normalized by the Rh photon counts. Elemental and Compton intensities were normalized by their corresponding maximum intensity.

3.2.4 Statistical analysis

Data collected 48 h after the application of the treatments were submitted to statistical analysis, in order to investigate if the fertilizers were absorbed, and whether the addition of glyphosate affected the manganese transport from the tested sources. Since the data did not show a normal distribution, they were analyzed using the non-parametric method of Kruskal-Wallis, which is a one-way analysis of variance by ranks. When the value of the Kruskal-Wallis test was calculated as statistically significant, the Nemenyi method of pairwise multiple comparisons was performed among the groups to locate the source of significance. All tests were applied at 5 % of significance.

3.2.5 Radiation damage assessment

A soybean plant at V3 phenological stage was settled in a sample holder as described above. Then, the plant was loaded within the Orbis PC X-ray spectrometer (ORBIS PC, EDAX, USA), where it was irradiated by a 40.5 W polychromatic 30 µm focused beam. In this experiment, no primary filter was employed. The plant petiole was exposed to ten successive shots of 120s each in the same spot. Rhodium Kα Compton scattering and potassium Kα fluorescence intensities were recorded to investigate possible damages caused by the radiation. Elemental and Compton intensities were normalized by their corresponding maximum intensity.

Simultaneously to Mn-K XANES measurements, we also recorded the fluorescence intensities for potassium Kα and the scattered radiation.

3.2.6 Chemical speciation

The Mn chemical environment during the redistribution process was monitored in vivo using X-ray absorption near edge structure (XANES) at Mn-K edge. The measurements were performed at the XRF beamline at the Brazilian Synchrotron Light Laboratory (LNLS). This station was equipped with a bending-magnet, Si (111) double crystal monochromator, KB mirror system resulting in a *ca.* 20 x 20 μm^2 diameter spot size and silicon drift detector (SDD; KETEK GmbH, Germany).

The treatments, 0.14 mL of spray mix, were spread on the upper half of the central leaflet of the older trifoliate leave, on both adaxial and abaxial surfaces. The measurements at the petiole were made at fluorescence geometry about 24 h after the application of the treatments. To avoid the backscattering of Mn fluorescence photons, the treated leaflet was covered with an aluminum foil. In order to prevent radiation damage in plant tissues, three points were analyzed for control plants and four points for treated plants. Since the beam size was ca. 20 x 20 μm^2, the points were spaced 0.1 mm from each other (Figure 3.1). We performed only one XANES scan per point, each required *ca.* 35 min to be accomplished. Between three to four XANES spectra were merged to improve the signal-to-noise ratio. The measurements were carried out in two biological replicates.

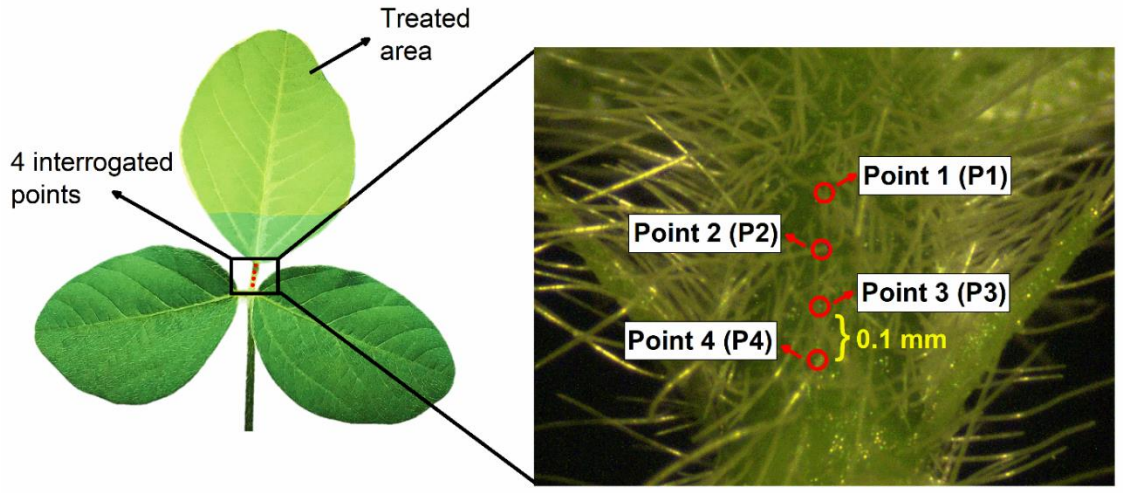

Figure 3.1. Scheme of the points where the XANES spectra were recorded. The treatments were applied on both abaxial and adaxial surfaces of the central leaflet, and the measurements were carried out on the petiole. Three points were analyzed for control plants and four points for treated plants. Since the beam size was *ca.* 20 x 20 μm^2, the points were spaced 0.1 mm from each other. One scan per point was performed, in a period of *ca.* 35 minutes each

Spectra for aqueous $MnSO_4$ (pH=4.62), Mn-EDTA (pH=6.06), and Mn-phosphite (pH=1.60) at 0.7 Mn wt% were recorded as reference compounds. The aqueous reference compounds were placed in 1 mm thick acrylic cells, covered with Kapton^{TM} tape. These measurements were performed at transmission mode.

The spectra were merged, energy calibrated using a Mn foil and then normalized using the Athena software within the IFEFFIT package (RAVEL; NEWVILLE, 2005).

3.2.7 Scanning electron microscopy (SEM) analysis

The SEM analysis were performed to evaluate the effects of the application of Mn sources, with and without glyphosate, on the leaf tissue. A 5 μL droplet of each treatment was dripped on the adaxial surface of soybean leaves. Besides the treatments, we also performed the application of a 5 μL droplet of distilled water, diluted glyphosate and an aqueous solution of H_2SO_4. The application of the acid solution was done in order to evaluate the acidity effects on the leaf surface, so, we adjusted the pH of the solution to 1.40, which is a value close to the more acidic Mn source ($MnHPO_3$).

Twenty-four hours after the application, pictures of the leaves' surfaces were taken using a Hirox digital microscope. Samples were then fixed in Karnovsky solution (KARNOVSKY, 1965) and washed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) and washed three times in distilled water. After this process, they were dehydrated in graded acetones (10% 1×, 30% 1×, 50% 1×, 70% 1×, 90% 1×, 100% 2×, for 15 min each), dried up to the critical point (LEICA CPD 300, Wetzlar, Germany) (HORRIDGE; TAMM, 1969), glued on aluminum stubs and sputter coated with gold (BAL-TEC SCD 050, New York, USA), and finally examined with a scanning electron microscope (JEOL JSM IT 300, Tokio, Japan) at 15 kV and digitally recorded at a working distance of 15 mm.

3.3 Results and Discussion

3.2.1 Radiation damage assessment

Depending on the exposure time and power, X-ray radiation can damage the plant tissue and consequently induce spectral artifacts. Figure 3.2 shows features obtained through an assay performed to intentionally induce damages by radiation. Therefore, we exposed the

petiole to a *ca.* 30 µm diameter polychromatic 40.5 W X-ray beam for 20 minutes, without any filter. Hereafter, two features associated with radiation damage could be found: scorching of the irradiated spot, found 18 h after a 20 min exposure (Figure 3.2a and 3.2b), and decrease of potassium XRF intensity, beginning 4 min after the beam exposure (Figure 3.2c).

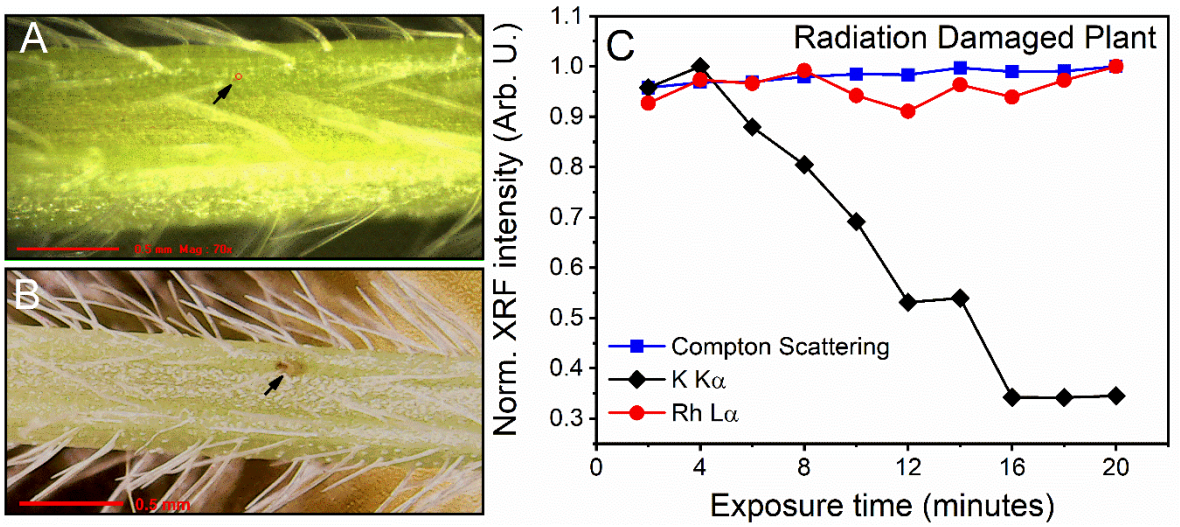

Figure 3.2. Radiation damage effects in soybean petioles exposed to a focused polychromatic X-ray beam with *ca.* 30 µm diameter (40.5 W without primary filter). Photograph (A) shows a soybean petiole immediately after 20 min of irradiation. The red circle indicates the irradiated area, no visual damaged was observed. Photograph (B) shows that scorching became visible past 18 hr since the irradiation. Figure (C) shows the XRF intensity of Compton scattering, rhodium Lα and potassium Kα; In addition to visual damages, potassium intensity decreases

Electrolyte leakage of cells is known as a plant response to several types of biotic and abiotic stresses. Potassium plays an important role in such leakage, whose efflux from plant cells can stimulate proteases, endonucleases, and even trigger programmed cell death (DEMIDCHIK et al., 2014). Therefore, this essay shows that monitoring K XRF intensity is an appropriate indicator to identify plants under stress by radiation. Note that depending on the dose, X-rays can damage living tissues. We believe that the decorrelation between the intensities of K Kα XRF and Rh Kα Compton scattering is related to difference in the volume probed by these two energies. We estimated that below 325 µm less than 1% of K Kα (3.31 keV) radiation would escape from the petiole (*ca.* 1000 µm thick). Conversely, more than 90% of Rh Kα Compton scattering (18.8 keV) would escape from 1000 µm depth. Hence, Rh Kα Compton was not enough sensitive to the water content.

Figure 3.3 presents the intensities for potassium and Compton scattering recorded during the Mn transport kinetic measurements. In this experiment, the irradiation conditions, i.e. 36 W, non-focused beam and the presence of a 250 µm primary Al filter, provided less harsh conditions for the petiole tissue. The data show that the intensities randomly oscillated during the analysis, without any trend. It is worth highlighting that the flux density of the 30 µm focused beam, without considering the absorption of the primary filter, is ca. 2400-fold higher than that of the 1 mm wide unfocused beam employed to monitor the transport of Mn. Hence, differently from Figure 1, there is no evidence of radiation damage while monitoring the transport of Mn.

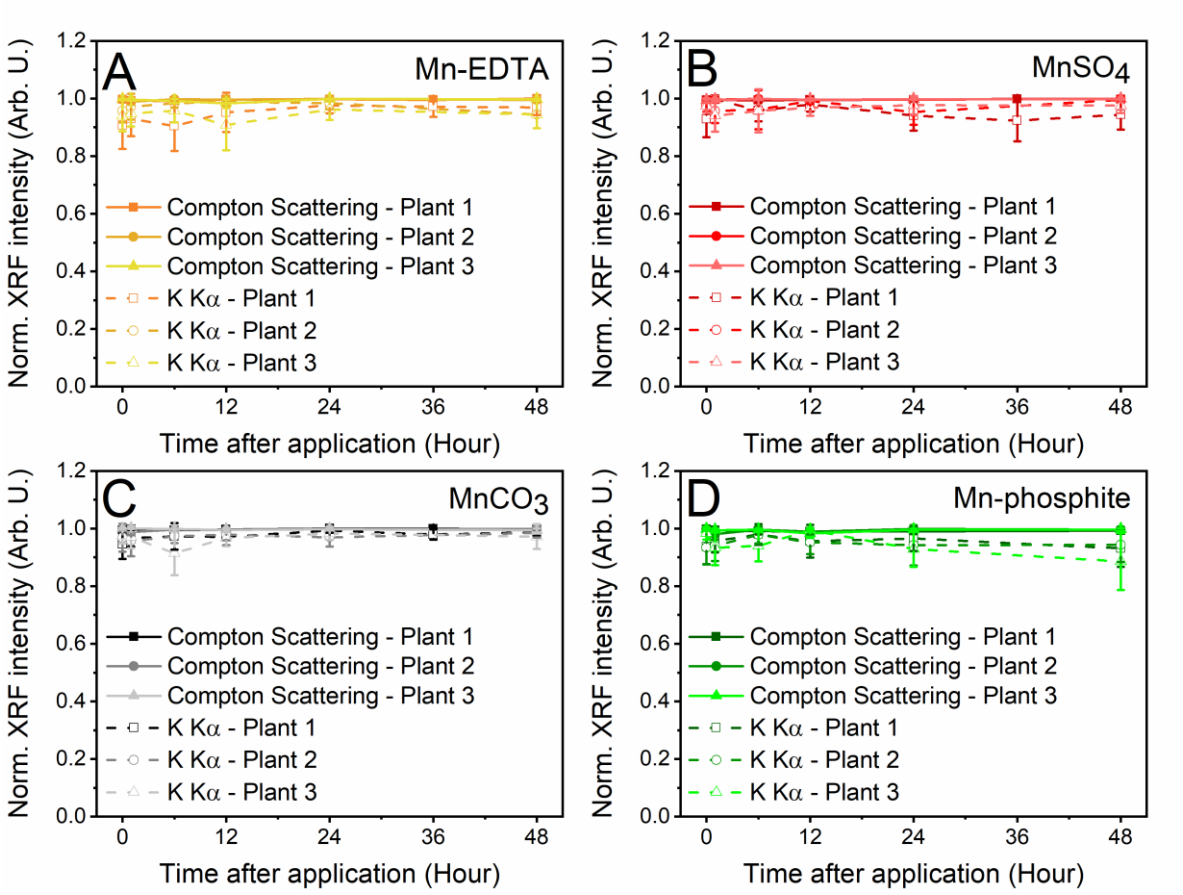

Figure 3.3. Compton scattering and potassium XRF intensities in soybean petioles whose leaves received the application of Mn based fertilizers. The petioles were irradiated for 6 minutes during each measurement (three shots of 2 min each), which was performed with a 1 mm wide polychromatic X-ray beam (36 W and 250 µm thick primary Al filter). By the end of the experiment, the plants were exposed to a total of 42 minutes to the X-rays beam. The plants were treated with (A) Mn-EDTA$_{(aq)}$; (B) $MnSO_{4(aq)}$; (C) $MnCO_{3(aq)}$; and (D) Mn-phosphite$_{(aq)}$. No evidence of radiation damage was found under the parameters employed in this study

Regarding synchrotron measurements, avoiding X-ray induced artifacts in biological samples under *in vivo* or fresh conditions is a major challenge. X-ray irradiation can promote

changes in the chemical environment of the analyte (SCHECKEL et al., 2004), dehydration (LOMBI et al., 2011) and scorching (SANISHVILI et al., 2011). Radiation damage was observed in the leaf tissue of Iberis intermedia that was analyzed *in vivo*. After consecutive XANES scans at the same spot, a significant reduction in the fluorescence intensity was noted.24 Even changes in the oxidation state can occur (KEMPSON; HENRY; FRANCIS, 2009). Arsenic speciation in rice grains also showed a decrease in As signal and reduction of As(V) to As(III), and further to As(0) (SMITH et al., 2009).

Even though synchrotron sources present X-ray brightness several orders of magnitude above laboratory anodes, XANES measurements are usually carried out using a monochromatic beam. It means the sample is subjected to a specific wavelength, in the present study the sample was scanned by the energy around the Mn K edge (6,539 eV).

To check the occurrence of radiation damage during the chemical speciation, a series of XANES spectra were recorded at three 0.1 mm distant points of the petioles of control plants and at four different points for the petioles used for chemical speciation analysis (Figures 3.1 and 3.4).

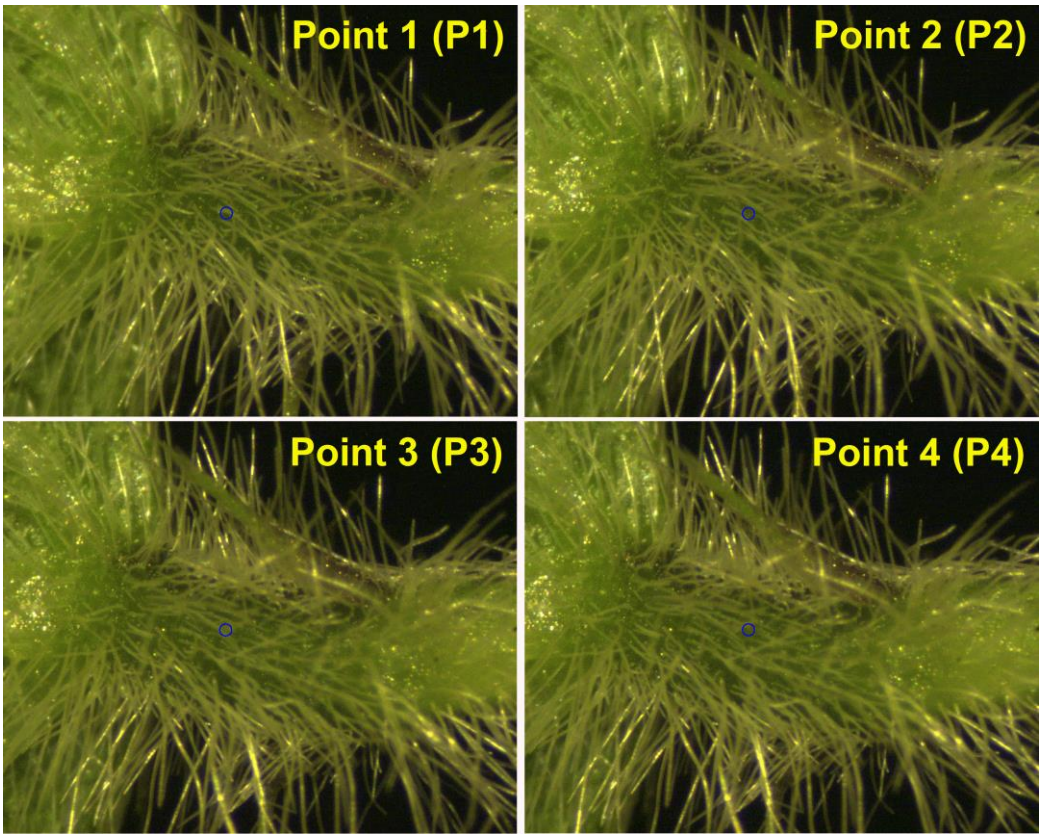

Figure 3.4. Pictures of the petiole of soybean plants. The blue circles indicate where the X-ray beam was probed. Any sign of scorching was observed

Figures 3.5a and 3.5 show the non-normalized Mn-K XANES spectra recorded for control plants. Despite the noisy spectra, due to the low Mn concentration in control plants, these measurements did not indicate any spectral changes that could cause redox reactions of Mn during the measurements.

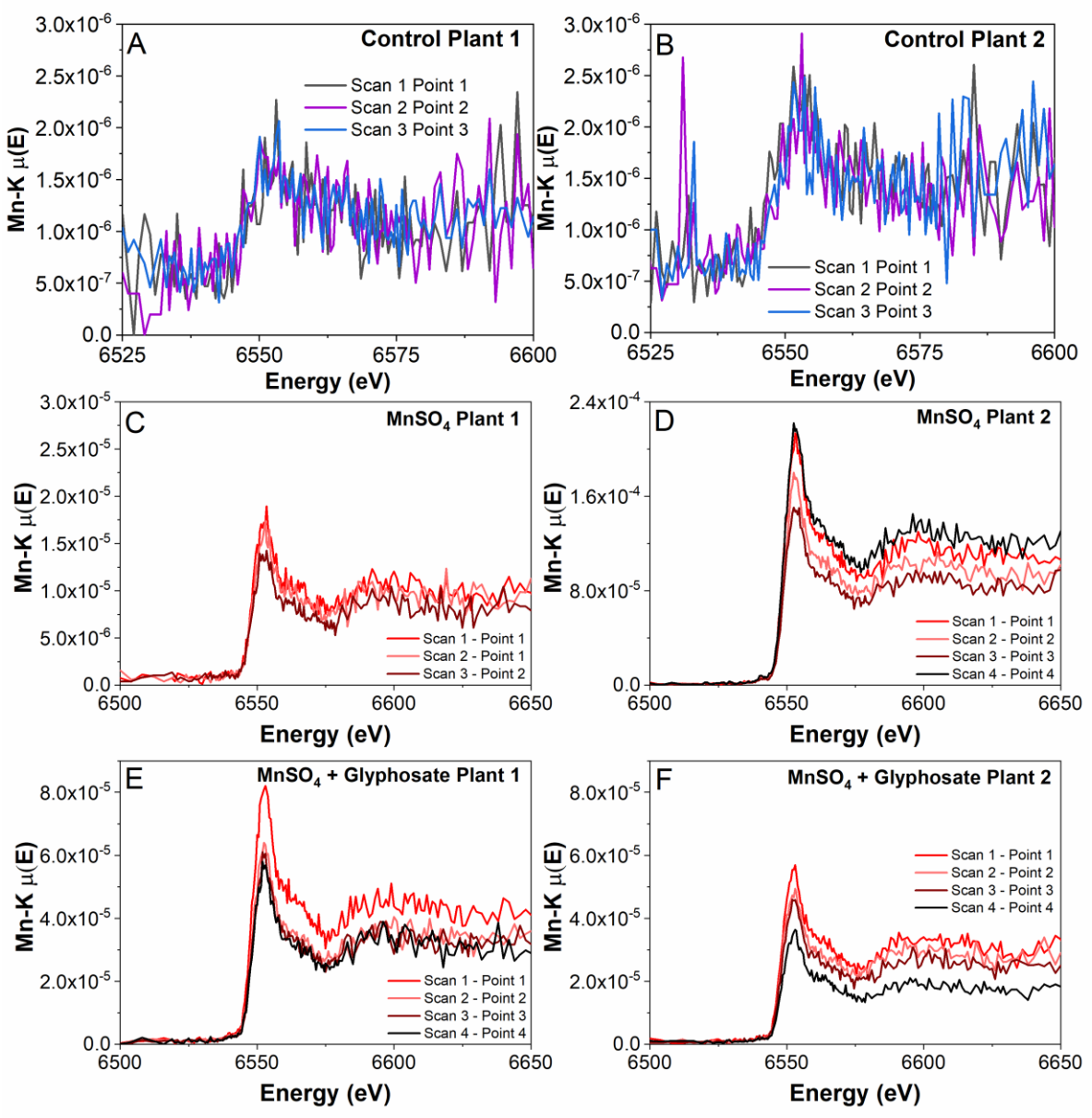

Figure 3.5. Non normalized Mn-K edge XANES spectra recorded at the petiole of control plants (A and B), and plants treated with $MnSO_4$ (C and D) and $MnSO_4$ + Glyphosate (E and F). Data show the number of scans performed per plant, as well as their investigated points. Despite the noise, no spectral changes were observed during the measurements

Additionally, Figures 3.5c to f and 3.6a to 3.6h present the non-normalized Mn-Kα edge XANES spectra recorded for the petioles of treated leaves. For some plants, we observe

that the XRF yield decreases from the leaf to stem direction. This might be a consequence of a dilution effect from the leaf that received the treatment to the stem where the ions will be unloaded.

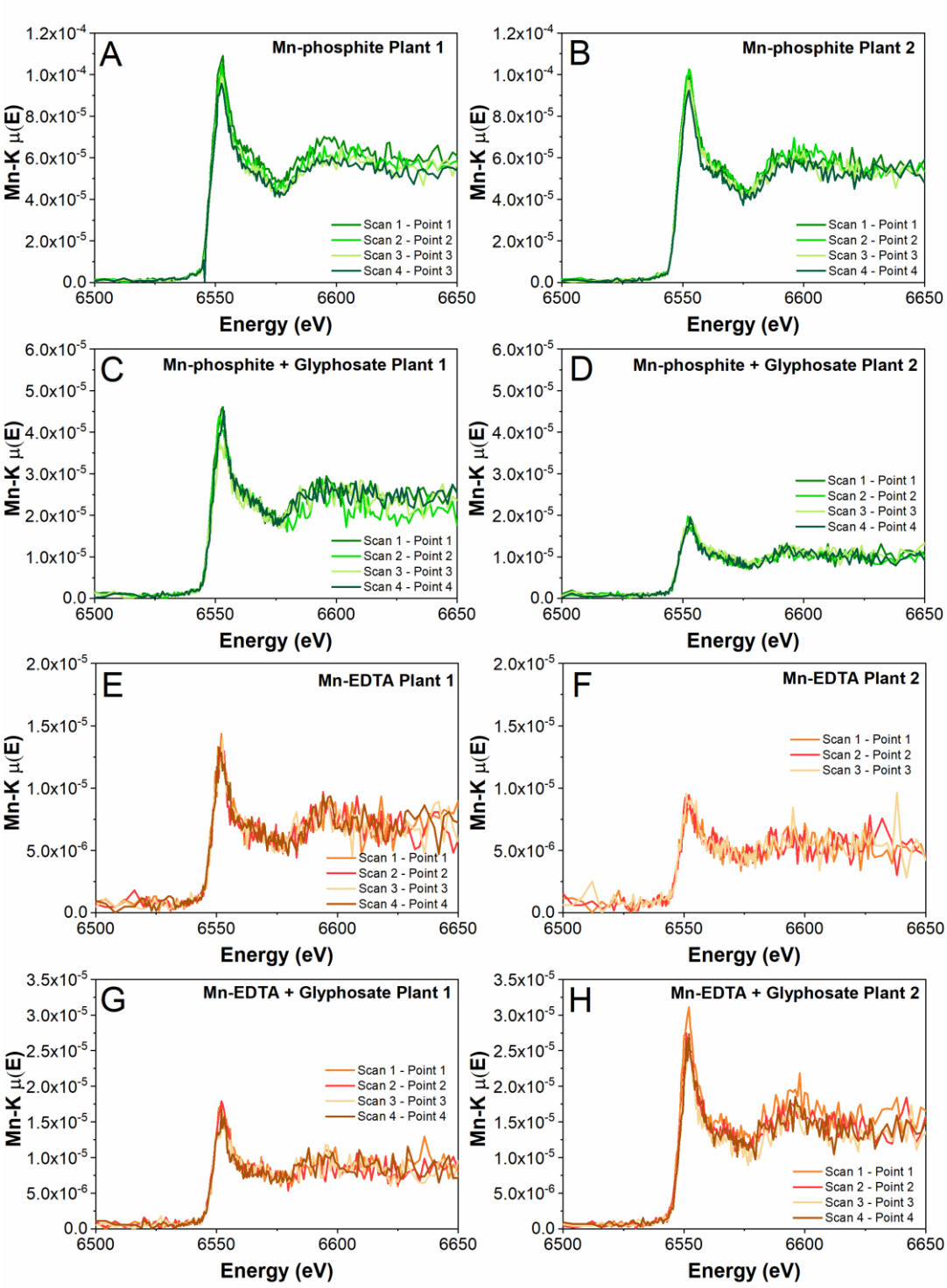

Figure 3.6. Non normalized Mn-K edge XANES spectra recorded at the petiole of plants treated with Mn-phosphite (A and B), Mn-phosphite + Glyphosate (C and D), Mn-EDTA (E and F) and Mn-EDTA + Glyphosate (G and H). Data show the number of scans performed per plant, as well as their investigated points, revealing no spectral changes during the measurements

Figure 3.7 presents the Compton scattering and potassium intensities recorded at petioles simultaneously to the XANES measurements. The data recorded at the XRF beamline of a second-generation synchrotron (2x108 ph s^{-1} mm^{-2} 100 mA-1) did not present potassium decreasing trend. Furthermore, differently from Figure 3.2, no signs of scorching are observable in the pictures of the points in which the X-ray beam hit the sample (Figure 3.4). Altogether, these results suggest that the X-ray beam provided by the XRF beamline of LNLS did not introduce artifacts to the chemical speciation.

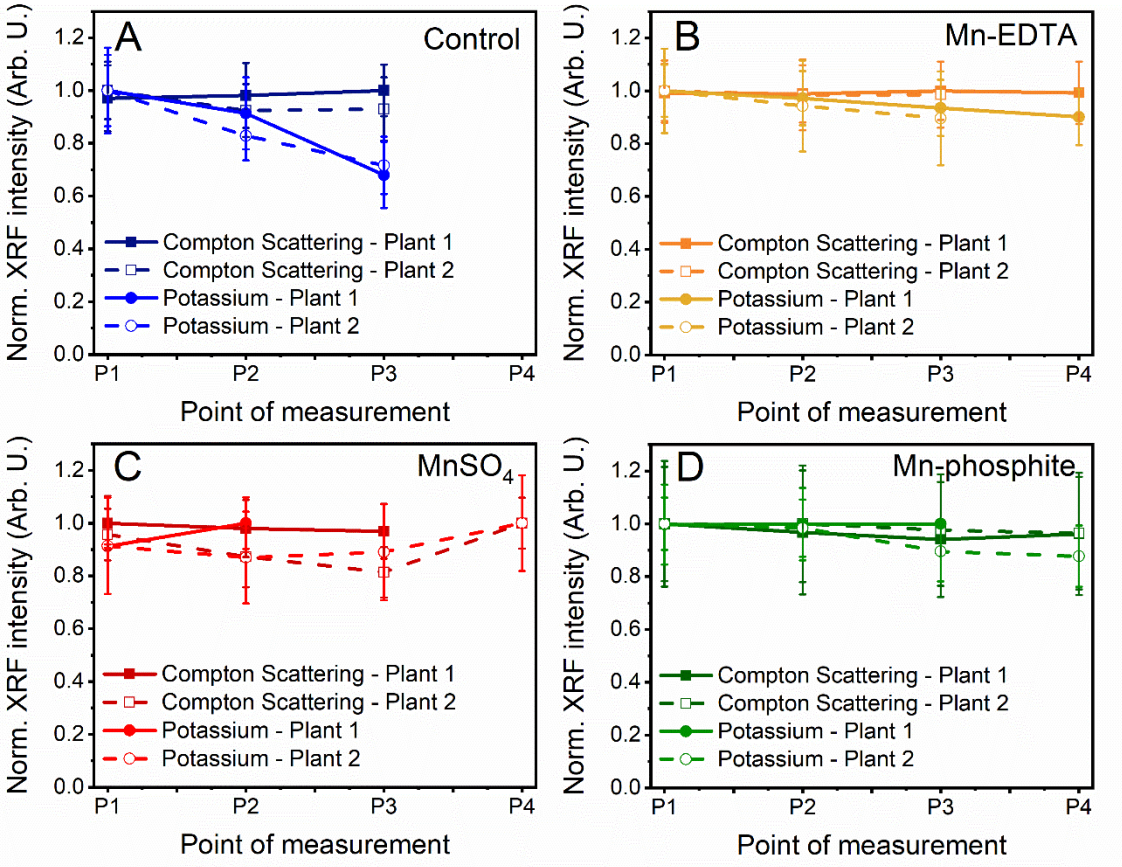

Figure 3.7. Mean Compton scattering and potassium intensity recorded at soybean petioles whose leaves received the application of Mn based fertilizers simultaneously to the XANES spectra. (A) two control plants; (B) two plants treated with Mn-$EDTA_{(aq)}$; (C) two plants treated with $MnSO_{4(aq)}$; and (D) two plants treated with Mn-$phosphite_{(aq)}$. The monochromatic beam neither influence the content of potassium nor the sample density (Compton scattering)

3.3.2 *In vivo* transport kinetics

Figure 3.8a presents a soybean leaf that received a Mn-containing treatment at the upper half of the abaxial surface of the central leaflet, whereas Figure 3.8b indicates the site of the petiole which was monitored by the X-ray beam. Figures 3.8c-f show the average content of Mn (n=3) as a function of time at the petioles of plants whose leaves were treated with Mn-phosphite$_{(aq)}$, $MnSO_{4(aq)}$, Mn-EDTA$_{(aq)}$, $MnCO_{3(aq)}$, respectively, with and without the addition of glyphosate, plus a control treatment. The Mn content was expressed as the number of Mn Kα photon counts normalized by the Rh Kα photon counts.

The results show that foliar application of Mn-phosphite$_{(aq)}$, $MnSO_{4(aq)}$ and Mn-EDTA$_{(aq)}$, with and without glyphosate, increased the Mn content in soybean petioles within the 48 h of analysis, while for $MnCO_{3(aq)}$, with and without glyphosate, the Mn content was low and close to the initial value.

The Mn-phosphite$_{(aq)}$ and $MnSO_{4(aq)}$ were the treatments that most increased the Mn content in the petioles. However, the addition of glyphosate decreased the amount of Mn transported from the leaves to other sink organs. Conversely, Mn-EDTA$_{(aq)}$ was not affected by the addition of glyphosate.

After 12 h the Mn content in the petioles of $MnSO_{4(aq)}$, $MnSO_{4(aq)}$ + glyphosate, Mn-phosphite$_{(aq)}$ and Mn-phosphite$_{(aq)}$ + glyphosate treated plants remained almost constant until reach the 48 h. Data showed high variability in the first hour of analysis for $MnSO_{4(aq)}$ treatments, but for Mn-EDTA$_{(aq)}$ treatments, the variability was high from the beginning to the end of the experiment.

µ-XRF analysis were previously employed to monitor *in vivo* root uptake of Mn, Fe, and Zn by common beans (*Phaseolus vulgaris*) (CRUZ et al., 2017; RODRIGUES et al., 2018). It also granted the investigation of mechanisms of sensitivity and tolerance to high Mn availability in the root environment of soybean, white lupin (*Lupinus albus*), narrow-leafed lupin (*Lupin angustifolius*), and sunflower (*Helianthus annuus*) (BLAMEY et al., 2015). XRF microanalysis traced the short-distance movement of Zn in tomato (*Solanum lycopersicum*) and citrus (*Citrus reticulatus*) leaves, and also to investigate Zn transport through mapping cross sections of treated sunflower leaves' petioles (TIAN et al., 2015). To the best of our knowledge, this is the first study employing such techniques to time-resolved monitoring the Mn transport through petioles of living soybean plants.

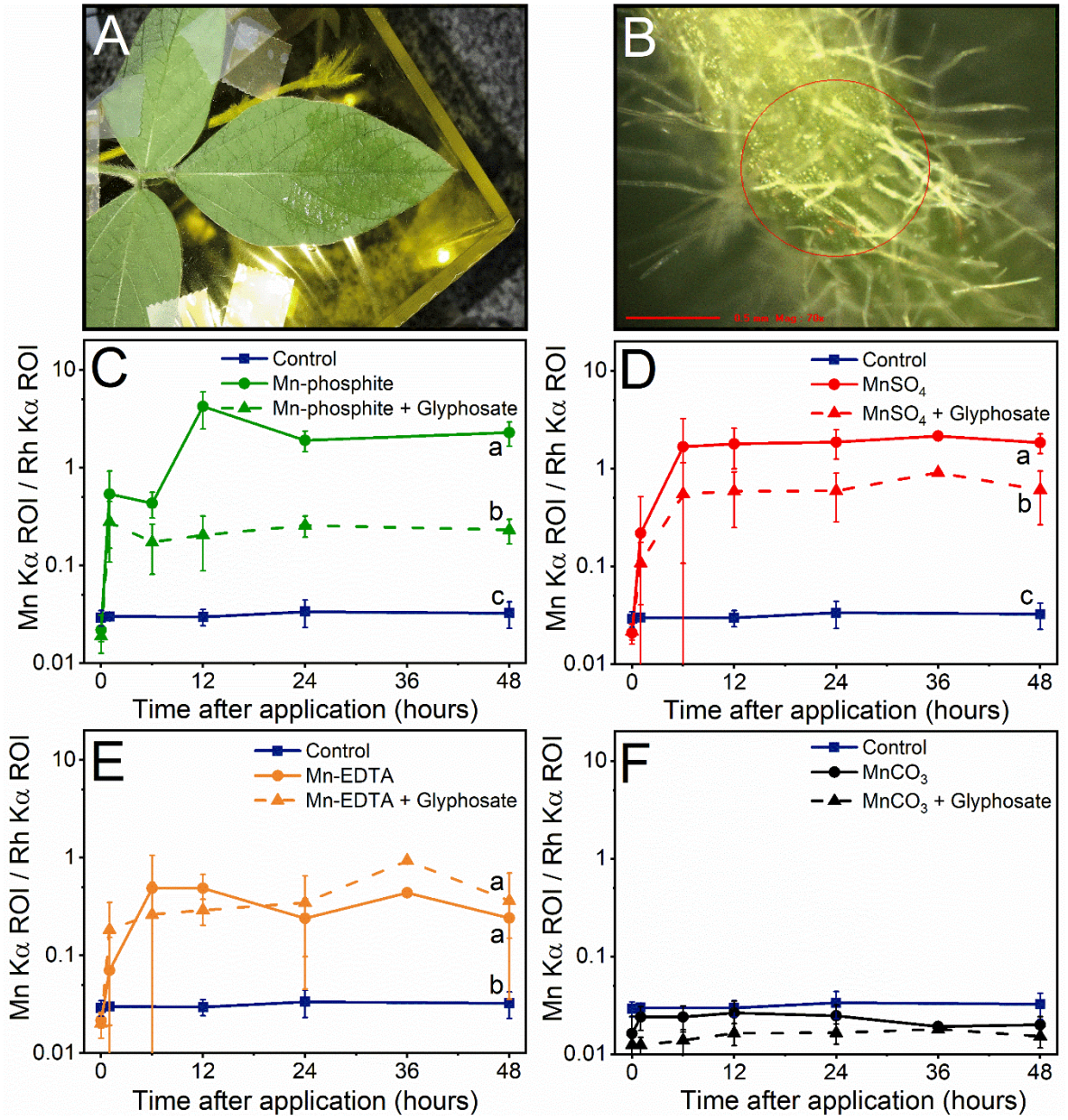

Figure 3.8. XRF monitoring the Mn intensity in the petiole of soybean plants as a function of time. The pictures illustrate (A) the treatment spread at the upper half of the abaxial surface of the central leaflet; (B) the petiole of the treated leaflet with the red circle indicating the region irradiated by the 1 mm X-ray beam; (C) the mean Mn content in the petioles of three soybean plants whose leaves were exposed to Mn-phosphite$_{(aq)}$, (D) $MnSO_{4(aq)}$, (E) Mn-EDTA$_{(aq)}$, and (F) $MnCO_{3(aq)}$. All treatments were also compared to the mean Mn content recorded for three control plants, which did not receive any foliar application. Different letters indicate significant differences regarding the Mn intensity in the petioles 48 hr after the application of the treatments ($p < 0.05$). Data show that, except for $MnCO_{3(aq)}$ with and without glyphosate, all treatments increased the Mn content at the petiole. Glyphosate decreased the Mn intensity in petioles treated with $MnSO_{4(aq)}$ and Mn-phosphite$_{(aq)}$

In agreement to our results, the evaluation of cuticular penetration of $MnSO_4$, Mn-EDTA, and $MnCO_3$ in isolated cuticles of tomato showed the highest penetration for soluble sources and the lowest for $MnCO_3$ (ALEXANDER; HUNSCHE, 2016). Additionally, for the same type of cuticle, higher foliar sorption was found for $MnSO_4$ than Mn-EDTA, but further translocation in the tissues was much higher for the chelated form (FERRANDON; CHAMEL, 1988). $MnSO_4$ was also more effective than Mn-EDTA to increase leaf Mn concentration in orange trees (PAPADAKIS et al., 2005) and sugar beet (LAST; BEAN, 1991).

The reduced Mn content in the petioles in plants treated with Mn-EDTA could be associated to a negative net charge of the complex, since the cuticle itself is negatively charged (FERNANDEZ; EICHERT, 2009; FERRANDON; CHAMEL, 1988). By analogy with zinc complexes (SINHA; PRASAD, 1977), one can expect that the diffusion coefficient of Mn-EDTA to about half of that for $MnSO_4$. These combined factors contributed to hindering the movement of the chelate within the cuticle pores.

In soybean that received foliar application of commercial fertilizer containing Mn, the addition of glyphosate affect neither the absorption nor the foliar Mn concentration (BASSO et al., 2011; CORREIA; DURIGAN, 2009). Nonetheless, from a contrary point of view, there is evidence that Mn applied as foliar fertilizer has the potential to interfere in glyphosate efficacy and reduce weed control. As Figure 3.8 shows, the interaction between Mn and glyphosate, and therefore Mn fate regarding absorption and transport, depends on the type of Mn source. In velvetleaf, foliar applied Mn-EDTA did not impair glyphosate efficacy, absorption, and translocation, but these factors were significantly reduced by MnSO4 (BERNARDS et al., 2005).

3.3.3 Chemical speciation

XAS was already employed to investigate the Mn chemical form in rice leaves (WATANABE et al., 1990), soybean, cowpea, sunflower and white lupin leaves from plants intoxicated by Mn (BLAMEY et al., 2018), and leaves of Mn hyperaccumulator woody species (FERNANDO et al., 2010; HERNDON; MARTÍNEZ; BRANTLEY, 2014). Additionally, living plants were already submitted to μ-XANES analysis to determine the speciation of thallium in leaves of Iberis intermedia (SCHECKEL et al., 2004).

Figure 3.9 presents the XANES spectra recorded for the reference compounds $MnSO_{4(aq)}$, $MnHPO_{3(aq)}$ and $Mn\text{-}EDTA_{(aq)}$. The spectral features of $MnSO_{4(aq)}$ and $MnHPO_{3(aq)}$ indicate that they present a similar structure, while Mn-EDTA(aq) presented a whiteline shift towards lower energy and the presence a defined second absorption minimum at 6,564 eV. The spectral resemblance between $MnSO_{4(aq)}$ and $MnHPO_{3(aq)}$, combined to the ionic nature of these compounds, suggest that they form aqueous outer sphere complex while it confirms the high stability of Mn-EDTA chelate.

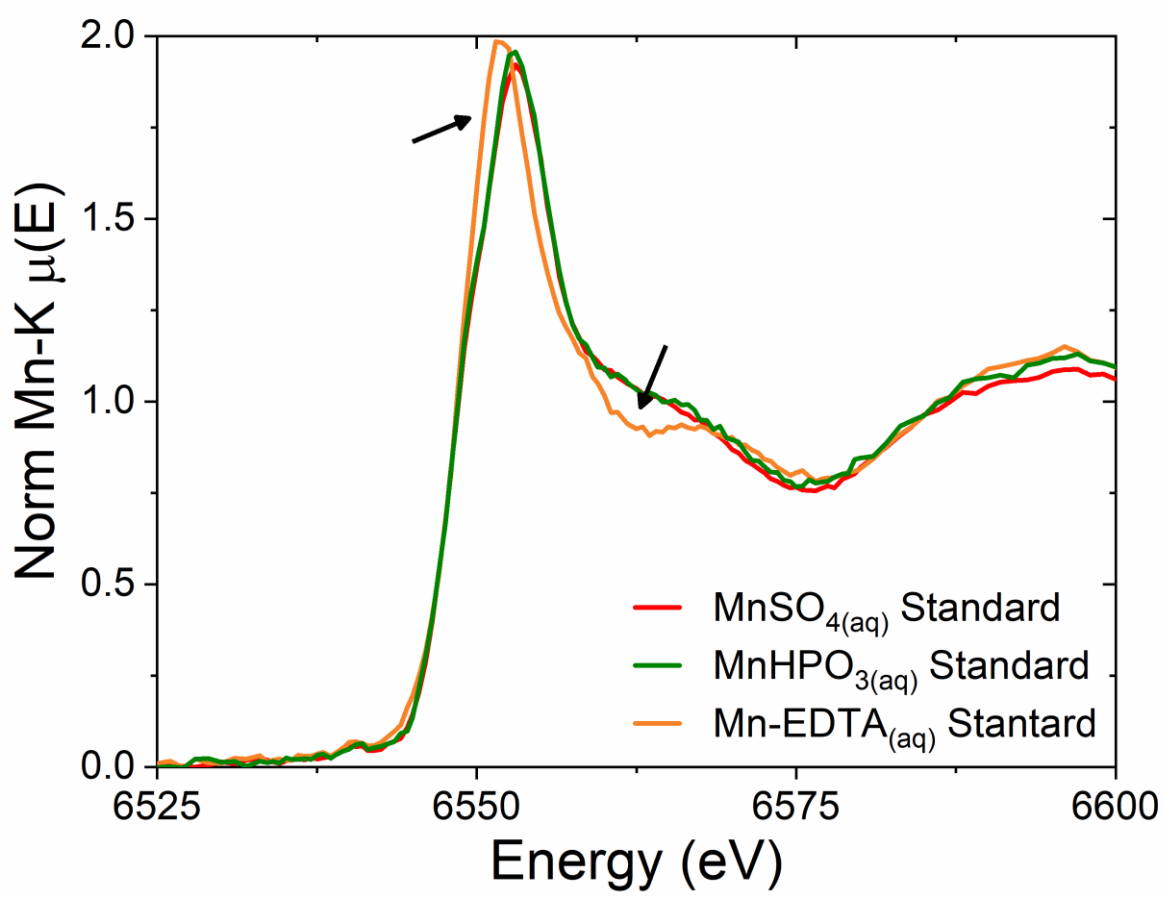

Figure 3.9. Mn K edge XANES spectra for MnSO4(aq), MnHPO3(aq) and Mn-EDTA(aq). The Mn chemical environment provided by MnSO4(aq) and MnHPO3(aq) in aqueous solutions was similar, while Mn-EDTA(aq) shows features (arrows) that differ from the others

Figure 3.10a and 3.10b show a soybean leaf assembled in a sample holder after the spreading of one of the tested materials and the plant loaded in the beamline for analysis, respectively. Figure 3.10c presents the merged XANES spectra recorded for petioles whose leaves were treated with $MnSO_{4(aq)}$, $MnHPO_{3(aq)}$ and $Mn\text{-}EDTA_{(aq)}$ with and without glyphosate, plus the spectra of their respective reference compounds.

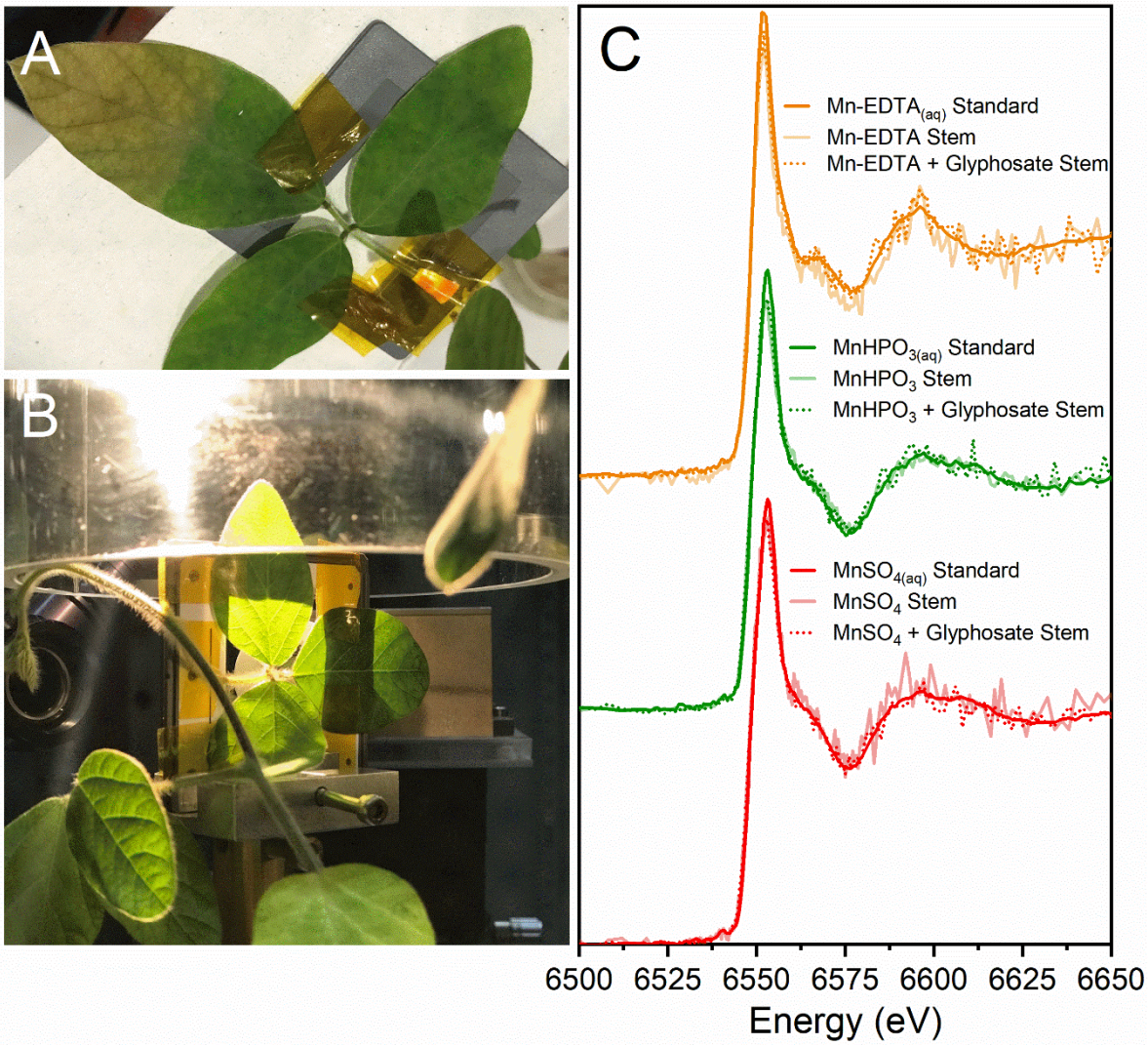

Figure 3.10. Manganese chemical speciation in soybean petioles using XANES. (A) Plant assembled on the acrylic sample holder with one of the tested materials applied at the upper half of the central leaflet; (B) Plant loaded in the beamline; (C) Mn-K XANES merged spectra recorded under *vivo* conditions for plants exposed to $MnSO_{4(aq)}$, $MnHPO_{3(aq)}$ and $Mn\text{-}EDTA_{(aq)}$ with and without glyphosate, and the spectra of their respective reference compounds. Glyphosate did not change Mn chemical environment within the plant tissue regardless of the applied Mn source. Manganese was transported as compounds similar to those applied to the leaf, including Mn-EDTA which was not transformed until its passage through the petiole

The XANES spectra recorded for the two biological replicates and the spectra for the reference compounds are presented in Figures 3.11, 3.12 and 3.13.

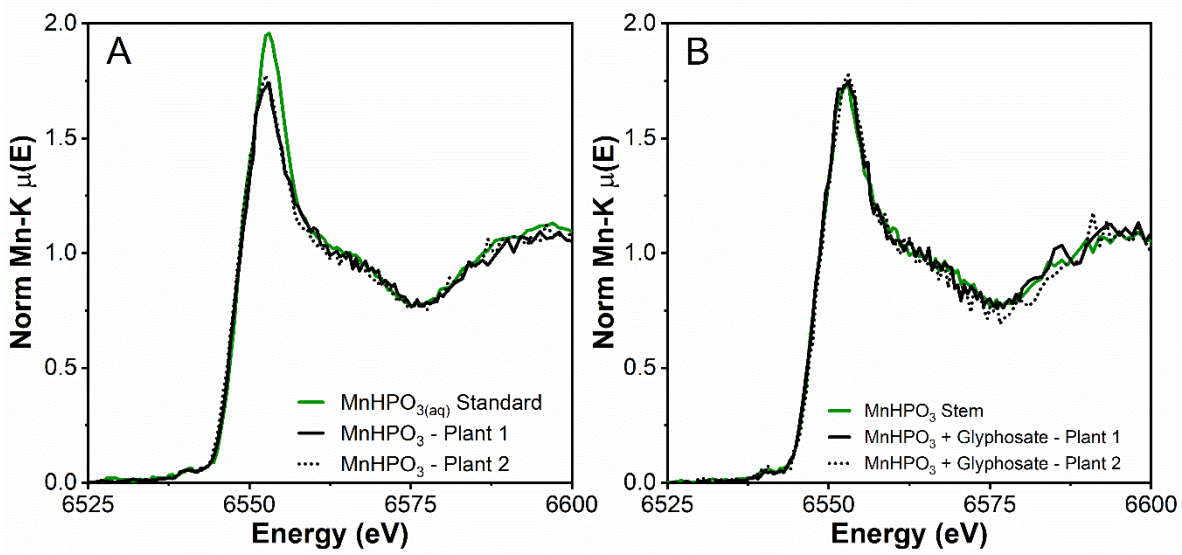

Figure 3.11. Mn-K edge XANES spectra recorded for the petioles of two plants after foliar application of (a) $MnHPO_3$ and (b) $MnHPO_3$ + glyphosate, plus a spectrum recorded for $MnHPO_{3(aq)}$ reference compound. The figure shows that the Mn applied as $MnHPO_3$ did not have its chemical environment affected by the glyphosate molecules within the plant tissue

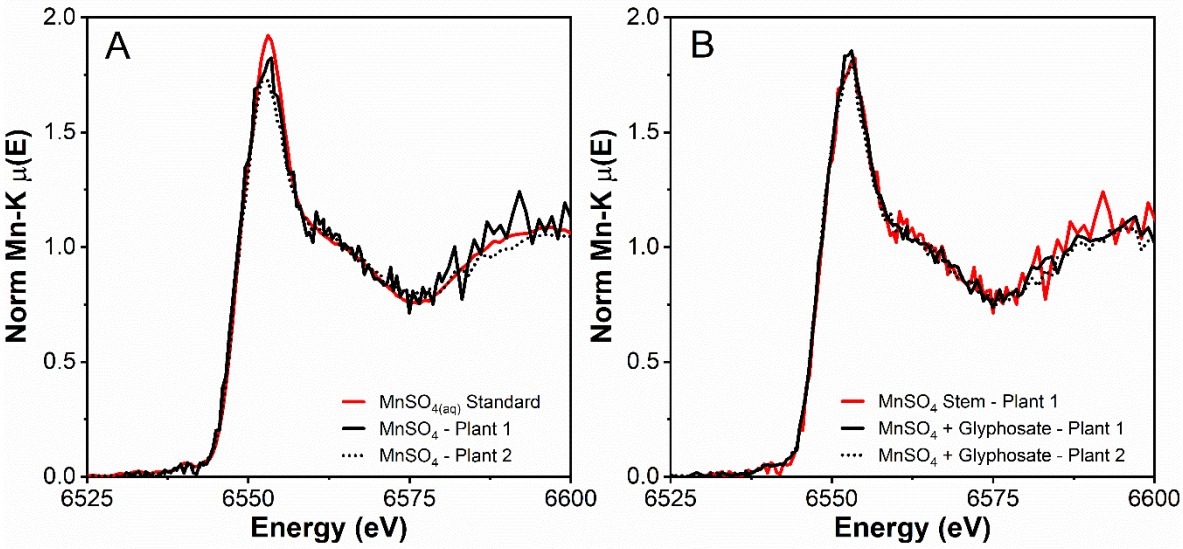

Figure 3.12. Mn-K edge XANES spectra recorded for the petioles of two plants after foliar application of (a) $MnSO_4$ and (b) $MnSO_4$ + glyphosate, plus a spectra recorded for $MnSO_{4(aq)}$ reference compound. The figure shows that the Mn applied as $MnSO_4$ did not have its chemical environment affected by the glyphosate molecules within the plant tissue

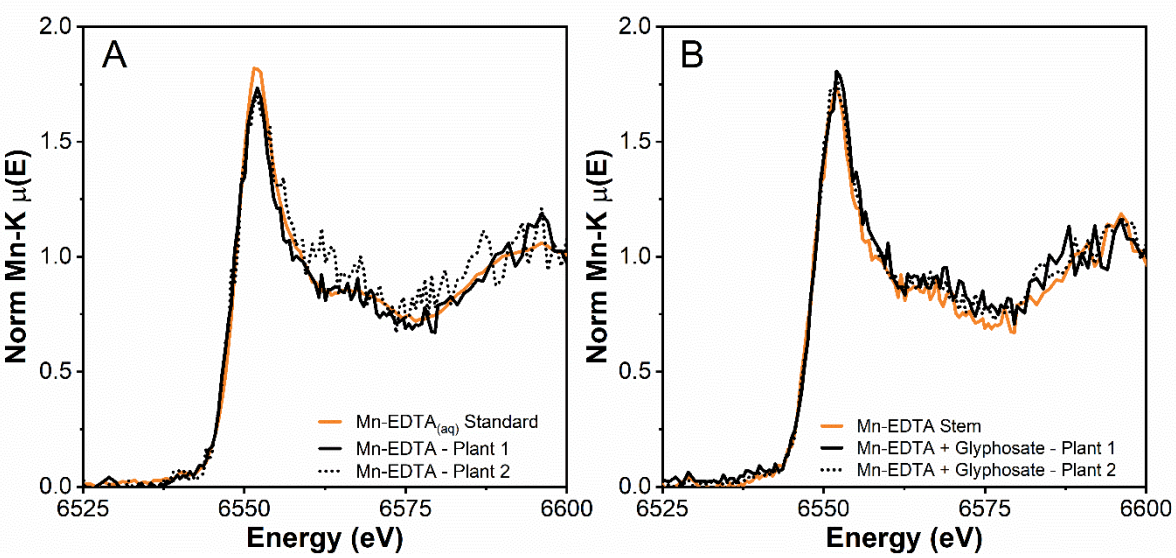

Figure 3.13. Mn-K edge XANES spectra recorded for the petioles of two plants after foliar application of (a) Mn-EDTA and (b) Mn-EDTA + glyphosate, plus a spectra registered for Mn-EDTA$_{(aq)}$ reference compound. It is possible to conclude that Mn is transported as Mn-EDTA and that the glyphosate molecules did not change the Mn-EDTA chemical environment within the plant tissue

The spectral features for the plants that received glyphosate perfectly match those observed for the plants without glyphosate. The data acquired for Mn-EDTA treated plants (Figure 3.13) overlapped that recorded for the reference compound. So far, it had not yet been demonstrated whether Mn-EDTA chelate could cross the leaf epidermis or not, which is a question previously raised (TIAN et al., 2015). Therefore, the results of our study showed that the Mn-EDTA can reach soybean petiole still in its pristine form.

Glyphosate present zwitterionic behavior, in solution it can complex divalent cationic nutrients via carboxyl, phosphoryl, and amine groups producing stable or low soluble complexes. Literature suggests that such coordination compounds present limited mobility in plants, compromising not only the physiological activity of the nutrient but also the glyphosate ability to control weed plants (CAKMAK et al., 2009; SHEALS; PERSSON; HEDMAN, 2001; SHKOLNIKOVA et al., 1983).

However, the results found in the present study suggest that, inside the plant, glyphosate did not interfere in Mn chemical species regardless of the source. Keeping in mind the elemental sensitivity of XANES, we can state that most of the Mn ions are not complexed by glyphosate within the plants. Therefore, on the contrary, that was previously suggested (BERNARDS et al., 2005; BERNARDS; THELEN; PENNER, 2005), the XANES measurements point out that yellowing symptoms on soybeans should not be caused by the unavailability of Mn due to the reaction with glyphosate.

3.3.4 Fertilizers and glyphosate are able to damage the leaf epidermis

Standard terrestrial application rates for spray mixtures range from 50 to 150 L ha^{-1} (JUSTINIANO, 2014). The reduction of the applied volume per hectare is a current trend aiming at increasing the operational capacity of the sprayers while decreasing production costs (SOUZA; CUNHA; PAVANIN, 2012).

In this study, we prepared Mn and glyphosate mixtures aiming at simulating the delivery of 50 L ha^{-1}, nevertheless, application rates of water-based tank mixtures may reach up to 30 L ha^{-1} during aerial spraying (GADANHA JUNIOR; MONTEIRO, 2005).

The 50 L ha^{-1} application rate provided concentrations of fertilizers and glyphosate that induced ultrastructural changes on the epidermal tissue (Figures 3.14 and 3.15). Based on SEM analyses, the treatments promoted four major alterations on G. max leaflet surface: i – epicuticular wax crystal (EWC) dissolution; ii – outer periclinal epidermal cell wall damage; iii – epidermal cell shrinking; iv – epidermal cell death.

Healthy soybean leaflet adaxial surface is light green with numerous trichomes (Figure 3.14a), and it presented turgid lens-shaped ordinary epidermal cells (Figures 3.14b-c). In outer cell wall, the epidermis is covered by epicuticular wax crystals (Figure 3.14c).

Firstly, we will introduce the epidermal ultrastructural modifications induced by the fertilizers, followed by the description of glyphosate effects and finally tank mixture. The $MnSO_4$ seems to accumulate and provide a dark appearance to the leaflet veins (Figure 3.14d), suggesting signs of phytotoxicity. It promoted the loss of the EWC integrity, and some epidermal cells have the collapse of the outer periclinal cell wall (Figures 3.14e-f). $MnHPO_3$ stained (brown color) the epidermis surface. Differently from the other fertilizers, $MnHPO_3$ did not promote EWC dissolution, on the other hand, it caused severe epidermal cell shrinking (Figures 3.14g-i). Since the application of H_2SO_4 solution at pH 1.40 also caused severe damages to leaf epidermis (Figure 3.15), in part, the cell shrinking can be attributed to the acidity of the $MnHPO_3$ solution (Table 3.1) and not only to the fertilizer itself. The dried Mn-EDTA droplet generated a whitish color area (Figure 3.14j). In detail, it was possible to verify damages on EWC, the formation of cuticle platelets, and the collapse of the outer periclinal wall of the epidermis (Figures 3.14k-l). In the latter, case the deleterious effects cannot be attributed to low the pH of the fertilizer solution. Finally, the dried $MnCO_3$ presented white color (Figure 3.14m). After the application, we observed the positive correlation of $MnCO_3$ crystals deposition site and EWC dissolution (Figures 3.14n-o).

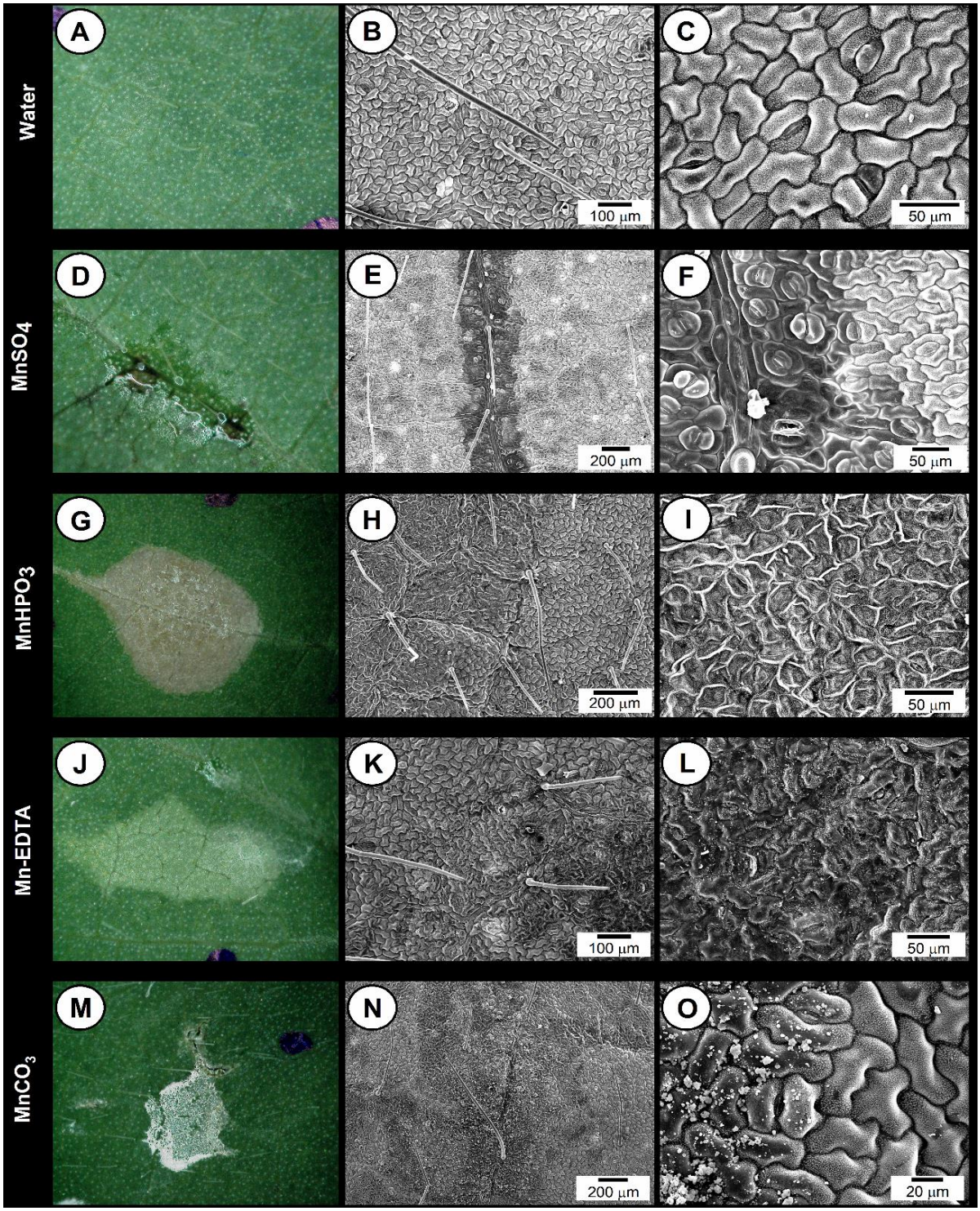

Figure 3.14. SEM micrographs of the adaxial surface of soybean leaves that received a 5 µL droplet of the respective treatments: (A, B and C) distilled water; (D, E and F) $MnSO_{4(aq)}$; (G, H and I) $MnHPO_{3(aq)}$; (J,K and L) Mn-$EDTA_{(aq)}$; (M, N and O) $MnCO_{3(aq)}$. All fertilizers were diluted in distilled water yielding a concentration of 7 g Mn L^{-1}

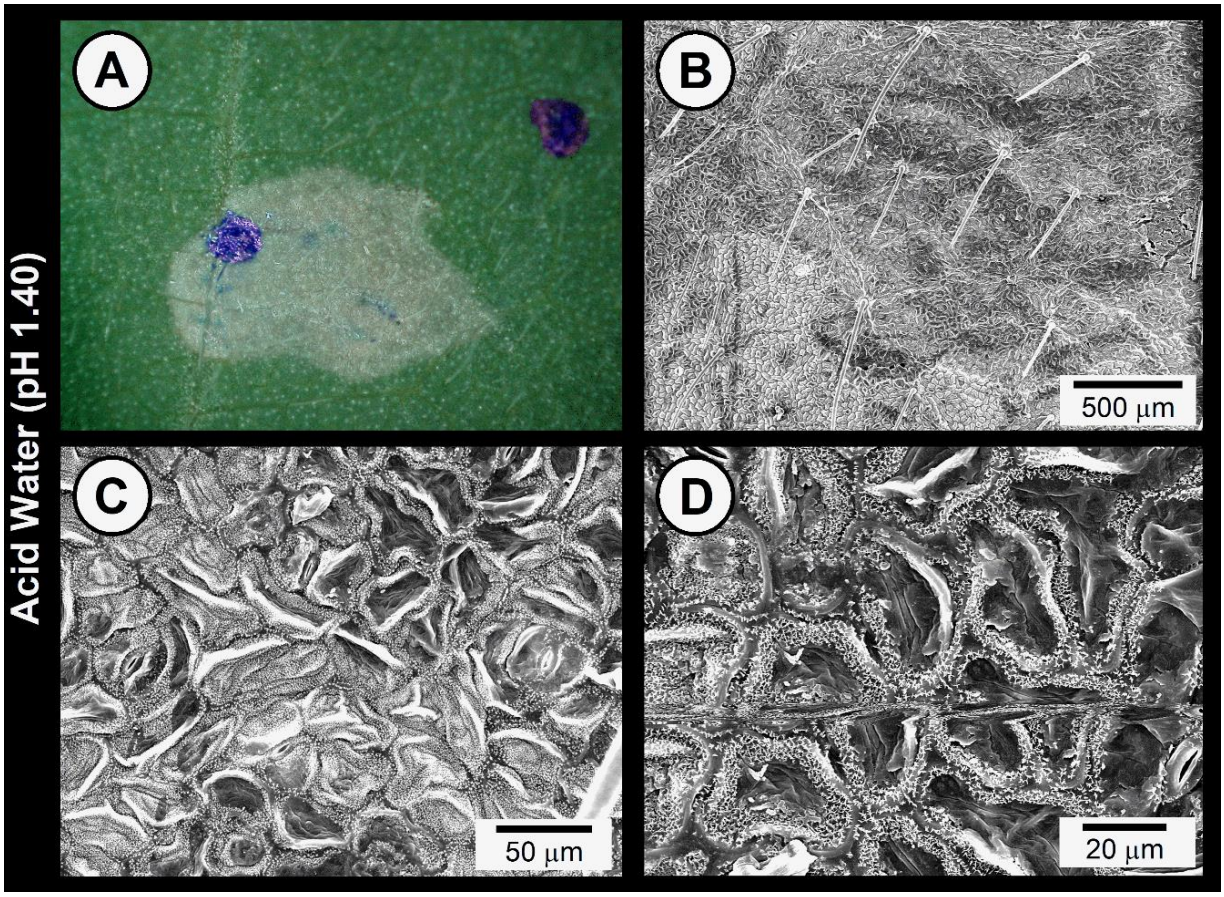

Figure 3.15. SEM micrographs of the adaxial surface of a soybean leaflet that received a 5μL droplet of distilled water acidified with H_2SO_4. The pH of the acid water was adjusted to 1.40, which is close to the pH provided by the Mn-phosphite solution

A whitish appearance was observed on the surface of the leaflets exposed to Glyphosate (Figure 3.16a). Epidermal cells exposed to this herbicide exhibited altered wax cuticle pattern with striate spaces between the EWC (Figures 3.16b-c). The loss of topography on some epidermal cells suggests the occurrence of a shrinking process (Figure 3.16b). $MnSO_4$ mix with glyphosate assumed white color when dried (Figure 3.16d), which can be attributed to the formation of insoluble and non-absorbable Mn-glyphosate complexes. SEM analysis did not show apparent ECW dissolution. We observed a high number of shrunk cells with collapsed periclinal outer cell walls (Figures 3.16e-f). For $MnHPO_3$ + glyphosate is a whitish spot was observed around the brownish spot (Figure 3.16g). Cell collapsing was the major characteristic of this treatment. In the area that received the treatment, the totality of cells was collapsed, and the epidermal anticlinal cell wall became evident (Figures 3.16h-i). Mn-EDTA + glyphosate did not present any ultrastructural differences from those observed for pristine Mn-EDTA (Figures 3.16j-l). $MnCO_3$ mix with glyphosate also presented white crystals deposited on the surface

(Figure 3.16m), in addition to striate EWC pattern and epidermal wall collapse (Figures 3.16n-o). No severe EWC degradation was observed in this treatment.

Hagedorn et al. (2017) reported that 1-triacontanol ($C_{30}H_{61}OH$) is the main component of soybean leaves EWC. Due to its hydrophobic nature, EWC repel water-based droplets, which tend to concentrate on leaf patches around veins, not covered by EWC (PUENTE; BAUR, 2011).

The dissolution of EWC observed for some treatments in this study took place in the same way as for surfactants. This dissolution capacity has been reported as an enhancement factor for uptake of spray mixtures by the leaf since it promotes the direct contact between the solutions and the leaf surface (MACISAAC; PAUL; DEVINE, 1991; PUENTE; BAUR, 2011).

On the other hand, some treatments triggered severe damages to the leaf epidermis, showing features of phytotoxicity in the treated area. It is possible to observe a certain positive correlation between damage and Mn intensity in the petiole. However, despite enabling Mn absorption, this study did not investigate to what extent the employment of concentrated spray mixtures is beneficial to boost crop development. Nevertheless, further studies are necessary, and special attention should be paid on tank mixtures concentrations, in order to avoid undesirable effects.

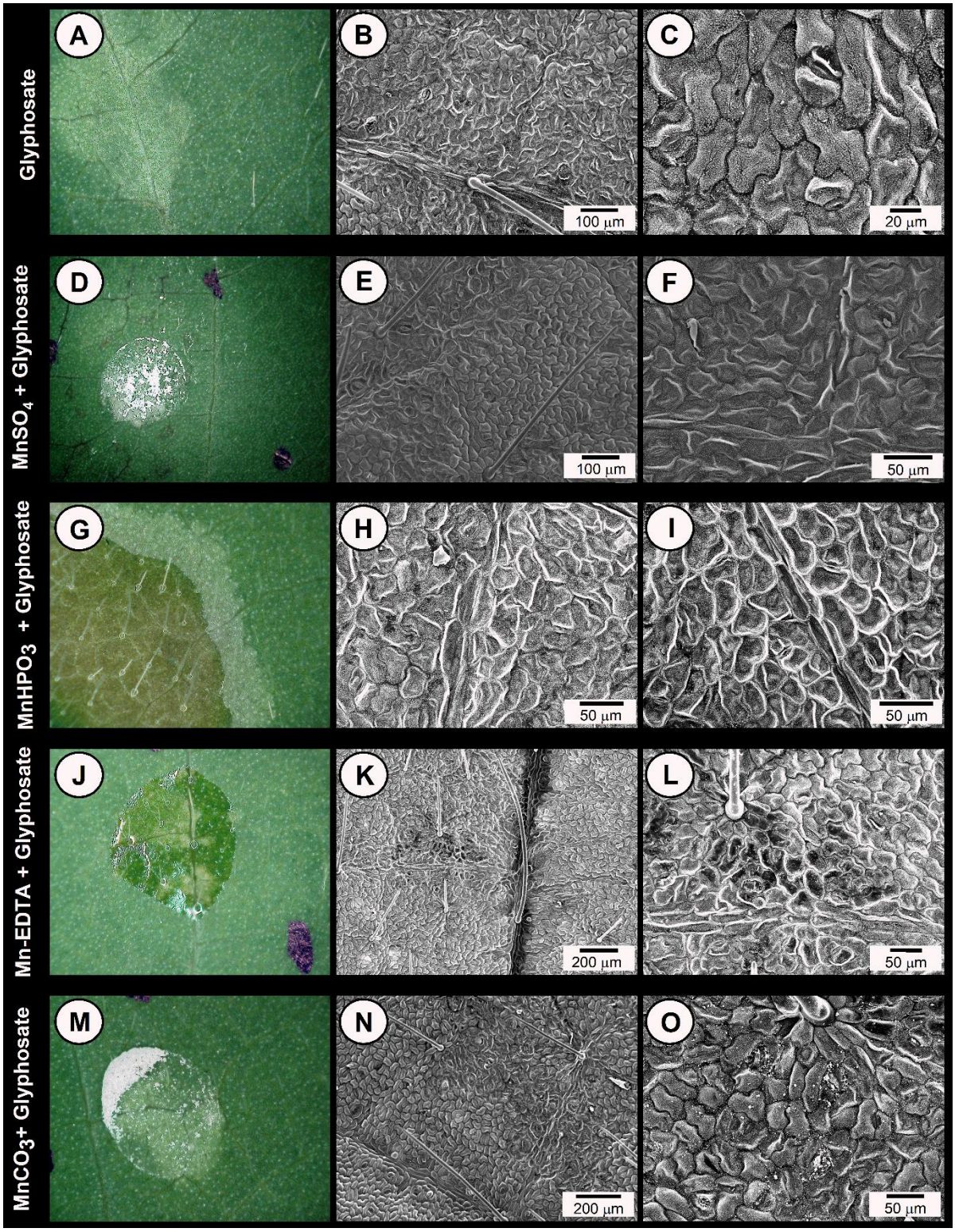

Figure 3.16. SEM micrographs of the adaxial surface of soybean leaves that received a 5 µL droplet of the respective treatments: (A, B and C) glyphosate; (D, E and F) $MnSO_{4(aq)}$ + Glyphosate; (G, H and I) $MnHPO_{3(aq)}$ + Glyphosate; (J, K and L) Mn-$EDTA_{(aq)}$ + Glyphosate; (M, N and O) $MnCO_{3(aq)}$ + Glyphosate. All treatments were diluted in distilled water yielding concentrations of 7 g Mn L^{-1} and 10.8 g glyphosate L^{-1} (acid equivalent)

3.4 Conclusions

We did not find pieces of evidence of beam induced radiation damage under the instrumental conditions explored in this study. Continuous efforts shall be made to promote the application of X-ray spectroscopy to the study of plants under in vivo conditions. Altogether, time-resolved XRF showed that MnSO4(aq) and MnHPO3(aq) are absorbed in higher amounts than Mn-EDTA and $MnCO_3$. This might be related to the ionic species provided by the salts which might have yielded outer sphere complexes that diffuse faster than the chelate. $MnCO_3$ might slowly dissolve and provide Mn^{2+} ions to the leaf. The absorption of $MnSO_4$ and $MnHPO_3$ were clearly impaired by glyphosate.

XANES showed that most Mn is transported as aqueous Mn^{2+}, except for Mn-EDTA which is still found as Mn-EDTA in the petiole. No evidence of Mn-Glyphosate complexation inside the plant was found.

Attention must be paid to the damage caused by the application of concentrated spray mixtures since it can induce ultrastructural changes on the leaf epidermal tissue. On the positive side, such damage can boost uptake of ionic nutrients by removing hydrophobic tissues that prevent nutrient uptake, but in opposite can increase the sensitivity to pests or diseases attack.

4 EFFECTS OF GLYPHOSATE ON FOLIAR ABSORPTION AND METABOLISM OF MANGANESE IN SOYBEAN PLANTS (*Glycine max* L.)

Abstract

Symptoms of yellowing of soybean leaves after glyphosate application is widely attributed to the complexation of intracellular Mn by glyphosate molecules. In this context, the present work employed XRF spectroscopy to unravel whether tank mixtures of $MnSO_4$, Mn-EDTA and $MnCO_3$ with glyphosate can influence on Mn foliar uptake and utilization by soybean plants. Glyphosate reduced the foliar $MnSO_4$ absorption in 25% when compared to $MnSO_4$ applied alone, while Mn-EDTA was not affected by the mixture with glyphosate. Reduction in foliar absorption is most likely related to the precipitation of low solubility Mn-glyphosate complexes on the leaves surface. Based on data of Mn concentration and content in the shoot, as well as antioxidant enzymes activity, $MnSO_4$ was the only source which efficiency was negatively affected by glyphosate.

Keywords: XRF. Foliar absorption. Tank mixtures. Antioxidant enzymes. SOD.

4.1 Introduction

Soybean covers more than 8 % of the whole world agricultural land (FAO, 2018). Brazil, United States of America and Argentina are, respectively, the greatest producers for the 2020/19 crop. In this season, these countries produced more than 270 million metric tons of grains (80% of the total), over an area of 84 million of hectares (69% of the total). This corresponds to an average yield of 3.2 tons per hectare (USDA, 2020).

More than 95% of the soybean cultivars planted in Brazil, United States of America and Argentina are resistant to glyphosate (JAMES, 2017). Glyphosate is a broad spectrum herbicide which inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (STEINRÜCKEN; AMRHEIN, 1980). This inhibition avoids the production of essential aromatic amino acids such as tryptophan, tyrosine and phenylalanine, in the shikimate pathway (DUKE et al., 2012a), which lead plants to death. The resistance to glyphosate was conferred thanks to the introduction of the CP4 EPSPS gene from *Agrobaterium tumefaciens* intro the soybean genome. This gene encodes the CP4 EPSP synthase enzyme, allowing the shikimate pathway to function in glyphosate's presence (DILL, 2005).

The release of glyphosate resistant soybean allowed the application of glyphosate in the fields without damaging the main crop. This simplified the weed control and reduced the costs involved with this management (DUKE, 2018). Additionally, it enabled the application of glyphosate in conjunction with micronutrients. Thus, tank mixtures became a practical alternative to alleviate nutritional deficiency while controlling weeds (BERNARDS et al., 2005).

Manganese (Mn) is a micronutrient widely applied in conjunction with glyphosate in soybean fields. This micronutrient is required for photosynthesis processes, nitrogen metabolism, hormonal regulation and enzymes activation. Manganese deficiency leads to decreased oxygen evolution, which results in lower rates in the production of photo assimilates and decreased plant growth (NABLE; BAR-AKIVA; LONERAGAN, 1984).

It is known that glyphosate can complex Mn^{2+} ions in solution, and previous studies had demonstrated that tank mixtures can diminish the herbicidal action of glyphosate (BERNARDS et al., 2005; BERNARDS; THELEN; PENNER, 2005). Likewise, some reports indicate that the complexation of Mn by glyphosate can extend to the cytoplasmatic environment (BERNARDS et al., 2005; EKER et al., 2006). Such interaction could make the intracellular Mn unavailable for accomplish its role on plant metabolism.

Manganese deficiency is characterized by the yellowing of new leaves, which is a visual symptom frequently reported by soybean growers following glyphosate's application in the fields. This symptom usually disappear within 21 days, being known as *yellowflash* (DUKE et al., 2012).

Although studies had demonstrated that the *yellowflash* symptoms occurs due to the toxicity of AMPA (aminomethylphosphonic acid), which is the first metabolite formed during glyphosate degradation (DUKE et al., 2012a; REDDY; RIMANDO; DUKE, 2004), many still attribute it to the complexation of intracellular Mn by glyphosate molecules. In this context, the present work aimed at unraveling whether tank mixtures with glyphosate can influence on Mn foliar absorption and metabolism. Therefore, X-ray fluorescence spectroscopy (XRF) was used to monitor the foliar absorption of $MnSO_4$, Mn-EDTA and $MnCO_3$, with and without glyphosate. In order to evaluate the utilization of Mn, its concentration and content were determined in treated and untreated leaves, as well as in the shoots of soybean plants foliar fertilized with $MnSO_4$, Mn-EDTA and $MnCO_3$, with and without glyphosate. The activity of antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPOX), were also investigated.

4.2 Material and Methods

4.2.1 Foliar absorption assay using x-ray fluorescence spectroscopy (XRF)

4.2.1.1 Plant growth

Soybean plants were cultivated in pots with substrate, in a growth room where the temperature was kept at ± 25ºC, with a photoperiod of 12 h provided by LED lamps (6500 K), using a photon flux of 250 µmol m^{-2} s^{-1}. At the V3 phenological stage, the plants were transferred to acrylic sample holders which enable their proper positioning inside the XRF spectrometer, besides allowing to keep the plants alive during the analysis and facilitate the treatments' application (Figure 4.1a).

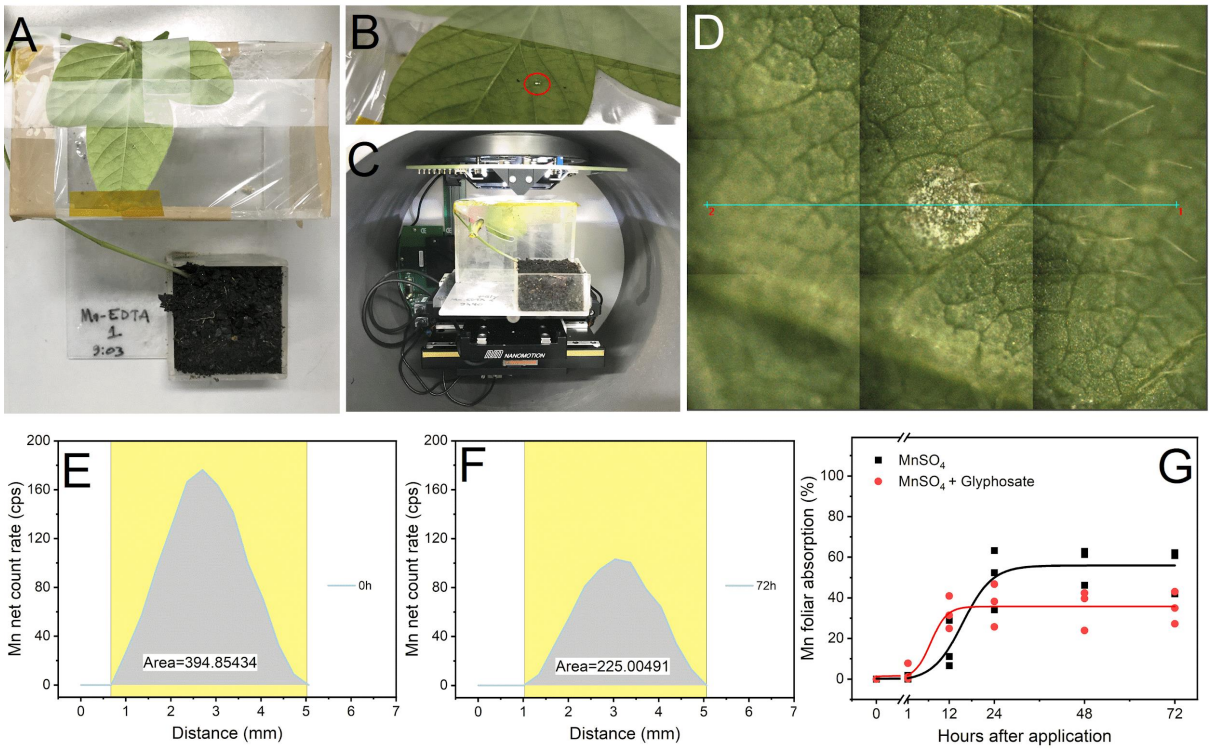

Figure 4.1. Steps of the droplet absorption assay. (A) soybean plant settled in the sample holder, (B) droplet deposition in the abaxial surface of the central leaflet, (C) plant ready for XRF analysis, (D) line scan on the droplet, (E) Mn net count rate at the 0h after the treatment application, (F) Mn net count rate 72h after the treatment application, (G) fitting curves determined to identify the absorption trends for each treatment

4.2.1.2 Treatments and application

The treatments consisted of 0.5 µL droplets of $MnSO_4$, Mn-EDTA and $MnCO_3$ solutions/dispersion, with and without glyphosate, applied on the abaxial surface of the central leaflet of the first completely expanded trefoil (Figure 4.1b). The concentrations of 1.15 g L^{-1} of Mn and 5 mL L^{-1} of glyphosate (commercial product) were employed to prepare the solutions/dispersion. Theses concentrations correspond to the application of 172 g ha^{-1} of Mn and 750 mL ha^{-1} of glyphosate (commercial product), considering an application volume of 150 L ha^{-1}. We employed half of the field recommendation dosage in order to enable the measurements at the XRF equipment.

The following Mn sources were employed: $MnSO_4.H_2O$ (31.85 wt% Mn, Synth), $MnCO_3$ nanopowder 80-100 nm (47.78 wt% Mn, Nanoshel), and Mn-ethylenediamine tetraacetic acid (Mn-EDTA) (13 wt% Mn, Stoller). The employed glyphosate was a N-(phosphonomethyl)glycine (48 wt% active ingredient and 36 wt% acid equivalent, Ameribrás).

4.2.1.3 XRF measurements

The Mn foliar absorption was investigated by monitoring the Mn XRF intensity on and around the deposited droplet (Figure 4.1c). It was assumed that Mn intensity would diminish as long as this nutrient would be absorbed. Therefore 5 mm line scans were performed at 0, 1, 12, 24, 48, and 72 hours after the droplets' application (Figure 4.1d).

The absorption kinetics assay was performed with the Orbis PC X-ray spectrometer (ORBIS PC, EDAX), operating with a rhodium anode at 36 W. The measurements were made using a 250 µm thick primary Al filter to improve the Mn signal to noise ratio. A 2 mm wide X-ray beam shaped by a collimator was employed to perform the line scans, which were composed by 16 points. Each point was analyzed for 30 seconds, generating a spectrum in which the Mn net XRF intensity was determined. Only the intensities above the instrumental limit of identification (ILOI) were considered in order to trace the Mn absorption (equation 3), being the following equation employed to determine the ILOI (MONTANHA et al., 2020a):

$$8.485 \times \sqrt{BG \div t} \qquad (3)$$

Where, BG is the background intensity (cps) and t is the acquisition time (seconds).

4.2.1.4 Absorption determination

Bell-shaped curves were obtained by plotting the Mn net intensity of each line scan performed on and around the droplet. The Mn absorption was determined by the integration of the curve obtained at each time of analysis (Figures S1E and S1F). The Mn net intensity at the time zero was considered as 0 % of absorption, and the relative Mn net intensity in the subsequent measurements determined the percentage of the applied Mn that was absorbed by the leaf (Figure 4.1g).

Scatter plots were generated with the percentage of Mn absorbed by the leaves as a function of the time. Then, it was determined the best fitting in order to identify the absorption trends for each treatment. The $MnSO_4$ and Mn-EDTA based treatments followed a Boltzmann sigmoidal model fitting (equation 4), while the $MnCO_3$ based treatments followed a first-degree linear equation fitting.

The sigmoidal model fits to the pattern where there is an initial low fertilizer absorption, with a sequential fast rate of absorption, followed by a transition point from where the absorption loses intensity and finally reaches a plateau. The modified Boltzmann sigmoidal model is shown in equation 4:

$$\hat{y} = \frac{a}{1 + e^{\left(\frac{t0 - t}{b}\right)}} \quad (4)$$

where, $\hat{y}$ is the Mn absorption in percentage, t is the time after the treatment application, a is the maximum Mn absorption, t_0 is a three interpretation parameter being the time when 50% of the maximum absorption occurs, the moment when the maximum rate of absorption occurs, and the inflection point of the function; b is the behavior of the slope of the process during the transition. The parameter b can also be used to estimate the maximum rate of absorption (R_{max}) at the t_0 moment, following the equation 5:

$$Rmax = \frac{a}{4b} \quad (5)$$

4.2.1.5 Statistical analysis

The linear and sigmoidal fit were estimated using the Origin software (ORIGIN, 2020), considering a p value < 0.05. Values of maximum absorption (a), time when 50% of

the maximum absorption occurs (t_0) and R_{max} were determined for each replicate. The results were submitted to analysis of variance (ANOVA), and means were compared using LSD ($p < 0.05$) considering a factorial 2x2 for $MnSO_4$ and Mn-EDTA based treatments.

4.2.2 Performance of foliar applied Mn sources + glyphosate in Mn starving plants

4.2.2.1 Plant growth

Soybean seeds (*Glycine max* L. Merril cv. M7739 IPRO, Monsoy) were germinated in substrate (Basaplant ®, Brazil), composed of a mixture of pat, vermiculite, charcoal, and pine bark. Ten days after, at the VC phenological stage, two plants were transferred to each 4 L plastic pot containing nutrient solution. For acclimation, the nutrient solution was gradually replaced at 20, 50 and 100 % strength on the second, fifth and seventh-day after the transfer, respectively. From the seventh day on, the plants continued to be supplied with the nutrient solution at 100 % strength, which was constantly aerated and renewed every seven days. The 100% strength nutrient solution was composed of 2 mM $Ca(NO_3)_2.4H_2O$, 0.6 mM KNO_3, 0.25 mM $NH_4H_2PO_4$, 0.65 mM $MgSO_4.7H_2O$, 12.5 µM KCl, 6.25 µM H_3BO_3, 0.5 µM $ZnSO_4.7H_2O$, 0.125 µM $CuSO_4 \cdot 5H_2O$, 0.125 µM $(NH_4)_6Mo_7O_{24}$, 53.7 µM NaFeEDTA and 0.5 µM MnSO4.H2O. The plants were cultivated under low Mn concentration (0.05 µM $MnSO_4.H_2O$) and positive control treatments (adequate Mn) received complete nutrient solution (0.5 µM $MnSO_4.H_2O$).

The plants were cultivated in a growth room, where the temperature was kept at ± 25 ºC with a 12 h photoperiod, supplied by led lamps (6500 K) with a photon flux of 250 µmol m^{-2} s^{-1}.

4.2.2.2 Treatments and experimental design

The treatments consisted of foliar application of four Mn sources, mixed or not with glyphosate, on soybean plants cultivated in nutrient solution with low Mn availability (10 % of the complete nutrient solution), plus a negative control (no foliar Mn application, low Mn availability in the nutrient solution) and a positive control (no foliar application, adequate Mn availability in the nutrient solution). Thus, the experiment was settled in a 6 x 2 factorial (6 types of Mn supply, with or without glyphosate), arranged in randomized blocks, with three replications.

4.2.2.3 Treatments application

Manganese dosage was based on the recommendation of 350 g ha^{-1} Mn (EMBRAPA, 2013), while the employed dosage of glyphosate (1.5 L ha^{-1}) meet the supplier recommendation for several types of weeds. We considered a field population of 250,000 plants per hectare to calculate a total delivering of 1.4 mg Mn per plant, which was split into three applications of 0.4, 0.4 and 0.6 mg per plant at the V5, V7 and R2 phenological stages, respectively. The full dosage of glyphosate (1.5 L ha^{-1}, or 6 µL per plant) was employed for the three applications.

The treatments were diluted in 10 mL of distilled water and sprayed in the entire plants' shoot, using a CO_2-pressurized sprayer. During the first two applications (V5 and V7) there were two plants per pot, while the third (R2 phenological stage) was performed after harvesting one of the plants, but still we kept the same volume of application (10 mL). Therefore, it was applied 0.8 mg of Mn in each vase in the first and second application, and 0.6 mg of Mn per vase in the third foliar application.

4.2.2.4 Manganese concentration in treated and untreated leaves

Ten days passed the second foliar application, at the R1 phenological stage, we collected one entire plant of each pot. From each of these plants, we sampled all new leaves emerged after the second foliar application (untreated leaves), and the one trifoliate leaf immediately below the untreated region (treated leaf). These leaves were washed in deionized water and HCl solution at 3 mL L^{-1}, according to Embrapa (2000). Then, they were placed in paper bags and taken for drying in a forced air oven at 65 ºC till reach constant mass. After dried, the samples were ground in ball mills, sieved through 150 µm sieve, and their Mn content were determined by X-ray fluorescence spectroscopy.

One hundred milligrams of the ground samples were assembled in XRF cuvettes for analysis. The measurements were performed on the Shimadzu EDX-720 spectrometer, which operated with a Rh X-ray tube working at a tension of 50 kV and a current of 1,000 µA with a 3-mm collimator. The fluorescent photons were detected by a Si(Li) detector with an acquisition live time of 200 seconds and death time close to 25 %. The Mn concentration % (m/m) was calculated by the fundamental parameters' method according to Van Grieken et al. (1993) considering a cellulose matrix. The results were verified analyzing two certified reference materials NIST-1573 and NIST-1515 (tomato and apple leaves).

4.2.2.5 Manganese concentration and content in the shoot

In the R6 phenological stage, we cut the plants at their base, being their pods separated from the shoot and counted. The first fully expanded trifoliate leaf was collected and stored in liquid nitrogen for further enzymatic analysis, while the remaining shoot biomass (leaves + stem + petioles) was washed in deionized water and HCl solution at 3 mL L^{-1} (EMBRAPA, 2000). The washed samples were dried, ground, and analyzed through X-ray fluorescence spectroscopy for Mn concentration determination just as above mentioned. The Mn content in the shoot was assessed by multiplying the shoot dry mass (kg) by the Mn concentration (mg kg^{-1}), resulting in mg of Mn in the shoot.

4.2.2.6 Determination of lipid peroxidation, hydrogen peroxide, protein content, and antioxidative enzymes activity

The lipid peroxidation, hydrogen peroxide, protein content and antioxidative enzymes activity determination were performed at the first fully expanded trifoliate leaf of plants at the R6 phenological stage. Therefore, the leaves were collected, immediately stored in liquid N_2, and then transferred to a -80 ºC freezer. Later, the samples were ground by hand in plastic mortars, using liquid nitrogen. The samples were stored at -80 ºC for further analysis.

4.2.2.6.1 Lipid peroxidation

The sample extraction procedure was made placing 200 mg of the ground samples inside plastic mortars soaked in ice bath. The samples were homogenized with 2 mL of trichloroacetic acid (TCA) 0.1 % (w/v) containing about 20 % of polyvinylpolypyrrolidone (PVPP). The homogenized samples were transferred to 2 mL vials and centrifuged at 2,200 *g*, for 20 minutes, at 4ºC. The supernatant was employed for determination of lipid peroxidation and hydrogen peroxide.

The lipid peroxidation evaluation was determined according to Heath and Packer (1968), thus, 250 µL of the extracted sample were pipetted in 1.5 mL vials with 1000 µL of TCA 20 % containing 0.5 % of thiobarbituric acid (TBA). Then, the vials were closed and left in water bath at 95ºC for 30 minutes. After this procedure, the vials were cooled in ice bath for 10 minutes and centrifuged at 16,000 *g* for 10 minutes. The readings were performed in a spectrophotometer employing 532-600 nm wavelength.

4.2.2.6.2 Protein content and antioxidative apparatus activity

The Azevedo et al. (1998) extraction procedure was employed for determining the soluble protein content and activity of the antioxidative enzymes superoxide dismutase (SOD), guaiacol peroxidase (GPOX) and ascorbate peroxidase (APX), and catalase (CAT). Therefore, we placed 500 mg of the ground leaves' samples in plastic mortars in ice bath, then, homogenized them with 4% PVPP and 1.5 mL of the extraction buffer, which consisted on 1 mM of EDTA and 3 mM of dithiothreitol (DTT) diluted in 100 mM potassium phosphate buffer (pH 7.5). The homogenized samples were transferred to 2 mL vials and centrifuged at 2,200 *g* for 20 minutes, at 4ºC. Later, the supernatants were split into 200 µL aliquots and frozen at -80ºC for further analysis.

We determined the protein content according to Bradford (1976), using a standard curve with bovine serum albumin (BSA) with concentrations from 0.1 up to 1 mg L^{-1}. The samples' preparation consisted of adding 1000 µL of the Bradford reagent on 20 µL of the plants' extracts diluted in distilled water. The mixing was made in plastic cuvettes, which were analyzed in a spectrophotometer 5 minutes after the solution's homogenization, employing 595 nm wavelength.

4.2.2.6.3 Superoxide dismutase (SOD)

The SOD activity was determined according to Giannopolitis and Ries (1977) and Cembrowska-Lech, Koprowski and Kepczyński (2015). In test tubes, we homogenized 1490 µL of a solution containing 50 mM of potassium phosphate buffer (pH 7.8), 13mM methionine, 63 mM nitroblue tetrazolium (NBT), 0.1 mM EDTA, and 1.3 mM riboflavin, with 10 µL of the leaves' extracts. Two blank samples (a dark and a light blank) were made for each six regular samples, following the same procedure described above, but using 10 µL of 50 mM potassium phosphate buffer (pH 7.8) in place of the plant extracts. After mixing the samples with the solution, the tubes were placed in a closed box, and a light was up inside the box for 5 minutes, in order to promote the formation of the blue formazan due to the photoreaction of the NBT. The light blank was placed inside the box together with the samples, while the dark blank was maintained in the dark. Passed 5 minutes in the light, the samples were placed in plastic cuvettes and analyzed in the spectrophotometer at 560 nm wavelength. The dark blank was employed to calibrate the equipment, while the light blank was measured for further calculations of SOD activity. SOD activity was expressed as SOD

unit mg^{-1} protein (SOD U mg $protein^{-1}$). One SOD unit corresponds to the SOD activity which inhibits 50% of the NBT reduction.

4.2.2.6.4 Ascorbate peroxidase (APX)

The APX activity was determined according to Moldes et al. (2008), with modifications. Thus, in quartz cuvettes, we homogenized 12.5 µL of the plant extract, 650 µL of 80 mM potassium phosphate buffer (pH 7), 100 µL of 5 mM ascorbate, 100 µL of 1mM EDTA, and 100 µL of 1mM H_2O_2. The potassium phosphate buffer, ascorbate and EDTA solutions were kept in water bath at 30 ºC during the samples' preparation. After homogenization, the reactions were monitored for 20 seconds in a spectrophotometer at 290 nm wavelength. The APX activity was expressed in nmol $minute^{-1}$ mg $protein^{-1}$.

4.2.2.6.5 Guaiacol peroxidase (GPOX)

The GPOX activity was determined according to Matsuno and Uritani (1972), standardized in the laboratory of vegetal physiology of the Embrapa Temperate Climate, Brazil, with modifications. Therefore, in test tubes, we homogenized 790 µL of phosphate-citrate buffer (0.2 M of dibasic sodium phosphate and 0.1 M of citric acid, pH 5), 10 µL of the plant extracts, 50 µL of 0.2 % guaiacol and 50 µL of H_2O_2 9.8 mM. The mixtures were placed in water bath at 30 ºC for 15 minutes, then, placed in ice bath, adding 50 µL of 2 % sodium metabisulphite to stop the reaction. After 10 minutes, the readings were performed in a spectrophotometer at 450 nm.

4.2.2.7 Data analysis

The variables were subjected to analysis of variance (ANOVA), and means were compared using LSD ($p < 0.05$).

4.3 Results and Discussion

4.3.1 Foliar absorption assay using X-ray fluorescence spectroscopy (XRF)

The Mn foliar absorption from $MnSO_4$, Mn-EDTA and $MnCO_3$, with and without glyphosate, was monitored *in vivo* using X-ray fluorescence spectroscopy. XRF spectroscopy was employed before to monitor the transport kinetics of radicular supplied nutrients through the stem (CRUZ et al., 2017; MONTANHA et al., 2020b), and foliar applied fertilizers through the petioles of treated leaves (GOMES et al., 2019; MACHADO et al., 2019).

The fertilizers absorption was evaluated by monitoring the Mn XRF intensity through subsequent XRF line scans over the droplets. The line scans resulted in bell shaped graphics based on the Mn net count rate (cps), as shown by Figure 4.2. Herein, only one of three replicates for each treatment is shown. Data provided information to estimate the percentage of the applied Mn which was absorbed and to propose fitting curves to describe the absorption trends along the time for each treatment.

One can observe that, for the first 72 hours after application, $MnSO_4$ and Mn-EDTA based treatments followed a Boltzmann sigmoidal fitting curve regarding Mn absorption (Figure 4.2c and 4.2f). Meanwhile, $MnCO_3$ absorption followed a linear trend of absorption, but with a discrete slope, which indicates a very slow absorption. The $MnCO_3$ + glyphosate did not present any absorption trend, so no model was fitted to this treatment. One can see in Tables 4.1 and 4.2 the parameters of the curve fittings.

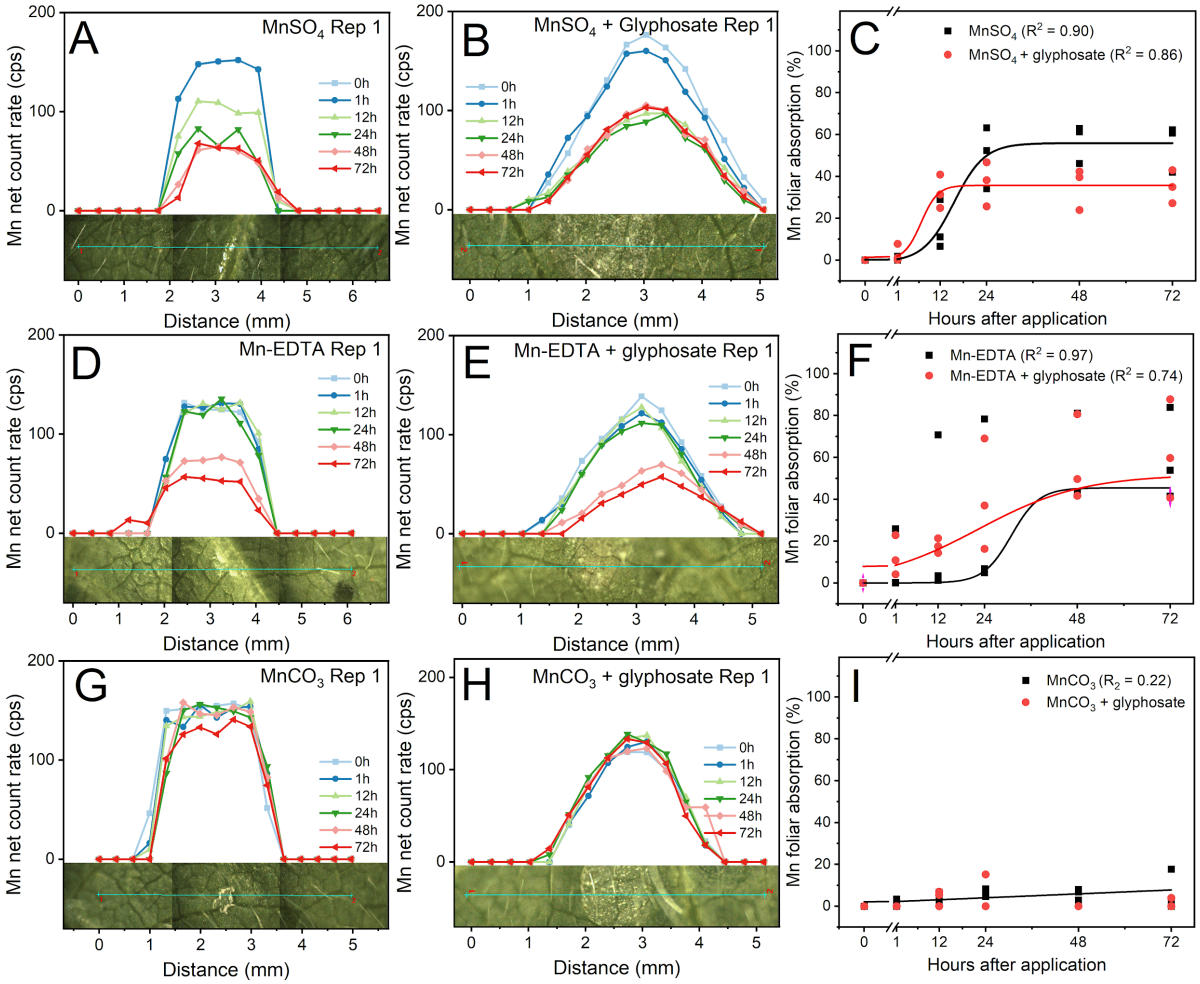

Figure 4.2. Mn net count rate (cps) on and around the droplets and percentage of Mn foliar absorption from a 0.5 µL droplet of $MnSO_4$, Mn-EDTA and $MnCO_3$, with and without glyphosate, during the first 72 hours after the application. (A) Mn net count rate for $MnSO_4$; (B) Mn net count rate for $MnSO_4$ + glyphosate; (C) Mn foliar absorption of $MnSO_4$ and $MnSO_4$ + glyphosate; (D) Mn net count rate for Mn-EDTA; (E) Mn net count rate for Mn-EDTA + glyphosate; (F) Mn foliar absorption of Mn-EDTA and Mn-EDTA + glyphosate; (G) Mn net count rate for $MnCO_3$; (H) Mn net count rate for $MnCO_3$ + glyphosate; (I) Mn foliar absorption of $MnCO_3$ and $MnCO_3$ + glyphosate

Table 4.1. Parameters of the linear fitting found for $MnCO_3$ foliar absorption

	Curve equation	**a**	**b**	**R-Square (COD)**	**p value**
$MnCO_3$	y = a + b*x	2,16	0,078	0,22	0,04

Table 4.2. Parameters of the sigmoidal fitting found for $MnSO_4$, $MnSO_4$ + glyphosate, Mn-EDTA and Mn-EDTA + glyphosate treatments

	$MnSO_4$	**$MnSO_4$ + glyphosate**	**Mn-EDTA**	**Mn-EDTA + glyphosate**
a	55.90	35.74	45.45	51.81
t_0	15.53	7.05	30.94	22.39
b	3.96	2.16	3.60	13.45
R-Square (COD)	0.91	0.86	0.98	0.81
Adj. R-Square	0.89	0.84	0.98	0.75
p-value	1.46E-09	3.52E-10	3.76E-10	7.65E-05

The maximum Mn absorption was influenced by the interaction between Mn supply and glyphosate. Nearly 60% of the $MnSO_4$ applied alone was absorbed, but the mixture of glyphosate significantly reduced this amount to 35%. Meanwhile, Mn-EDTA absorption was not affected by glyphosate (52 and 48 % of absorption with and without glyphosate, respectively). Comparing the two Mn sources, $MnSO_4$ was more absorbed in glyphosate's absence, but Mn-EDTA overcame $MnSO_4$ in matters of the total Mn absorbed when glyphosate was added (Figure 4.3).

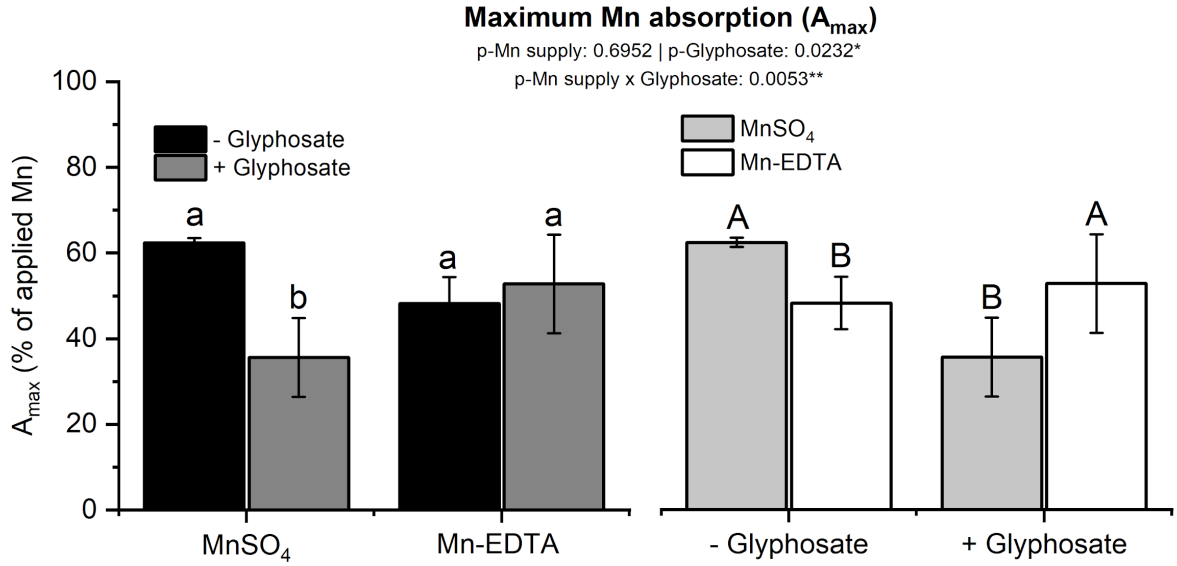

Figure 4.3. Maximum Mn foliar absorption (% of applied Mn) as function of Mn supply and glyphosate mixture. Means followed by the same letters are not significantly different for Mn supply (lower case letters) and glyphosate application (capital letters) by the LSD test ($p < 0.05$). Glyphosate decreased Mn absorption from MnSO4. In glyphosate absence, MnSO4 is more absorbed than Mn-EDTA, but when glyphosate is tank mixed Mn-EDTA is more absorbed than MnSO4. *significant at $p < 0.05$. **significant at $p < 0.01$

The hour of maximum absorption (T_{max}) was influenced by the Mn supply and glyphosate application, with no interaction between these factors. The Mn-EDTA took longer (28h) to reach the hour of maximum absorption than $MnSO_4$ (10h), which suggests that this source is absorbed more slowly. Meanwhile, the mixing of glyphosate decreased the T_{max}, most likely due to the adjuvants present in glyphosate's formulation which can improve the droplets penetration into the leaves (Figure 4.4a).

The R_{max} is the percentage of the applied Mn which is absorbed at the T_{max} moment. This parameter was influenced only by the Mn supply. One can observe a significantly higher hourly absorption rate for $MnSO_4$ (4.7%) than for Mn-EDTA (2.5%) (Figure 4.4b). The glyphosate mixture did not affect the absorption rate.

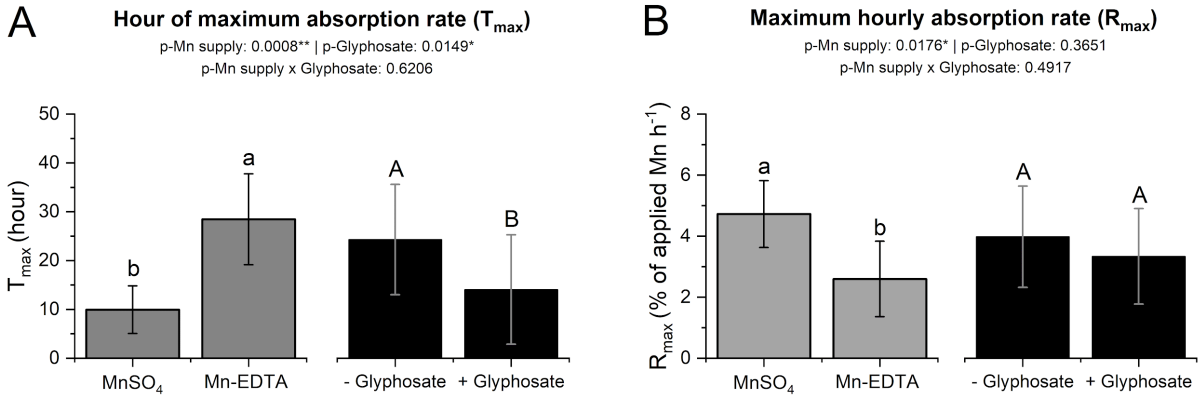

Figure 4.4. Hour of maximum absorption rate (A) and maximum hourly absorption rate (B) as function of Mn supply and glyphosate. Means followed by the same letters are not significantly different for Mn supply (lower case letters) and glyphosate application (capital letters) by the LSD test ($p < 0.05$). Mn-EDTA and glyphosate delayed the hour of maximum Mn absorption. The maximum hourly absorption rate for Mn-EDTA is smaller than that of MnSO4. *significant at $p < 0.05$. **significant at $p < 0.01$

The results found herein for $MnSO_4$, Mn-EDTA and $MnCO_3$ foliar absorption, with and without glyphosate, match with that observed by Machado et al. (2019), which monitored the Mn transport kinetics in petioles of soybean leaves that received the same treatments. Likewise, Machado et al. (2019) found no Mn increment in petioles of leaves treated with $MnCO_3$. Higher Mn intensity was observed when $MnSO_4$ were applied in comparison to Mn-EDTA, and the mixture of glyphosate diminished the absorption of $MnSO_4$, but it did not affect the Mn-EDTA efficiency. These studies are complementary because they show that the decreasing in Mn transport kinetics observed for $MnSO_4$ + glyphosate treated plants are

due to the precipitation of Mn-glyphosate complexes on the leaf surface rather than to the complexation of intracellular Mn by glyphosate.

Figure 4.5 shows the foliar region of a replicate which received the application of a $MnSO_4$ droplet during the 72 hours of analysis. One can observe the appearance of a brownish aspect in the leaf veins beginning 48 hours after application, which is most likely due to Mn phytotoxicity. Meanwhile, the application of $MnSO_4$ + glyphosate caused the formation of a white precipitate on the leaf surface, beginning 12 hours after application (Figure 4.6). This observation match with the sigmoidal fitting found for this treatment since the absorption kinetics reached a plateau from 12 hours after the droplet application (Figure 4.2c). That is, the low solubility Mn-glyphosate complexes are not absorbed by the leaves.

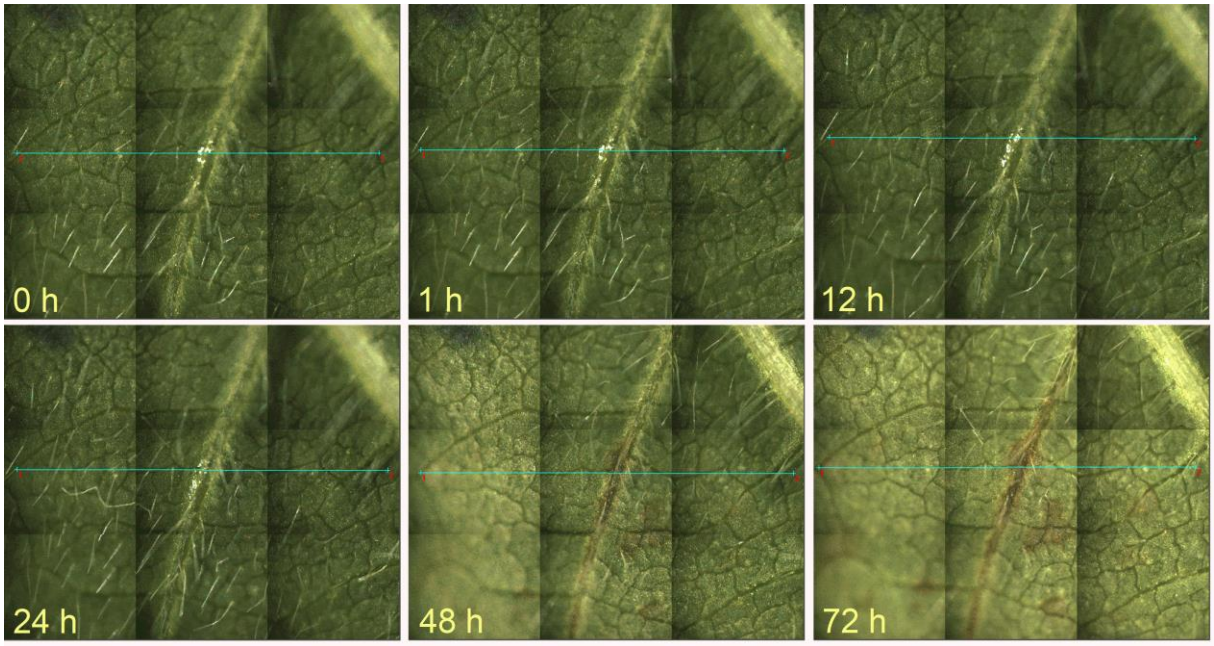

Figure 4.5. Monitoring of the foliar region treated with $MnSO_4$ solution. One can observe the appearance of a brownish aspect in the leaf veins beginning 48 hours after application, which is most likely due to Mn phytotoxicity

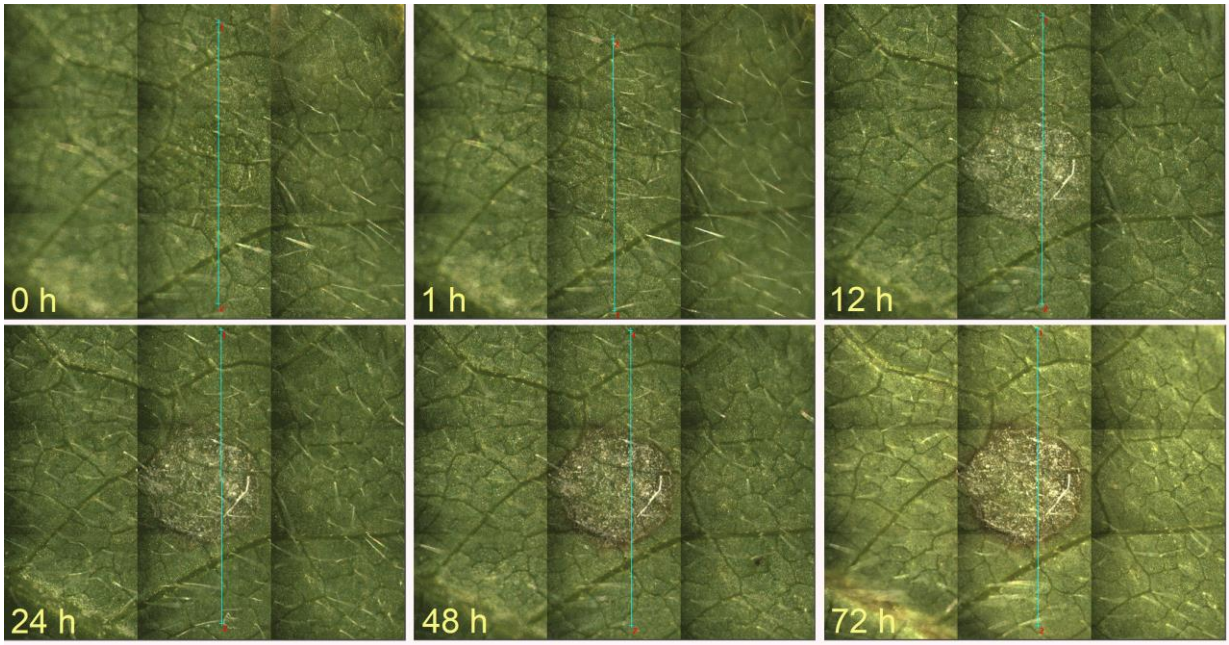

Figure 4.6. Monitoring of the foliar region treated with $MnSO_4$ + glyphosate. One can observe the appearance of a white precipitate beginning 12 hours after the treatment application

4.3.2 Mn concentration and content

Figure 4.7a shows the Mn concentration in a trifoliate leaf already expanded when the second foliar application was done. This leaf was sampled 10 days after the spraying. The data show that Mn concentration was influenced by the Mn source and glyphosate application, without interaction between these factors. As expected, the negative control presented lower Mn concentration than the other treatments. Meanwhile, all plants which received foliar fertilization showed Mn rates as high as the positive control. These results point out two possibilities: (1) the fertilizers are being absorbed and immobilized in the treated leaves, or (2) the washing procedure is not enough to remove all the applied fertilizers from the leaves' surface.

One can notice that leaves that received glyphosate application showed higher Mn concentration. This response also suggests some possibilities. One can state that Mn concentration was improved because glyphosate complexed intracellular Mn (BERNARDS et al., 2005; EKER et al., 2006), preventing its transport to sink organs, however, the intracellular complexation hypothesis was already refuted before (BOTT et al., 2008; MACHADO et al., 2019; ROSOLEM et al., 2010). Therefore, since it was demonstrated that glyphosate can cause striate fissures on the epicuticular waxes crystals of soybean leaves

(MACHADO et al., 2019), its presence may improve the adherence of the fertilizers on the leaves cuticle through these fissures, impairing their removal by the washing procedure.

Figure 4.7b shows the Mn concentration in leaves that developed passed 10 days of the second foliar application. We verified interaction between the Mn supply and glyphosate application. Glyphosate affected the positive control only, increasing the Mn concentration in its presence.

Regarding the Mn supply effects in glyphosate's absence, data show that $MnSO_4$ and Mn-EDTA improved the Mn concentration in the new leaves, just as the positive control. Although $MnCO_3$ did not present statistically difference from the positive control, this is the only treatment which did not differ from the negative control also, suggesting inefficiency on Mn transport from treated leaves to new developed ones.

In the presence of glyphosate, none of the foliar applied fertilizers matched with the positive control regarding Mn concentration in new leaves. $MnSO_4$ and Mn-EDTA improved Mn concentration in comparison to the negative control. While $MnCO_3$ treated plants are statistically equal to the negative control. Glyphosate application increased the Mn concentration in the new leaves for the positive control treatment. It is known that glyphosate is rapidly transported to root and shoot tips (MCALLISTER; HADERLIE, 1985), and this perhaps has influenced the root to shoot Mn transport, but further studies are necessary to understand this response.

Other effects, such as the $MnCO_3$ treatment presenting lower Mn concentration can be related to the low solubility of this source, turning its Mn content less available for leaf uptake, thus, decreasing its transport to new structures. Migliavacca (2018) also cultivated soybean plants in nutrient solution with low Mn availability (0.1 µM Mn), and found no Mn concentration improvement in new shoot structures of plants foliar fertilized with $MnCO_3$. The application of $MnSO_4$, on the other hand, was able to improve the Mn concentration in new leaves.

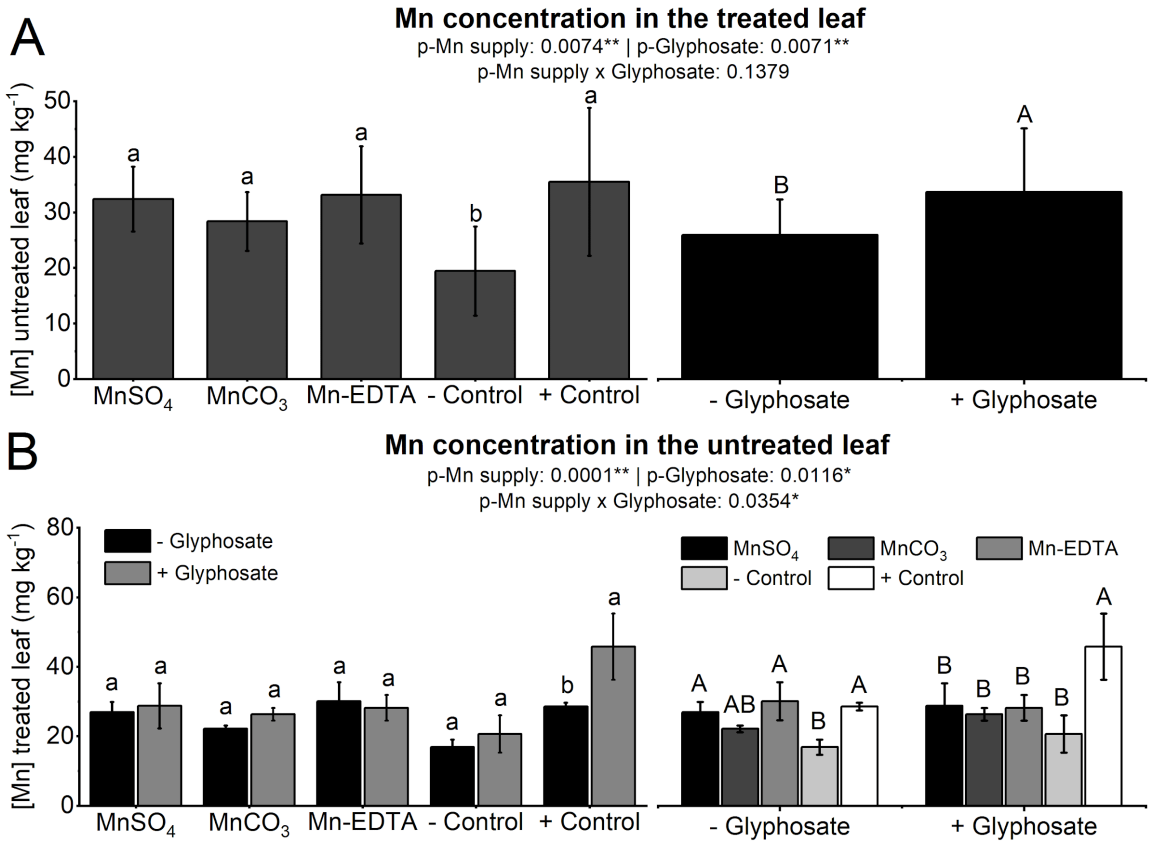

Figure 4.7. Mn concentration in treated (A) and untreated (B). Means followed by the same letters are not significantly different for Mn supply (lower case letters) and glyphosate application (capital letters) by the LSD test ($p < 0.05$). Glyphosate and foliar Mn applications increased the Mn concentration in the treated leaves. The Mn concentration in the untreated leaves responded to the interaction between Mn supply and glyphosate's application. *significant at $p < 0.05$. **significant at $p < 0.01$

Even though the fertilizers presented different performances, only the negative control presented Mn concentration below 20 mg kg^{-1}, which is considered the threshold for adequate soybean development and production (MALAVOLTA; VITTI; OLIVEIRA, 1997). We did not observe visual Mn deficiency symptoms for any treatment (Figures 4.8, 4.9 and 4.10), nor yellowing of the leaves occurred following glyphosate's application, as occasionally reported in some field (BROCH; RANNO, 2012) and greenhouse experiments (ROSOLEM et al., 2010). It seems that the yellowing is highly associated with the soybean cultivar (BROCH; RANNO, 2012).

Figure 4.8. Aspect of three replicates of treatments (A) MnSO4; (B) MnSO4 + Glyphosate; (C) MnCO3; (D) MnCO3 + Glyphosate; (E) Mn-EDTA; (F) Mn-EDTA + Glyphosate. One can notice no visual symptoms of Mn deficiency neither visual biomass differences among the treatments

Figure 4.9. Aspect of three replicates of treatments (A) Negative control; (B) Negative control + Glyphosate; (C) Positive control; and (D) Positive control + Glyphosate, before the first foliar spraying. One can notice no visual symptoms of Mn deficiency neither visual biomass differences among the treatments

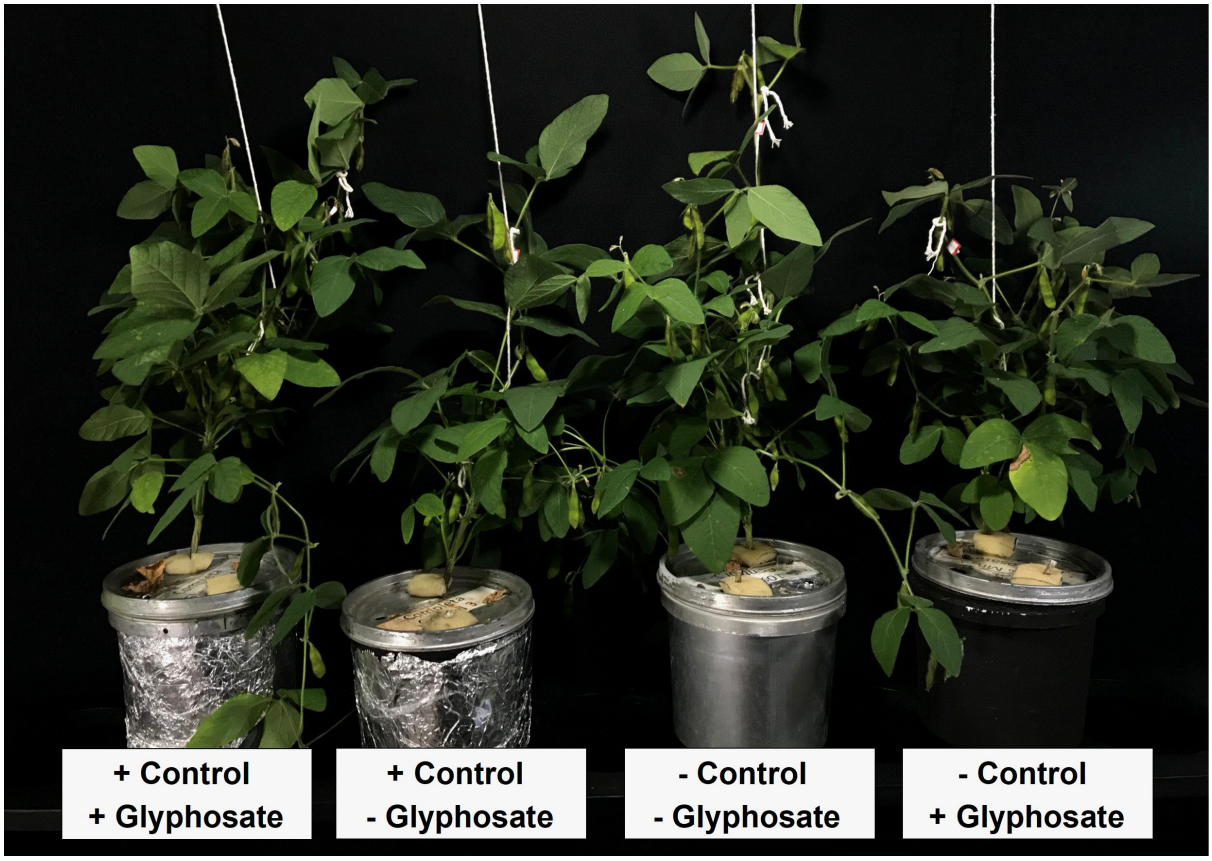

Figure 4.10. Plants at R6 phenological stage. No visual Mn deficiency symptoms were detected nor visual differences in plants' biomass

Migliavacca (2018) found increasing in shoot dry mass when Mn concentration in the nutrient solution was increased from 0.1 to 1 µM, Rosolem et al. (2010) did not found alteration in the biomass using Mn doses from 1 up to 20 µM, and Santos et al., 2017 reported decreasing in shoot biomass as function of the Mn increasing from 2 up to 300 µM. For plants cultivated under Mn deficiency, foliar fertilization with $MnSO_4$ was able to improve the shoot biomass, while $MnCO_3$ was not (MIGLIAVACCA, 2018). In our investigation, neither Mn supply nor glyphosate influenced on shoot dry biomass (Table 4.3).

Table 4.3. Dry mass of the shoot (leaves + stem + pods) at R6 phenological stage as function of the Mn supply and glyphosate addition. No significant differences were found among the factors

Mn supply	Shoot dry mass (g)		
	Glyphosate		Mean
	Without	With	
	Mean value + standard error (n = 3)		
$MnSO_4$	25.33 ± 2.99	24.61 ± 1.10	24.97 ± 2.05
$MnCO_3$	24.93 ± 5.63	24.26 ± 2.58	24.60 ± 3.93
Mn-EDTA	23.08 ± 3.24	23.50 ± 2.05	23.29 ± 2.43
Mn-EDTA + Zn-EDTA	25.75 ± 1.57	24.07 ± 2.50	24.91 ± 2.08
- Control	24.61 ± 4.61	26.84 ± 3.02	25.72 ± 3.69
+ Control	24.06 ± 2.19	25.98 ± 3.69	25.02 ± 2.91
Mean	24.63 ± 3.19	24.88 ± 2.50	24.75 ± 2.83
p-Mn supply	0.86824		
p-Glyphosate	0.82081		
p-Mn supply x Glyphosate	0.88234		
CV (%)	13.26		

Figure 4.11 shows the Mn concentration and content in the entire shoot (leaves + stem + petioles). Since no significant differences were detected for shoot dry mass (Table 4.1), the Mn concentration (Figure 4.11a) and content (Figure 4.11b) presented the same behavior. In both cases, we found interaction between Mn supply and glyphosate. Glyphosate affected only the plants treated with $MnSO_4$, reducing the Mn concentration and content in the shoot when mixed to this fertilizer. In glyphosate's absence, the $MnSO_4$ fertilized plants presented the highest Mn concentration and content. An intermediate Mn status were found for Mn-EDTA, which presented Mn concentration and content equal to the positive control. Plants that received the foliar application of $MnCO_3$ did not show improvement in the Mn status, since they are statistically equal to the negative control.

In the presence of glyphosate, plants fertilized with Mn-EDTA presented the highest Mn concentration and content, being statistically equal to the positive control. Meanwhile, $MnSO_4$ did not differentiate statistically from both positive and negative controls, presenting lower Mn concentration and content than Mn-EDTA. $MnCO_3$ presented the lowest Mn concentration and content, being statistically equal to the negative control and to the plants fertilized with $MnSO_4$.

These results agree with previous reports which found that foliar fertilization with $MnSO_4$ (MACHADO et al., 2019; MIGLIAVACCA, 2018; OHKI et al., 1987) and Mn-EDTA (CORREIA; DURIGAN, 2009; MACHADO et al., 2019; OHKI et al., 1987; STEFANELLO et al., 2011) can raise the Mn status of soybean plants, while $MnCO_3$ seems to be less efficient on providing Mn for leaf uptake (MACHADO et al., 2019; MIGLIAVACCA, 2018). Our data point that Mn foliar application using $MnSO_4$ and Mn-EDTA can be as effective as Mn supply by nutrient solution on improving the Mn concentration in the shoot of soybean plants.

Glyphosate's ability to complex metal cations is well known (BERNARDS et al., 2005b; SUBRAMANIAM; HOGGARD, 1988; SUNDARAM; SUNDARAM, 1997a), and it is the reason why there is a concern about the possibility of glyphosate complexation with intracellular Mn (BERNARDS et al., 2005b; BOTT et al., 2008; EKER et al., 2006). This hypothesis was refuted by some studies (BOTT et al., 2008; MACHADO et al., 2019; ROSOLEM et al., 2010), and occasional deleterious effects of glyphosate application on soybean fields has been attributed to the soybean cultivar (BROCH; RANNO, 2012) and to toxic effects of AMPA (aminomethylphosphonic acid), a metabolite formed through glyphosate's degradation (DUKE et al., 2012b; REDDY; RIMANDO; DUKE, 2004). GR crops are not necessarily resistant to AMPA.

Nevertheless, the intracellular Mn complexation by glyphosate is still a widespread hypothesis mistakenly employed to explain the yellowing of soybean leaves occasionally followed by glyphosate application. In this context, tank mixtures of glyphosate with Mn sources are widely employed in soybean fields, not only because the timing for glyphosate's application coincides with that of Mn, but also as an erroneous alternative to mitigate possible intracellular interactions between Mn and glyphosate. However, attention must be paid for choosing the right Mn source for tank mixtures.

It was found that Mn-ethylaminoacetate, Mn-lignin sulfonate, and $MnSO_4$ can decrease glyphosate's herbicidal action on velvetleaf (*Abutilon theophrasti*). On the other hand, Mn-EDTA, did not affect glyphosate's efficiency (BERNARDS et al., 2005). Since EDTA molecule possess higher stability constant (log K = 13.81) than glyphosate (log K = 5.53) (MADSEN et al., 1978), Mn-EDTA is unlikely to react with glyphosate. The affinity between Mn and EDTA is so high that they were still found bind together even inside the soybean petiole, after foliar uptake, regardless glyphosate mixture (MACHADO et al., 2019).

Our data showed lower shoot Mn concentration at the expense of applying $MnSO_4$ together with glyphosate, pointing out that glyphosate also affects $MnSO_4$ efficiency, as previously reported (MACHADO et al., 2019). But no evidence leads to confirm the intracellular complexation hypothesis. The lower efficiency can be attributed to the formation of Mn-glyphosate complexes in the tank mixtures, which became Mn less available for leaf uptake. The absence of tank mixtures interactions between glyphosate and Mn-EDTA can also explain why glyphosate did not influence Mn-EDTA efficiency on providing Mn for foliar absorption in our study.

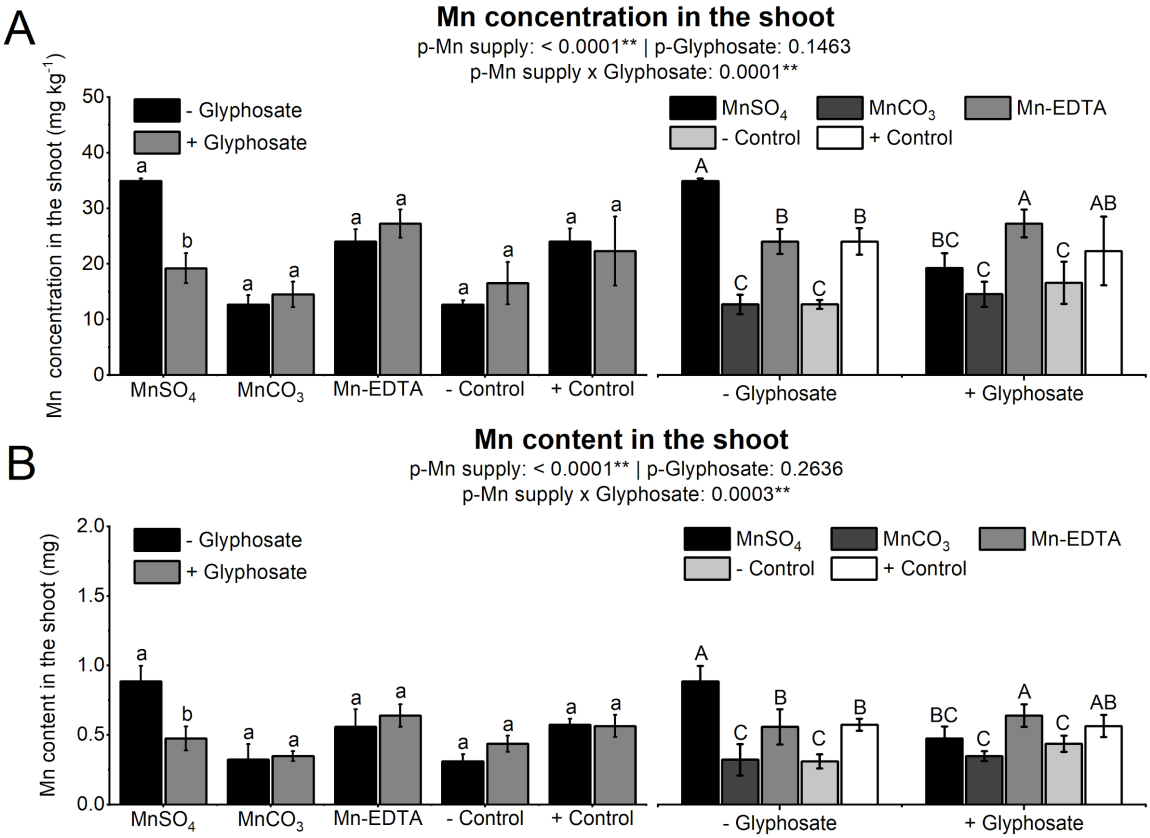

Figure 4.11. Mn concentration (A) and content (B) in the shoot. Means followed by the same letters do are not significantly different for Mn supply (lower case letters) and glyphosate application (capital letters) by the LSD test ($p < 0.05$). Mn concentration and content responded to the interaction between Mn supply and glyphosate's application. *significant at $p < 0.05$. **significant at $p < 0.01$

4.3.3 ROS and antioxidant system

The aerobic cellular metabolism leads to the generation of byproducts derived from the O_2 reduction, known as reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH^-$). About 1-

2% of the whole O_2 consumed are naturally converted to ROS, which can play an ambiguous role on plants' metabolism, depending on their production rate. In low concentrations, ROS work as intracellular signaling agents, while high concentrations can induce the interaction with organic molecules, triggering oxidative damages (MITTLER, 2017). In this context, plants developed a complex antioxidant (AOX) system, composed by enzymatic and non-enzymatic agents that work out to face ROS overproduction and manage cell redox homeostasis (SOARES et al., 2019).

High ROS content and the consequent increasing of AOX enzymes activity can be indicators of plants facing oxidative-induce damages caused by biotic and abiotic stresses, as previously demonstrated for common beans grown in Mg deficient medium (CAKMAK; MARSCHNER, 1992), soybeans affected by white mold (*Sclerotinia sclerotiorum*) (NOVAES et al., 2019), soybeans cultivated under moderate boron toxicity (HAMURCU et al., 2013), and soybeans grown under low (MIGLIAVACCA, 2018) and high (SANTOS et al., 2017) Mn availability.

Herein, we assumed that (1) plants cultivated under low Mn availability present intensive physiological stress indicators; (2) Mn foliar fertilization can reverse stressful conditions related to inadequate Mn supply via roots; and (3) glyphosate can affect Mn foliar fertilization efficiency on mitigating oxidative stresses.

4.3.3.1 Lipid peroxidation

Lipid peroxidation (LP) is closely related to oxidative stress. It basically consists of the oxidative degradation of lipids caused by several events resulted from ROS activity, which compromises cellular membranes' integrity. We quantified the LP through determining the concentration of one of its sub-products, the malondialdehyde (MDA). Data show that both manganese supply and glyphosate application influenced on MDA concentration, but without interaction between these factors (Figure 4.12). Plants treated with $MnSO_4$ and Mn-EDTA exhibit higher MDA concentrations in the leaves. Considering that both $MnSO_4$ and Mn-EDTA were previously demonstrated as effective sources on delivering Mn to soybeans (MACHADO et al., 2019; MIGLIAVACCA, 2018; OHKI et al., 1987) one should expect that these fertilizers could mitigate oxidative stresses caused by Mn deficiency. However, it was also demonstrated that both fertilizers are able to cause visual damages to the leaves (MACHADO et al., 2019; OHKI et al., 1987), in which scanning electron microscopy (SEM) analysis unraveled being caused due to the dissolution of the leaves'

epicuticular wax crystals, and to the collapsing of outer periclinal cell walls (MACHADO et al., 2019). On the other hand, although $MnCO_3$ has shown low efficiency on Mn delivering, SEM analysis showed that this source does not cause severe damages to the leaf epidermis (MACHADO et al., 2019). Hence, the increased MDA concentration in $MnSO_4$ and Mn-EDTA treatments might be linked to damages caused to the membrane integrity after interactions between these sources and the epidermal cells.

Regarding to glyphosate, plants that received this herbicide application presented lower levels of MDA. Moldes et al. (2008) found no significant impact on lipid peroxidation in resistant and susceptible soybean plants exposed to glyphosate, but further studies are necessary to conclude why it can reduce the lipid peroxidation as our results indicate.

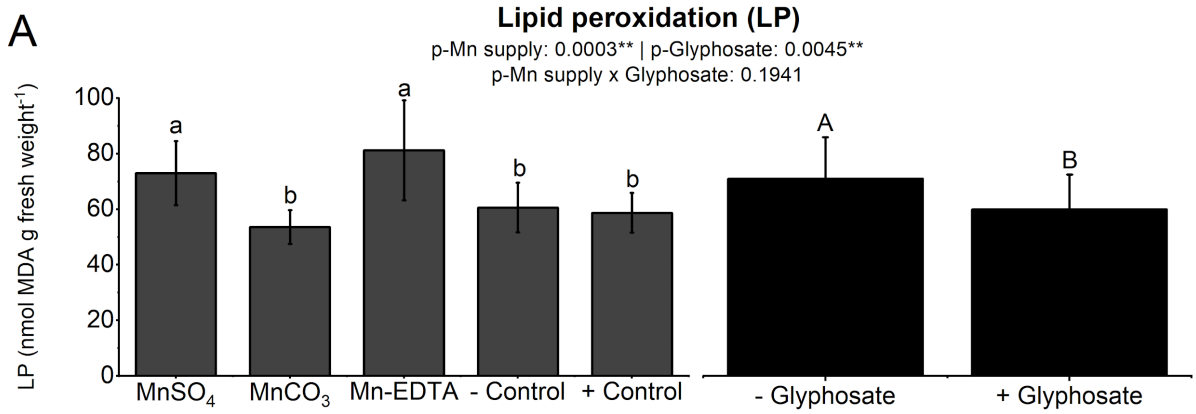

Figure 4.12. Lipid peroxidation in the leaves. Means followed by the same letters are not significantly different for Mn supply (lower case letters) and glyphosate application (capital letters) by the LSD test ($p < 0.05$). One can see higher rates of lipid peroxidation for plants foliar fertilized with $MnSO_4$ and Mn-EDTA. Glyphosate application decreased the lipid peroxidation rate. *significant at $p < 0.05$. **significant at $p < 0.01$

4.3.3.2 Total soluble proteins

The total soluble proteins concentration in the leaves was investigated in order to determine the enzymatic activity. This parameter was influenced only by the Mn supply (Figure 4.13). The negative control plants and those whose where fertilized with $MnCO_3$ presented the lowest concentrations. Plants fertilized with Mn-EDTA and $MnSO_4$ treated plants were not statistically different from both positive and negative controls. A previous proteomic study was able to identify 54% of the total soybean leaves' proteins, pointing out that most of them (37%) are involved in photosynthesis, respiration, and metabolism, while 7% are related to oxidative stresses (MESQUITA et al., 2012). Thus, protein content in the

leaf can indicate high physiological activity. As expected, no glyphosate effect was detected on protein content. Glyphosate acts on preventing the synthesis of aromatic amino acids, slowing down the protein synthesis in susceptible plants (TAN; EVANS; SINGH, 2006), but this response should not be observed in glyphosate resistant soybeans.

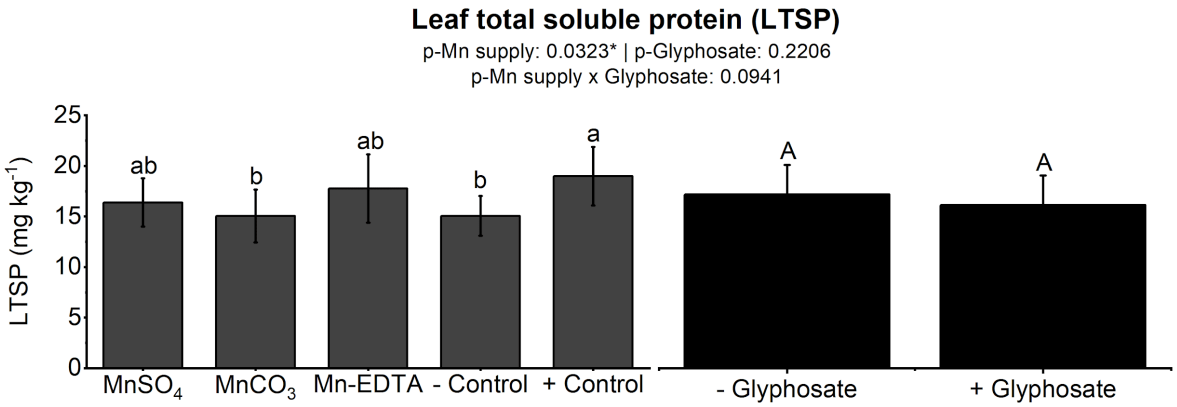

Figure 4.13. Total soluble proteins in the leaves. Means followed by the same letters are not significantly different for Mn supply (lower case letters) and glyphosate application (capital letters) by the LSD test ($p < 0.05$). $MnCO_3$ and the negative control treatments presented lower concentration on total soluble proteins in the leaves. *significant at $p < 0.05$. **significant at $p < 0.01$

4.3.3.3 Antioxidant enzymes activity

There are lots of enzymatic and non-enzymatic components which face reactive species of oxygen in order to avoid potential severe damages to plant cells. In this study, we investigated the activity of three antioxidant enzymes: superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPOX).

4.3.3.3.1 Superoxide dismutase

Superoxide dismutase is found in almost all cellular compartments (MITTLER, 2002). This is an AOX enzyme that acts in the frontline against ROS, though the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. There are three distinct SOD isoforms, each one modulated by a different metal cofactor: Mn, Fe, or Cu/Zn, but here we did not evaluate their isolated forms. Data show significative interaction between Mn supply and glyphosate application in SOD activity (Figure 4.14a).

One can see that glyphosate mixture with $MnSO_4$ increased SOD activity if compared with $MnSO_4$ applied alone. This response suggests that higher SOD activity may be linked to a less availability of Mn due to the formation of low solubility Mn-glyphosate complexes which cannot be absorbed. Thus, tank mixtures of $MnSO_4$ + glyphosate diminish the MnSO4 hability to circumvent oxidative stresses caused by Mn starvation.

In glyphosate absence, $MnSO_4$ is the only source which SOD activity is not significantly different from positive control. Meanwhile, Mn-EDTA and MnCO3 present SOD activity similar to the negative control. When mixed with glyphosate, Mn-EDTA showed itself as the only Mn source which can reduce SOD activity in Mn starved plants.

Our results suggest that Mn-starved plants are facing more stressing conditions, since higher SOD activity was found when Mn supply was low, however, Santos et al. (2017) and Migliavacca (2018) did not find the same. These authors did not observe responses for SOD activity among soybeans cultivated in nutrient solution from 0 up to 2 μmol L^{-1} Mn. Regarding to foliar fertilization, Migliavacca (2018) found no decreasing in SOD activity when Mn-starved soybeans were sprayed with $MnSO_4$ and $MnCO_3$, agreeing with our observations, but this author did not evaluate Mn-EDTA application, which seems to alleviate some stressful conditions.

4.3.3.3.2 Ascorbate peroxidase

Ascorbate peroxidase (APX) catalyzes H_2O_2 into water and monodehydroascorbate (MDHA). Due to its high affinity for H_2O_2 (μM range) this enzyme might be responsible for the fine adjustment of ROS for signaling, which contributes to its presence in almost all cellular compartments (MITTLER, 2002). Little is found about APX activity in soybeans under different types of Mn supply, but increasing in this enzyme activity was found in soybeans' leaves inoculated with white mold (NOVAES et al., 2019) and in leaves of soybeans cultivated in soil with high concentration of CuO nanoparticles (25 nm) (YUSEFI-TANHA et al., 2020).

Our data did not point glyphosate effect on APX activity (Figure 4.12b), but the Mn supply with $MnCO_3$ promoted it. Curiously, the negative control did not differ statistically from the $MnCO_3$ treatment nor from other types of Mn supply. In our study, we believe that the higher rates of APX activity was found mainly due to the low Mn status of the plants (which $MnCO_3$ was not capable to revert) rather than to possible deleterious effects in consequence of nanoparticulate $MnCO_3$ (80 ~ 100 nm) employment. Regarding to

glyphosate, Moldes et al. (2008) did not find any major alterations in APX activity in leaves of susceptible and resistant soybean cultivars 72 hours after glyphosate application, which corroborates our findings.

4.3.3.3.3 Guaiacol peroxidase

Guaiacol peroxidase is present in all living organisms. It can be found in several plant organs and organelles, especially in cytosol, vacuoles and cell wall. This is a heme containing protein which regulates H_2O_2 levels through the oxidation of aromatic electron donors, such as guaiacol and pyragallol (SHARMA et al., 2012).

None of the factors tested in our study influenced GPOX activity (Figure 4.14c), but previous reports found GPOX response for both deficiency (MIGLIAVACCA, 2018) and toxicity of Mn (SRIVASTAVA; DUBEY, 2011). Increased GPOX activity was found in rice seedlings cultivated in substrate with toxic Mn concentrations(SRIVASTAVA; DUBEY, 2011). Migliavacca (2018) found higher GPOX activity in Mn-starved soybean plants, even after foliar fertilization with $MnCO_3$, while the application of $MnSO_4$ was able to decrease GPOX activity. Glyphosate application was found to increase GPOX activity in only one of two glyphosate resistant soybean cultivars evaluated by Moldes et al. (2008).

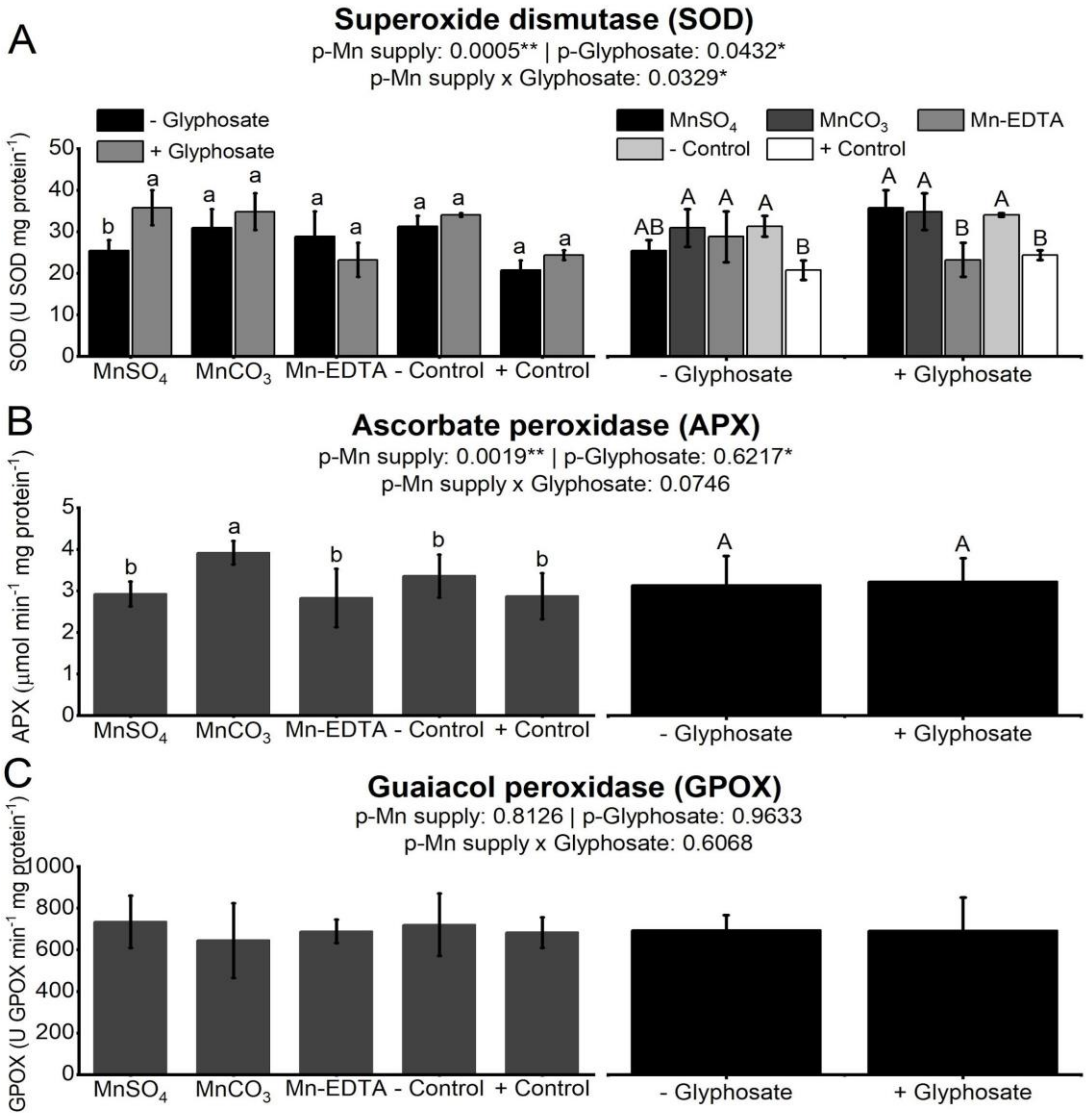

Figure 4.14. Superoxide dismutase (A), Ascorbate peroxidase (B), Guaiacol peroxidase (C) activities in the soybeans' leaves. Means followed by the same letters do are not significantly different for Mn supply (lower case letters) and glyphosate application (capital letters) by the LSD test ($p < 0.05$). SOD activity was higher for $MnSO_4$, $MnCO_3$, negative control, and plants that received glyphosate's application. APX activity was increased for the $MnCO_3$ and negative control treatments. GPOX was not influenced by Mn supply or glyphosate's application. CAT activity responded to the interaction between Mn supply and glyphosate's application. *significant at $p < 0.05$. **significant at $p < 0.01$

4.4 Conclusions

Our results indicate that the loss of Mn efficiency on foliar fertilization due to tank mixtures with glyphosate can be avoided by using an adequate Mn source. The XRF monitoring of foliar absorption shows that, within 72 hours after application, $MnSO_4$ is more absorbed than Mn-EDTA, but $MnSO_4$ + glyphosate mixture presents a significantly lower

efficiency on providing Mn due to precipitation with glyphosate on the leaves. Meanwhile, the performance of Mn-EDTA is not affected by the mixture with glyphosate. Within 72 hours of monitoring, $MnSO_4$ is absorbed more rapidly than Mn-EDTA, regardless of glyphosate mixture. The $MnCO_3$ supplied Mn in the slowest rate. Finally, oxidative stresses caused by Mn starvation can be attenuated by foliar application with soluble sources of Mn, such as $MnSO_4$ and Mn-EDTA, but the mixture of glyphosate with a reactive source like $MnSO_4$ must be avoided, since it diminish the amount of Mn available for leaf uptake.

5 FINAL REMARKS AND OUTLOOK

This study allowed us to draw some conclusions about the effects of glyphosate on soybean manganese foliar uptake. The tank mixture investigation showed that glyphosate can complex free Mn^{2+} ions provided by soluble Mn sources, such as $MnSO_4$ and Mn-glycine, which present lower stability constant than that of Mn-glyphosate. Nearly 30% of the employed Mn can be lost due to precipitation with glyphosate using recommended field doses, being the precipitates found with a Mn:glyphosate molar ratio of nearly 2:1.

The use of a chelated source, such as Mn-EDTA, prevented the formation of precipitates due to its higher stability constant. Meanwhile, decreasing of the pH of the mixture to values below 2.5, or the use of an acid Mn source, such as Mn-phosphite, circumvented the precipitation events because glyphosate molecules remain protonated in low pH conditions. However, one should be attentive whether the final mixture pH will be adequate for agricultural purposes.

The results on foliar uptake, leaf concentration and transport kinetcs of Mn through soybean petioles revealed that tank mixtures with glyphosate can decrease the efficiency of the Mn fertilization when tank reactive sources are employed, such as $MnSO_4$. The use efficiency of Mn-EDTA was not affect by the glyphosate mixture.

Data from XRF, XANES and antioxidant enzymes activity show no evidence of intracelullar Mn-Glyphosate complexation. The results suggests that manganese is transported in a similar chemical environment regardless of the source, likely complexed to organic acids. The exception was Mn-EDTA, which was absorbed and transported in its pristine form. Interferences of glyphosate seem related to complexations in the tank mixture rather than affecting the metabolism of Mn inside plant tissue. Finally, the results showed that $MnCO_3$ precipitated on the leaf surface regardless of glyphosate mixture, providing Mn in the slowest rate due to its low solubility.

6 REFERENCES

ALCÁNTARA-DE LA CRUZ, R. et al. Herbicide resistance in Brazil: status, impacts, and future challenges. London: IntechOpen, 2020. doi: 10.5772/intechopen.91236.

ALEXANDER, A.; HUNSCHE, M. Influence of formulation on the cuticular penetration and on spray deposit properties of manganese and zinc foliar fertilizers. **Agronomy**, v. 6, n. 3, p. 39, 2016. doi: 10.3390/agronomy6030039.

ALMEIDA, L. A. DE et al. Melhoramento da soja para regiões de baixas latitudes. In: QUEIROZ, M. A. DE; GOEDERT, C. O.; RAMOS, S. R. R. (Ed.). **Recursos genéticos e melhoramento de plantas para o Nordeste brasileiro**. Petrolina: Embrapa Semiárido, 1999.

ALT, D. et al. Seed yield and quality of transgenic high- oleic and conventional soybean as influenced by foliar manganese application. **Crop Science**, v. 58, n. 2, p. 874-879, 2018.

ANDRESEN, E.; PEITER, E.; KÜPPER, H. Trace metal metabolism in plants. **Journal of Experimental Botany**, v. 69, n. 5, p. 909-954, 2018.

BARRETT, K. A.; MCBRIDE, M. B. Oxidative degradation of glyphosate and aminomethylphosphonate by manganese oxide. **Environmental Science and Technology**, v. 39, n. 23, p. 9223–9228, 2005.

BASSO, C. J. et al. Foliar application of manganese in transgenic soybean tolerant to glyphosate. **Ciência Rural**, v. 41, p. 1726–1731, 2011.

BELBIN, F. E. et al. Plant circadian rhythms regulate the effectiveness of a glyphosate-based herbicide. **Nature Communications**, v. 10, art. n. 3704, 2019.

BENBROOK, C. M. Trends in glyphosate herbicide use in the United States and globally. **Environmental Sciences Europe**, v. 28, n. 1, p. 3, 2016. doi: 10.1186/s12302-016-0070-0.

BERNARDS, M. L.; THELEN, K. D.; PENNER, D. Glyphosate Efficacy Is Antagonized by Manganese. **Weed Technology**, v. 19, n. 1, p. 27–34, 2005.

BERNARDS, M. L. et al. Glyphosate interaction with manganese in tank mixtures and its effect on glyphosate absorption and translocation. **Weed Science**, v. 53, n. 6, p. 787–794, 2005.

BLAMEY, F. P. C. et al. Manganese distribution and speciation help to explain the effects of silicate and phosphate on manganese toxicity in four crop species. **New Phytologist**, v. 217, n. 3, p. 1146–1160, 2018.

BLAMEY, P. et al. Synchrotron-based techniques shed light on mechanisms of plant sensitivity and tolerance to high manganese in the root environment. **Plant Physiology**, v. 169, p. 2006-2020, 2015. doi: 10.1104/pp.15.00726.

BOTT, S. et al. Glyphosate-induced impairment of plant growth and micronutrient status in glyphosate-resistant soybean (Glycine max L.). **Plant and Soil**, v. 312, n. 1–2, p. 185–194, 2008.

BROCH, D. L.; FERNANDES, C. H. **Resposta da soja à aplicação de micronutrientes**. Maracaju, MS: Fundação MS, 1999. 56 p. (Informativo Técnico, 2).

BROCH, D. L.; RANNO, S. K. Fertilidade do solo, adubação e nutrição da cultura da soja. **Tecnologia e Produção: Soja e Milho 2011/2012**, p. 3–39, 2012. Available at: https://www.fundacaoms.org.br/base/www/fundacaoms.org.br/media/attachments/132/132/newarchive-132.pdf.

BROOKES, G.; BARFOOT, P. Global income and production impacts of using GM crop technology 1996-2014. **GM Crops & Food. Biotechnology in Agriculture and the Food Chain**, v. 7, n. 1, p. 38-77, 2016. doi: 10.1080/21645698.2016.1176817.

BROOKES, G.; TAHERIPOUR, F.; TYNER, W. E. The contribution of glyphosate to agriculture and potential impact of restrictions on use at the global level. **GM Crops & Food. Biotechnology in Agriculture and the Food Chain**, v. 8, n. 4, p. 216-228, 2017. doi: 10.1080/21645698.2017.1390637.

BUHLER, D. D.; BURNSIDE, O. C. Effect of water quality, carrier volume, and acid on glyphosate phytotoxicity. **Weed Science**, v. 31, n. 58, p. 163–169, 1983.

CAIRES, E. F.; FONSECA, A. F. Absorção de nutrientes pela soja cultivada no sistema de plantio direto em função da calagem na superfície. **Bragantia**, v. 59, n. 2, p. 213–220, 2000.

CAKMAK, I. et al. Glyphosate reduced seed and leaf concentrations of calcium, manganese, magnesium, and iron in non-glyphosate resistant soybean. **European Journal of Agronomy**, v. 31, n. 3, p. 114–119, 2009.

CAKMAK, I.; MARSCHNER, H. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. **Plant Physiology**, v. 98, n. 4, p. 1222–1227, 1992.

CHAHAL, G. S. et al. Influence of Water Quality and Coapplied Agrochemicals on Efficacy of Glyphosate. **Weed Technology**, v. 26, n. 2, p. 167–176, 2012.

COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB. **Acompanhamento da safra brasileira**. Brasília, DF, 2020a.

COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB. **Produção agrícola**. **Área plantada e produção nacional**. Brasília, DF, 2020b. Available at: <https://portaldeinformacoes.conab.gov.br/safra-serie-historica-graos.html>.

CORREIA, N. M.; DURIGAN, J. C. Glyphosate and Foliar Fertilization Using Manganese in Transgenic Soybean Crop. **Planta Daninha**, v. 27, n. 4, p. 721–727, 2009.

CRUZ, T. N. M. DA et al. Shedding light on the mechanisms of absorption and transport of ZnO nanoparticles by plants via in vivo X-ray spectroscopy. **Environmental Science: Nano**, v. 4, p. 2367–2376, 2017.

DE ALMEIDA, E. et al. EDXRF for elemental determination of nanoparticle-related agricultural samples. **X-Ray Spectrometry**, v. 48, n. 2, p. 151–161, 2019.

DEFARGE, N.; SPIROUX DE VENDÔMOIS, J.; SÉRALINI, G. E. Toxicity of formulants and heavy metals in glyphosate-based herbicides and other pesticides. **Toxicology Reports**, v. 5, p. 156–163, 2018.

DEMIDCHIK, V. et al. Stress-induced electrolyte leakage: The role of K+-permeable channels and involvement in programmed cell death and metabolic adjustment. **Journal of Experimental Botany**, v. 65, n. 5, p. 1259–1270, 2014.

DILL, G. M. Glyphosate-resistant crops: History, status and future. **Pest Management Science**, v. 61, n. 3, p. 219–224, 2005.

DORDAS, C. Foliar application of manganese increases seed yield and improves seed quality of cotton grown on calcareous soils. **Journal of Plant Nutrition**, v. 32, n. 1, p. 160–176, 2009.

DUKE, S. O. et al. Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. **Journal of Agricultural and Food Chemistry**, v. 60, n. 42, p. 10375–10397, 2012.

DUKE, S. O. The history and current status of glyphosate. **Pest Management Science**, v. 74, n. 5, p. 1027–1034, 2018.

DUKE, S. O.; POWLES, S. B. Glyphosate: a once-in-a-century herbicide. **Pest management science**, v. 64, n. 4, p. 319-325, 2008.

EKER, S. et al. Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (Helianthus annuus L.) plants. **Journal of Agricultural and Food Chemistry**, v. 54, n. 26, p. 10019–10025, 2006.

EMBRAPA SOJA. **Tecnologias de produção de soja - Região Central do Brasil 2014**. 1. ed. Londrina, PR: Embrapa Soja, 2013.

FAGERIA, N. K. et al. Foliar fertilization of crop plants. **Journal of Plant Nutrition**, v. 32, n. 6, p. 1044–1064, 2009.

FAGERIA, N. K. et al. Biofortification of trace elements in food crops for human health. **Communications in Soil Science and Plant Analysis**, v. 43, n. 3, p. 556–570, 2012.

FAO. **FAOSTAT**. Crops and livestock products. Rome, 2020. Available at: <http://www.fao.org/faostat/en/#data/TP>.

FERNANDEZ, V.; EICHERT, T. Uptake of hydrophilic solutes through plant leaves: Current state of knowledge and perspectives of foliar fertilization. **Critical Reviews in Plant Sciences**, v. 28, n. 1–2, p. 36–68, 2009.

FERNANDO, D. R. et al. Characterization of foliar manganese (Mn) in Mn (hyper) accumulators using X-ray absorption spectroscopy. **New Phytologist**, v. 188, n. 4, p. 1014–1027, 2010.

FERRANDON, M.; CHAMEL, A. R. Cuticular retention, foliar absorption and translocation of fe, mn and zn supplied in organic and inorganic form. **Journal of Plant Nutrition**, v. 11, n. 3, p. 247–263, 1988.

FIGUEIRA, B. A. M.; ANGÉLICA, R. S.; SCHELLER, T. Síntese e caracterização de óxido hidróxido de manganês do tipo manganita (γ -MnOOH). **Cerâmica**, v. 54, n. 330, p. 193-197, 2008.

GADANHA JUNIOR, C. D.; MONTEIRO, M. V. M. Aplicação de fitossanitário requer tecnologia específica. **Visão Agrícola**, v. 5, p. 119–123, 2005.

GLASS, R. L. Metal Complex Formation by Glyphosate. **Journal of Agricultural and Food Chemistry**, v. 32, n. 5, p. 1249–1253, 1984.

GOMES, M. H. F. et al. In vivo evaluation of Zn foliar uptake and transport in soybean using X-ray absorption and fluorescence spectroscopy. **Journal of Agricultural and Food Chemistry**, v. 67, n. 44, p. 12172-12181, 2019.

GRAHAM, R. D.; HANNAM, R. J.; UREN, N. C. **Manganese in soils and plants**. Dordrecht: Springer Netherlands, 1988.

GROSSBARD, E.; ATKINSON, D. **The herbicide glyphosate**. London: Butterworths, 1985.

HAGEDORN, O. et al. Surfactant-induced enhancement of droplet adhesion in superhydrophobic soybean (Glycine max L.) leaves. **Beilstein Journal of Nanotechnology**, v. 8, p. 2345–2356, 2017.

HALL, G. J.; HART, C. A.; JONES, C. A. Plants as sources of cations antagonistic to glyphosate activity. **Pest Management Science**, v. 56, n. 4, p. 351–358, 2000.

HAMURCU, M. et al. Induced anti-oxidant activity in soybean alleviates oxidative stress under moderate boron toxicity. **Plant Growth Regulation**, v. 70, n. 3, p. 217–226, 2013.

HEM, J. D. **Chemical equilibria and rates of manganese oxidation** - Chemistry of Manganese in Natural Water. Washington, DC: US Department of the Interior, 1963.

HERNDON, E. M.; MARTÍNEZ, C. E.; BRANTLEY, S. L. Spectroscopic (XANES/XRF) characterization of contaminant manganese cycling in a temperate watershed. **Biogeochemistry**, v. 121, n. 3, p. 505–517, 2014.

HINCHEE, M. A. W. et al. Production of transgenic soybean plants using agrobacterium-mediated DNA transfer. **Nature Biotechnology**, v. 6, n. 8, p. 915–922, 1988.

HORRIDGE, G. A.; TAMM, S. L. Critical point drying for Scanning electron microscopic study of ciliary motion. **Science**, v. 163, n. 3869, p. 817–818, 1969.

INSTITUTO BRASILEIRO DO MEIO AMBIENTE E DOS RECURSOS NATURAIS RENOVÁVEIS - IBAMA. **Os 10 ingredientes ativos mais vendidos**. Brasília, DF, 2018. (Boletim, 2018). Available at: <https://www.ibama.gov.br/relatorios/quimicos-e-biologicos/relatorios-de-comercializacao-de-agrotoxicos#boletinsanuais>. Accessed at: 17 ago. 2020.

JAMES, C. Global Status of Commercialized Biotech/GM Crops in 2017: Biotech Crop Adoption Surges as Economic Benefits Accumulate in 22 years. **Isaaa**, n. 53, p. 1–143, 2017.

JUSTINIANO, W. **Tecnologia de aplicação de defensivos agrícolas**. Campinas, SP: Monsanto, 2014. 9 p. (Informativo de Desenvolvimento Tecnológico, 12). Available at: <http://www.roundupreadyplus.com.br/site/wp-content/themes/rrplus/assets/boletins/boletim_12.pdf>.

KADACHI, A. N.; AL-ESHAIKH, M. A. Limits of detection in XRF spectroscopy. **X-Ray Spectrometry**, v. 41, n. 5, p. 350–354, 2012.

KARIM, M. R. et al. Alleviation of drought stress in winter wheat by late foliar application of zinc, boron, and manganese. **Journal of Plant Nutrition and Soil Science**, v. 175, n. 1, p. 142–151, 2012.

KARNOVSKY, M. J. A Formaldehyde-Glutaraldehyde Fixative of High Osmolality for Use in Electron Microscopy. **The Journal of Cell Biology**, v. 27, p. 137–138, 1965.

KEMPSON, I. M.; HENRY, D.; FRANCIS, J. Characterizing arsenic in preserved hair for assessing exposure potential and discriminating poisoning. **Journal of Synchrotron Radiation**, v. 16, n. 3, p. 422–427, 2009.

KOPITTKE, P. M. et al. Synchrotron-Based X-Ray Fluorescence Microscopy as a Technique for Imaging of Elements in Plants. **Plant Physiology**, v. 178, n. 2, p. 507–523, 2018.

LAST, P. J.; BEAN, K. M. R. Controlling manganese deficiency in sugarbeet with foliar sprays. **The Journal of Agricultural Science**, v. 116, n. 3, p. 351–358, 1991.

LOMBI, E. et al. Fast X-ray fluorescence microtomography of hydrated biological samples. **PLoS ONE**, v. 6, n. 6, p. 4–8, 2011.

LOPES, A. S.; GUILHERME, L. R. G. Fertilidade do solo e produtividade agrícola. In: NOVAIS, R. F. et al. (Ed.). **Fertilidade do solo**. Viçosa, MG: Sociedade Brasileira de Ciência do Solo, 2007. p. 1-64.

MACHADO, B. A. et al. X-ray Spectroscopy Fostering the Understanding of Foliar Uptake and Transport of Mn by Soybean (Glycine max L. Merril): Kinetics, Chemical Speciation, and Effects of Glyphosate. **Journal of Agricultural and Food Chemistry**, v. 67, n. 47, p. 13010–13020, 2019.

MACISAAC, S. A.; PAUL, R. N.; DEVINE, D. M. A Scanning Electron Microscope Study of Glyphosate Deposits in Relation to Foliar Uptake. **Pesticide Science**, v. 31, p. 53–64, 1991.

MADSEN, H. E. L. et al. Stability Constants of Copper(II), Zinc, Manganese(II), Calcium, and Magnesium Complexes of N-(Phosphonomethyl)glycine (Glyphosate). **Acta Chemica Scandinavica**, v. 32a, p. 79–83, 1978.

MALAVOLTA, E.; VITTI, G. C.; OLIVEIRA, S. A. DE. **Avaliação do estado nutricional das plantas**: princípios e aplicações. 2. ed. Piracicaba: Associação Brasileira para Pesquisa da Potassa e do Fósforo, 1997.

MARSCHNER, P. A. **Marschner's mineral nutrition of higher plants**. 3. ed. Amsterdam: Elsevier, 2012. doi: 10.1016/C2009-0-63043-9.

MASCAGNI, H. J.; COX, F. R. Effective rates of fertilization for correcting manganese deficiency in soybeans1. **Agronomy Journal**, v. 77, n. 3, p. 363-366, 1985.

MCALLISTER, R. S.; HADERLIE, L. C. Translocation of 14C-Glyphosate and CO2-Labeled Photoassimilates in Canada Thistle (Cirsium arvense). **Weed Science**, v. 33, p. 153–159, 1985.

MESQUITA, R. O. et al. Method optimization for proteomic analysis of soybean leaf: Improvements in identification of new and low-abundance proteins. **Genetics and Molecular Biology**, v. 35, p. 353–361, 2012. Suppl. 1.

MIGLIAVACCA, R. A. **Absorção foliar de fontes insolúveis de manganês em soja [Glycine max (L .) Merrill]**. 2018. Tese (Doutorado) - Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, 2018.

MITTLER, R. Oxidative stress, antioxidants and stress tolerance. **Trends in Plant Science**, v. 7, n. 9, p. 405–410, 2002.

MITTLER, R. ROS Are Good. **Trends in Plant Science**, v. 22, n. 1, p. 11–19, 2017.

MOLDES, C. A. et al. Biochemical responses of glyphosate resistant and susceptible soybean plants exposed to glyphosate. **Acta Physiologiae Plantarum**, v. 30, n. 4, p. 469–479, 2008.

MONTANHA, G. S. et al. X-ray fluorescence spectroscopy (XRF) applied to plant science: Challenges towards: In vivo analysis of plants. **Metallomics**, v. 12, n. 2, p. 183–192, 2020a.

MONTANHA, G. S. et al. Zinc uptake from ZnSO4 (aq) and Zn-EDTA (aq) and its root-to-shoot transport in soybean plants (Glycine max) probed by time-resolved in vivo X-ray spectroscopy. **Plant Science**, v. 292, p. 110370, 2020b. doi: 10.1016/j.plantsci.2019.110370

MORAES, M. F.; ABREU JUNIOR, C. H.; LAVRES JUNIOR, J. Micronutrientes. In: PROCHNOW, L. I.; CASARIN, V.; STIPP, S. R. **Boas práticas para uso eficiente de fertilizantes**. Piracicaba, SP: International Plant Nutrition Institute, 2010, p. 207-278.

SUBTIL, J. B.; DALL AGLIO, F. Apresentação PLD (Programa de Levantamento de Deficiências) e do PAP (Programa de Avaliação de Produtos) para auxiliar o suprimento nutricional e aumento da produtividade da soja. In: SIMPÓSIO SOBRE ROTAÇÃO SOJA/MILHO NO PLANTIO DIRETO, 1., 2000, Piracicaba, SP. **Anais...** Piracicaba, SP: Associação Brasileira para Pesquisa da Potassa e do Fosfato, 2000.

MOTEKAITIS, R. J.; MARTELL, A. E. Metal chelate formation by n-phosphonomethylglycine and related ligands. **Journal of Coordination Chemistry**, v. 14, n. 2, p. 139–149, 1985.

NABLE, R. O.; BAR-AKIVA, A.; LONERAGAN, J. F. Functional manganese requirement and its use as a critical value for diagnosis of manganese deficiency in subterranean clover (Trifolium subterraneum L. cv. Seaton Park). **Annals of Botany**, v. 54, n. 1, p. 39–49, 1984.

NEUMANN, G. et al. Relevance of glyphosate transfer to non-target plants via the rhizosphere. **Journal of Plant Diseases and Protection**, v. 20, p. 963–969, 2006.

NOVAES, M. I. C. et al. Physiological and biochemical responses of soybean to white mold affected by manganese phosphite and fluazinam. **Acta Physiologiae Plantarum**, v. 41, n. 12, p. 1–16, 2019.

NOWACK, B.; STONE, A. T. Degradation of nitrilotris(methylenephosphonic acid) and related (amino)phosphonate chelating agents in the presence of manganese and molecular oxygen. **Environmental Science and Technology**, v. 34, n. 22, p. 4759–4765, 2000.

OHKI, K. et al. Foliar manganese application to soybeans. **Communications in Soil Science and Plant Analysis**, v. 18, n. 3, p. 243–253, 1987.

PAPADAKIS, I. E. et al. Foliar treatment of Mn deficient “Washington navel” orange trees with two Mn sources. **Scientia Horticulturae**, v. 106, n. 1, p. 70–75, 2005.

PUENTE, D. W. M.; BAUR, P. Wettability of soybean (Glycine max L.) leaves by foliar sprays with respect to developmental changes. **Pest Management Science**, v. 67, n. 7, p. 798–806, 2011.

RAVEL, B.; NEWVILLE, M. ATHENA, ARTEMIS, HEPHAESTUS: Data analysis for X-ray absorption spectroscopy using IFEFFIT. **Journal of Synchrotron Radiation**, v. 12, n. 4, p. 537–541, 2005.

REDDY, K. N.; RIMANDO, A. M.; DUKE, S. O. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. **Journal of Agricultural and Food Chemistry**, v. 52, n. 16, p. 5139–5143, 2004.

RESENDE, A. V. **Adubação da soja em áreas de Cerrado:** micronutrientes. Planaltina, DF: Embrapa Cerrados, 2004. 27 p. (Documentos, 115). Available at: <https://www.embrapa.br/busca-de-publicacoes/-/publicacao/569152/adubacao-da-soja-em-areas-de-cerrado-micronutrientes>.

RODRIGUES, E. S. et al. Laboratory microprobe X-ray fluorescence in plant science: Emerging applications and case studies. **Frontiers in Plant Science**, v. 871, p. 1–15, 2018.

ROSOLEM, C. A. et al. Manganese Uptake and Redistribution in Soybean As Affected By Glyphosate. **Revista Brasileira de Ciência do Solo**, v. 34, n. 1, p. 1915–1922, 2010.

SANISHVILI, R. et al. Radiation damage in protein crystals is reduced with a micron-sized X-ray beam. **Proceedings of the National Academy of Sciences of the USA**, v. 108, n. 15, p. 6127–6132, 2011.

SANTOS, E. F. et al. Physiological highlights of manganese toxicity symptoms in soybean plants: Mn toxicity responses. **Plant Physiology and Biochemistry**, v. 113, p. 6–19, 2017.

SAWAN, Z. M. et al. Direct and Residual Effect of Foliar Applications of Copper and Manganese on Cottonseed Yield and Quality. **Journal of Agricultural and Food Chemistry**, v. 41, n. 10, p. 1756–1759, 1993.

SCHECKEL, K. G. et al. In vivo synchrotron study of thallium speciation and compartmentation in Iberis intermedia. **Environmental Science and Technology**, v. 38, n. 19, p. 5095–5100, 2004.

SFREDO, G. J.; BORKERT, C. M.; OLIVEIRA JUNIOR, A. de; OLIVEIRA, F. A. de; CASTRO, C. de F. Estimativa do nível crítico de manganês trocável, em solos do Paraná. In: REUNIÃO DE PESQUISA DE SOJA DA REGIÃO CENTRAL DO BRASIL, 28., 2006, Uberaba, MG. **Resumos...** Londrina, PR: Embrapa Soja; Fundação Meridional; Fundação Triângulo, 2006a. p. 432-433. (Documentos, 272).

SFREDO, G. J. **Soja no Brasil**: calagem, adubação e nutrição mineral. 1. ed. Londrina, PR: Embrapa Soja, 2008.

SHAHI, S.; SRIVASTAVA, M. Influence of foliar application of manganese on growth, pigment content, and nitrate reductase activity of Vigna radiata (L.) R. Wilczek under salinity. **Journal of Plant Nutrition**, v. 41, n. 11, p. 1397–1404, 2018.

SHARMA, P. et al. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. **Journal of Botany**, v. 2012, art. ID 217037, p. 1–26, 2012. doi: 10.1155/2012/217037.

SHEA, P. J.; TUPY, D. R. Reversal of Cation-Induced Reduction in Glyphosate Activity by EDTA. **Weed Science**, v. 32, n. 6, p. 802–806, 1984.

SHEALS, J.; PERSSON, P.; HEDMAN, B. IR and EXAFS spectroscopic studies of glyphosate protonation and copper(II) complexes of glyphosate in aqueous solution. **Inorganic Chemistry**, v. 40, n. 17, p. 4302–4309, 2001.

SHILLING, D. G.; HALLER, W. T. Interactive effects of diluent pH and calcium content on glyphosate activity on Panicum repens L. (torpedograss). **Weed Research**, v. 29, n. 6, p. 441–448, 1989.

SHKOLNIKOVA, L. M. et al. X-ray structural study of organic-ligands of the complexone type. III. Crystal and molecular-structure of phosphonomethylglycine and iminodiacetic-monomethylphosphonic acid. **Journal of Structural Chemistry**, v. 23, n. 5, p. 737–746, 1983.

SINHA, M. K.; PRASAD, B. Effect of chelating-agents on kinetics of diffusion of zinc to a simulated root-system and its uptake by wheat. **Plant and Soil**, v. 48, p. 599–612, 1977.

SMITH, E. et al. Localization and speciation of arsenic and trace elements in rice tissues. **Chemosphere**, v. 76, n. 4, p. 529–535, 2009.

SOARES, C. et al. Plants facing oxidative challenges - A little help from the antioxidant networks. **Environmental and Experimental Botany**, v. 161, p. 4–25, 2019.

SOCHA, A. L.; GUERINOT, M. LOU. Mn-euvering manganese: The role of transporter gene family members in manganese uptake and mobilization in plants. **Frontiers in Plant Science**, v. 5, p. 1–16, 2014.

SOLTANI, N.; SHROPSHIRE, C.; SIKKEMA, P. H. Short communication: Influence of manganese on efficacy of glyphosate in glyphosate-resistant soybean. **Canadian Journal of Plant Science**, v. 91, n. 6, p. 1061–1064, 2011.

SOUZA, L. A. DE; CUNHA, J. P. A. R. DA; PAVANIN, L. A. Deposição do herbicida 2,4-D Amina com diferentes volumes e pontas de pulverização em plantas infestantes. **Revista Ciência Agronômica**, v. 43, n. 1, p. 78–85, 2012.

SPRANKLE, P.; MEGGITT, W. F.; PENNER, D. Adsorption, mobility, and microbial degradation of glyphosate in the soil. **Weed Science**, v. 23, n. 3, p. 229–234, 1975.

SRIVASTAVA, S.; DUBEY, R. S. Manganese-excess induces oxidative stress, lowers the pool of antioxidants and elevates activities of key antioxidative enzymes in rice seedlings. **Plant Growth Regulation**, v. 64, n. 1, p. 1–16, 2011.

STEFANELLO, F. F. et al. Efeito de glyphosate e manganês na nutrição e produtividade da soja transgênica. **Semina: Ciencias Agrarias**, v. 32, n. 3, p. 1007–1014, 2011.

STEINRÜCKEN, H. C.; AMRHEIN, N. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. **Biochemical and Biophysical Research Communications**, v. 94, n. 4, p. 1207–1212, 1980.

STEPIEN, A.; WOJTKOWIAK, K. Effect of foliar application of Cu, Zn, and Mn on yield and quality indicators of winter wheat grain. **Chilean Journal of Agricultural Research**, v. 76, n. 2, p. 220–227, 2016.

SUBRAMANIAM, V.; HOGGARD, P. E. Metal Complexes of Glyphosate. **Journal of Agricultural and Food Chemistry**, v. 36, n. 6, p. 1326–1329, 1988.

SUNDARAM, A.; SUNDARAM, K. M. S. Solubility products of six metal-glyphosate complexes in water and forestry soils, and their influence on glyphosate toxicity to plants. **Journal of Environmental Science and Health, Part B**, v. 32, n. 4, p. 583–598, 1997.

TAN, S.; EVANS, R.; SINGH, B. Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. **Amino Acids**, v. 30, n. 2, p. 195–204, 2006.

TIAN, S. et al. Supplemental macronutrients and microbial fermentation products improve the uptake and transport of foliar applied zinc in sunflower (Helianthus annuus L.) plants. Studies utilizing micro X-ray florescence. **Frontiers in Plant Science**, v. 5, p. 1–9, 2015.

TRAULSEN, M. L. et al. The effect of electrical polarization on electronic structure in LSM Electrodes: An operando XAS, RIXS and XES study. **Journal of the Electrochemical Society**, v. 164, n. 10, p. F3064–F3072, 2017.

USDA. **World agricultural production**. Washington, DC, 2020. Available at: <https://apps.fas.usda.gov/psdonline/circulars/production.pdf>. Accessed at: 17 Ago. 2020.

VANLAEYS, A. et al. Formulants of glyphosate-based herbicides have more deleterious impact than glyphosate on TM4 Sertoli cells. **Toxicology in Vitro**, v. 52, p. 14–22, 2018.

WATANABE, H. et al. Non-destructive determination of manganese state in rice leaf by x-ray absorption spectrometry. **Soil Science and Plant Nutrition**, v. 36, n. 1, p. 149–152, 1990.

YUSEFI-TANHA, E. et al. Particle size and concentration dependent toxicity of copper oxide nanoparticles (CuONPs) on seed yield and antioxidant defense system in soil grown soybean (Glycine max cv. Kowsar). **Science of the Total Environment**, v. 715, art. ID 136994, 2020. doi: 10.1016/j.scitotenv.2020.136994.