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**Foliar uptake of CuO and CeO<sub>2</sub> nanoparticles by soybean (*Glycine max* L.)**

**Piracicaba**

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*To my son Pedro Henrique Moraes Costa,  
to my parents Maria Isabel Correa Costa and Geovani Tadeu Costa,  
and to my brother Matheus Correa Costa.*



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## ABSTRACT

COSTA JUNIOR, G. T. **Foliar uptake of CuO and CeO<sub>2</sub> nanoparticles by soybean (*Glycine max* L.)**. 2020. 92 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

Nanotechnology presents a huge potential for plant nutrition. In this sense, foliar application of fertilizers has the advantages of delivering nutrients, reducing the environmental impact in relation to soil application. This study has investigated the effects of foliar application of CuO and CeO<sub>2</sub> nanoparticles on soybean plants. Copper was chosen due to its importance as a nutrient for higher plants and all the processes the element participates. Cerium is the most abundant rare earth element in the Earth's crust and its abundance is not that different compared with Zn and Cu, which are much more involved in environmental investigations than Ce. During the study, it was attempted to develop an X-ray spectrometric method to trace the uptake and redistribution of nanoparticles in living plants. Even though this was not possible, such method allowed to access the nutritional status of soybean under vivo conditions. The deposition of copper sources on leaves, such as nano and micro CuO and copper sulphate, caused damages, inducing to absence or scarce presence of platelets crystals. Shoot dry mass of plants was positively affected by the treatments, especially by the 40 nm nCuO. Copper speciation showed that the element undergoes redox reactions from Cu<sup>1+</sup> to Cu<sup>2+</sup> after 14 days of application. The nanoparticles were able to supply Cu and Ce to soybean since these elements were found in other plant tissues that were not directly exposed to the nanoparticles. However, it is not clear whether the particles are dissolved outside the leaves or taken up entirely. The use of cerium nanoparticles did not interfere in agronomic parameters such as biomass production and number of pods.

Keywords: *Glycine max* (L.) Merrill. Mineral nutrition. CuO nanoparticles. CeO<sub>2</sub> nanoparticles. X-ray absorption. ICP OES. ICP-MS.



## RESUMO

COSTA JUNIOR, G. T. **Absorção foliar de nanopartículas de CuO e CeO<sub>2</sub> em soja (*Glycine max* L.)**. 2020. 92 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

A nanotecnologia possui potencial aplicação à nutrição mineral de plantas. A aplicação foliar de fertilizantes tem como uma de suas principais vantagens a aplicação específica do nutriente, reduzindo assim o impacto ambiental em relação à aplicação no solo. Este estudo investigou os efeitos da aplicação foliar de nanopartículas de CuO e CeO<sub>2</sub> em plantas de soja. O cobre foi escolhido devido à sua importância como nutriente e por todos os processos metabólicos que o elemento participa. O cério é o elemento terra rara mais abundante na crosta terrestre, e é sujeito de várias investigações ambientais, mas sua essencialidade no metabolismo ainda não foi demonstrada. Durante este estudo, tentou-se desenvolver um método de espectrometria de raios X para rastrear a absorção e redistribuição de nanopartículas em plantas vivas. Mesmo não sendo possível, tal método permitiu acessar o estado nutricional de plantas de soja sob condições *in vivo*. A deposição de fontes de cobre, como nano e micro CuO e sulfato de cobre, nas folhas causou danos, induzindo a ausência ou presença escassa de cristais. Massa seca da parte aérea foi afetada positivamente pelos tratamentos, especialmente pela nanopartícula de óxido de cobre de 40 nm. A especiação de cobre mostrou que o ele é transformado de Cu<sup>1+</sup> em Cu<sup>2+</sup> após 14 dias de exposição. As nanopartículas foram capazes de fornecer Cu e Ce à soja, uma vez que esses elementos foram encontrados em outros tecidos vegetais que não foram diretamente expostos às nanopartículas. No entanto, não está claro se as partículas são dissolvidas fora das folhas ou absorvidas inteiramente. O uso de nanopartículas de cério não interferiu em parâmetros agrônômicos como produção de biomassa e número de vagens.

Palavras-chave: *Glycine max* (L.) Merrill. Nutrição mineral. Nanopartículas de CuO. Nanopartículas de CeO<sub>2</sub>. Absorção de raios X. ICP OES. ICP-MS



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## 1 INTRODUCTION

There are several definitions for nanomaterials depending on the scientific community that addresses the subject. According to the IUPAC glossary, nanoparticles are those with dimensions of approximately 1 to 100 nm (IUPAC, 2018). Some researchers, however, consider that a material can only be labeled "nano" if, due to its size, the material has different properties from those so-called bulk form, i.e., excitation, emission, chemical reactivity and stability, which are size-dependent properties (KOLAHALAM et al., 2019).

While increasing the surface-to-volume ratio, i.e., decreasing size, gives rise to new properties with technological potential, on the other hand, studies also show that undesirable characteristics such as toxicity may arise. In general, the reactivity of nanomaterials depends on size and shape (SANTEN, 2009).

According to the inventory maintained by the Nanotech Project, around 1,600 commercial products containing nanomaterials are currently available on the market. Table 1 highlights some applications of nanomaterials in agriculture and the food industry.

Table 1. Some examples of commercial products containing nanomaterials used in the food industry and agriculture. Data extracted from Mukhopadhyay (2014)

<b>Product</b>	<b>Application</b>	<b>Institution</b>
Nanocides	Pesticides encapsulated in nanoparticles for controlled release nanoemulsions for greater efficiency	BASF
Buckyball fertilizer	Ammonia from buckyballs	Kyoto University
Food packaging	Airtight plastic packaging with silicate nanoparticles	Bayer AG
Use of agricultural waste	Nanofibers from cotton waste for improved strength of clothing	Cornell University
Nanosensors	Contamination of packaged food	Nestle, Kraft
Precision farming	Nanosensors linked to a global positioning system tracking unit for real-time monitoring of soil conditions and crop growth	US Department of Agriculture

The potential application of nanotechnology in agriculture encompasses gene transfer methods as well as fertilizer and chemical pesticide technology. The oldest and most comprehensive application of nanotechnology is the size reduction of previously existing pesticide emulsions (GREEN; BEESTMAN, 2007).

Except for the nanosuspension and nanodispersion formulations, the use of nanomaterials in agriculture is indeed still modest. However, the possibilities are enormous, and scientific production in this area increases each year. We can highlight the implantation of carbon nanotubes in chloroplasts, leading to a significant increase in electron transport and, consequently, an improvement in photosynthesis yield (GIRALDO et al., 2014); and the treatment of seeds or seedlings with carbon nanotubes that result respectively in increased germination rate and root elongation (HAGHIGHI; SILVA, 2014; TRIPATHI; SONKAR; SARKAR, 2011).

Over the past decade, there has been a fierce debate in the literature about the effects of nanomaterials on living beings. Despite the possible benefits mentioned above, many studies report that nanomaterials can cause alterations in DNA (CUI et al., 2014; GHOSH et al., 2010; RICO et al., 2011; KUMARI; MUKHERJEE; CHANDRASEKARAN, 2009; KUMARI et al., 2011), decreased chlorophyll content (SADIQ et al., 2017) and inhibition of root growth (TAYLOR et al., 2014; DIMKPA et al., 2013; MA et al., 2011; ZHANG et al., 2012; CASTIGLIONE et al., 2011; ASLI; NEUMANN, 2009; LEE et al., 2010).

Leaf absorption of minerals was first confirmed in the 19th century. Since then, foliar application of minerals has been studied in various agricultural crops. One of the main advantages of foliar absorption is the specific application of the mineral, which reduces the environmental impact in relation to soil application. Moreover, the use of foliar fertilization has been increasing in different regions. Factors inherent to the leaf, nutrients, applied solution and external parameters influence the nutrient absorption by the leaves.

Nutrient uptake depends on structure (number of stomata, thin cuticles, hairiness), chemical composition (quality and quantity of wax) and leaf age (as new leaves absorb much more nutrients than adult ones) (EICHERT; FERNANDEZ, 2011). Other factors inherent to the leaf are cuticle moisture and internal ionic concentration (MALAVOLTA, 2006).

Regarding nutrients, leaf absorption of nutrients is regulated by ion mobility and its metabolization, solubility, ion diameter and hydration. Table 2 shows the classification of nutrients as to their mobility in phloem (mobility as redistribution). Mobile ions are rapidly absorbed and transported to other parts of the leaf and redistributed from the residence organ to other parts of the plant.

Table 2. Nutrients classification regarding their mobility in plants (WHITE, 2011)

<b>Mobility</b>		
<b>High</b>	<b>Intermediate</b>	<b>Low</b>
Potassium	Iron	Calcium
Magnesium	Zinc	Manganese
Phosphorus	Copper	Boron*
Sulfur	Boron*	
Nitrogen	Molybdenum	
Chlorine		

\*Boron mobility varies depending on the species.

Nutrient application by foliar spraying should be done in uniform sprays and in tiny droplets, and the solutions should be sprayed on the most absorbed leaf face. The excess causes dripping or dripping of the solution to the lower leaves and the soil, causing poor distribution of the solution on the leaves, waste of nutrients that will not be used by the foliage, and washing, which removes nutrients from the leaves. However, the results are often varied and not reproducible due to the lack of knowledge of various factors that affect the absorption process (FERNANDEZ; EICHERT, 2009).

Copper (Cu) is an essential element for plants, participating in a series of enzymatic processes such as prosthetic group. Cu gets in contact with the root predominantly by mass flow, with  $\text{Cu}^{2+}$  being the form readily available for absorption (MALAVOLTA, 2006). Chelating agents, such as citric, tartaric, malic, oxalic acids and phenols, may also help copper absorption (MALAVOLTA, 2006).

The absorption process occurs in a metabolically active manner (membrane transporters).  $\text{Zn}^{2+}$  inhibits the process competitively, which is reduced by respiratory inhibitors and by  $\text{H}_2\text{PO}_4^-$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{NH}_4^+$ . Root and leaf absorption processes occur by similar mechanisms (MALAVOLTA, 2006).

Cu has high affinity for sulfhydryl, peptide, carboxylic and phenolic groups and, therefore, 98% of the copper found in the cytoplasm is complexed and the free  $\text{Cu}^{2+}$  and  $\text{Cu}^+$  contents are very low (BROADLEY et al., 2011a). Redistribution occurs via phloem, with copper bound to organic compounds such as amino acids. In case of deficiency, copper has low mobility for organ drains, and therefore, deficiency symptoms appear primarily in younger leaves (strong drains) (MALAVOLTA, 2006).

Malavolta (2006) and Broadley et al. (2011a) report that the main functions of copper in plants are:

- (i) Participate in the structure of proteins such as azurine, stercyanin, umecianin, glycoproteins
- (ii) Constituent of enzymes such as ascorbate oxidase, polyphenol oxidase, laccase, plastocyanine, diamine oxidase, cytochrome oxidase, ribulose diphosphate carboxylase
- iii) Participates in physiological processes such as electron flux in the light phase and the activation of ribulose diphosphate carboxylase in the dark phase of photosynthesis, respiration, hormonal regulation, indirect effect of biological nitrogen fixation and metabolism of secondary compounds.

In addition to these functions, Cu plays an important role in disease resistance, which may be due to the fungistatic role of the element or its function in lignin synthesis, pollen grain structure and uniform grain maturation, thus aiding the harvest process (MALAVOLTA, 2006; BROADLEY et al., 2011a).

Cerium (Ce) is one of the elements known as rare earth elements (REE). This name was given due to the supposed scarcity of these elements in the earth's crust, however it is already known that certain REEs are more abundant than some metals, such as silver (ENGHAG, 2004). Among the REEs, cerium is the most abundant and the 25th most abundant element in the earth's crust (ROCIO et al., 2012).

Although abundant, these elements are difficult to extract from the rocks. Modern techniques of cation exchange, fractional crystallization and liquid-liquid extraction are used, which makes production more expensive (ROCIO et al., 2012).

The most relevant applications of cerium include oxidizing agent, polishing powder, glass and ceramic dyes, catalysts in self-cleaning furnaces and oil refinery fluids (ROCIO et al., 2012). In 2012, the estimated annual production of cerium oxide nanoparticles ( $n\text{CeO}_2$ ) was 1,000 tons (PICCINNO et al., 2012). China, which has the largest natural deposit of these elements (37%) (ROCIO et al., 2012), was the first to use this and other REEs as fertilizers in their crops (HU et al., 2002).

The study of the effects of REE is still incipient in the literature. An example of this is that the book Marschner's Mineral Nutrition of Higher Plants, Broadley et al. (2011b) devote only one paragraph to the use of cerium in agriculture.

Even though there are controversies about the biological effects of REEs, it is a fact that traces of these elements are found in phosphate fertilizers marketed in Brazil and in low concentrations they may be beneficial depending on the species. However, studies with cerium applications in Brazilian crops, including soybean, are still few.

The first idea of this study was to develop a method to detect the nanoparticles in plants under *in vivo* conditions. Such method was not able for this purpose but it was possible to achieve the plant nutritional status for some plant nutrients. Methods of direct analysis of solids diminish the number of steps in the analytical sequence and minimize or even eliminate the generation of chemical waste. In this context, X-ray fluorescence spectroscopy (XRF) arises as an alternative and promising technique for foliar diagnosis, presenting attractive features such as multielemental and simultaneous capabilities, non-destructive analysis, minimal or no sample preparation and equipment portability.

### 1.1 Hypotheses

This study has tested the following hypotheses:

- i) Cu and Ce elements from nanoparticulate sources may be absorbed by soybean leaves.
- ii) The plant transports these elements as complexes or chelates instead of oxides.
- iii) The element applied to the leaf is redistributed to other parts of the plant.
- iv) The application of nanoparticulate Cu and Ce influence soybean agronomic parameters such as shoot mass gain and number of pods.

### 1.2 Objectives

This study aimed at investigating the absorption, effects on the plant and the assimilation of nanomaterials absorbed by the leaves of plants. To this end, it was used CuO and CeO<sub>2</sub> nanoparticles as materials, and *Glycine max* (L.) Merrill as a model organism.

Specifically, it was investigated:

- i) What were the effects of nanomaterials applications on plant development.
- ii) If Cu and Ce elements present in nanomaterials were absorbed by the leaves.
- iii) If absorbed by the leaves, were they redistributed to different leaves/organs.

It was also aimed at developing a spectrometric method to monitor the movement of the foliar applied nutrients under *in vivo* conditions.

### 1.3 Structure of the thesis

In addition to the introductory text, this thesis contains more three chapters:

Chapter 2: Direct determination of mineral nutrients in soybean leaves under vivo conditions by portable X-ray fluorescence spectroscopy. This chapter was issued from our attempts to develop a method to detect the redistribution of Cu after foliar application. However, it did not work since the amount of Cu redistributed was below the limits of detection of the technique. On the other hand, the method allowed assessing the concentration of several nutrients in the plant tissue. Thus, due to its novelty and importance to assess the nutritional status of soybean plants the results were included in this thesis. The data was published in the *Journal X-Ray Spectrometry* in 2019. DOI: 10.1002/xrs.3111.

Chapter 3: Foliar uptake of nano copper oxide in soybean plants. [This chapter will be sent to publication]. In this chapter it was carried out a chemical, physiological and morphological study in order to evaluate if Cu nanoparticles can improve copper uptake and possible conjugation with organic acids comparing to soluble sources, such as CuSO<sub>4</sub>

Chapter 4: Foliar uptake of nano cerium oxide in soybean plants. [This chapter will be sent to publication]. This chapter regards the study about the effects of a rare earth element on soybean plants. Although Ce is not considered essential to plants, some studies have shown that in a proper concentration Ce can improve plant growth and development, increase yields and alleviate environmental stresses.

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## 2 DIRECT DETERMINATION OF MINERAL NUTRIENTS IN SOYBEAN LEAVES UNDER *VIVO* CONDITIONS BY PORTABLE X-RAY FLUORESCENCE SPECTROSCOPY<sup>1</sup>

### Abstract

A portable energy dispersive X-ray fluorescence spectrometer furnished with a Rh X-ray tube was evaluated for the determination of macro and micronutrients in soybean leaves (*Glycine max* (L.) Merrill). XRF instrumental parameters were optimized in a univariate way and emission intensities were measured for 60 s and under vacuum for macronutrients, and during 180 s, under air and 305 Al + 25.1  $\mu\text{m}$  Ti filter, for micronutrients. Fresh and dried leaves were irradiated and it was possible to identify P, K, Ca, S, Mn, Fe, Cu and Zn  $K\alpha$  emission lines. For comparative purpose, the samples were also microwave-assisted digested and analyzed by ICP OES. In general, linear correlations between K, Ca, Mn, Fe, Cu and Zn mass fractions in the tested samples and the corresponding pXRF intensities were obtained. The linear determination coefficients ( $R^2$ ) ranged from 0.42 to 0.86. In addition, the limits of detection were suitable for plant nutrient diagnosis. It is demonstrated that pXRF is a simple and powerful tool for analysis of plant materials.

**Keywords:** foliar diagnosis, mineral nutrition, soybean leaves, portable X-Ray fluorescence spectrometry

### 2.1 Introduction

Foliar diagnosis has been used for a long time (LUNDEGARDH, 1943; THOMAS, 1937) to evaluate plant nutritional status and still is a matter of study nowadays, partly due to promising recent advances in analytical techniques allowing multielemental (GUERRA et al., 2015; 2018; OLIVEIRA; RAPOSO; GOMES NETO, 2009), direct (GUERRA et al., 2015; 2018) and non-destructive (GUERRA et al., 2018) analysis.

Usually, essential elements determination in most routine laboratories requires sample pre-treatments. The samples are washed, dried and grinded with further wet acid decomposition in open or closed systems (GUERRA et al., 2015; TREVISAN et al., 2008). Finally, the solution can be analyzed using spectroscopic techniques such as visible spectrophotometry

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for B, P and S, atomic absorption spectrometry for K, Ca, Mg, Fe, Cu, Mn, Zn and Na, or inductively coupled plasma optical emission spectrometry (ICP OES) for multielemental and simultaneous P, K, Ca, Mg and S as well as Mn, Fe, Cu, Zn, Mo and B determinations. Some of the main drawbacks faced by this approach regard the great number of steps involved in the process and the time elapsed from all analytical procedures, i.e. collecting the samples, and getting the results may take in practice from one to two weeks.

Precision agriculture presents potential to increase crop yield. Hence, efforts have been made towards the development of quick, precise and accurate methods that allow assessing the plant and soil (GEBBERS; SCHIRRMANN, 2015; MCLAREN et al., 2012) nutritional status in field. This analytical strategy will permit the readily correction of nutritional deficiency. Methods of direct analysis of solids diminish the number of steps in the analytical sequence and minimize or even eliminate the generation of chemical waste. In this context, X-ray fluorescence spectroscopy (XRF) arises as an alternative and promising technique for foliar diagnosis, presenting attractive features such as multielemental and simultaneous capabilities, non-destructive analysis, minimal or no sample preparation and equipment portability. The samples can be analysed as powders, pellets or even under *vivo* conditions (DURAN et al., 2017; TOWETT; SHEPHERD; LEE, 2016).

XRF is available in wavelength or energy dispersive detection modes (JENKINS, 1999). It includes millimeter range and micro-focused excitation beam benchtop units, total reflection geometry and last but not least, the portable devices. The main differences between these variants regard the lateral resolution, limits of detection and portability (WEST et al., 2017). Portable XRF (pXRF) equipment can be manipulated in field and in principle; it may be even boarded to agricultural machinery. In addition to faster results, in field XRF measurements might become a tool for precision agriculture, allowing farmers to improve the sampling grid and therefore, leading to site-specific nutrient management.

Laser-induced breakdown spectroscopy (LIBS) is another complementary tool that presents promising features aiming at the direct analysis of solid samples. It is fast, multielemental, allows simultaneous determinations and the instruments are also available as portable devices (GUERRA et al., 2015; CAMPOS et al., 2015; CARVALHO et al., 2015a; 2015b; GOMES et al., 2013; GUERRA et al., 2013; 2014; SOUZA et al., 2013).

LIBS (GUERRA et al., 2015; CARVALHO et al., 2015b; AWASTHI et al., 2017; EL-DEFTAR et al., 2015; GONDAL et al., 2016) and XRF (GUERRA et al., 2018; ABEROUMAND, 2009; BLONSKI et al., 2007; CHUPARINA; AISUEVA, 2011; DALY; FENELON, 2018; DEMIR et al., 2010; KHRAMOVA et al., 2012; KHUDER et al., 2009; MCLAREN; GUPPY; TIGHE, 2012; NAYAK et al., 2011) have been employed for the determination of macro and micronutrients in different plant leaves, e.g., sugarcane (GUERRA et al., 2015; CARVALHO et al., 2015b; MELQUIADES et al., 2012), *cannabis* (EL-DEFTAR et al., 2015), black tea (GONDAL et al., 2016), tomato (CARVALHO et al., 2015b; DEMIR et al., 2010), medicinal plants (ABEROUMAND, 2009; CHUPARINA; AISUEVA, 2011; KHUDER et al., 2009), orange (BLONSKI et al., 2007), lemon (BLONSKI et al., 2007), wheat (MCLAREN; GUPPY; TIGHE, 2012), soybean (MCLAREN; GUPPY; TIGHE, 2012), cotton (MCLAREN; GUPPY; TIGHE, 2012), maize (MCLAREN; GUPPY; TIGHE, 2012) and grass (DALY; FENELON, 2018). However, in all the above-mentioned studies, prior to analysis, the leaves were either detached from the plants (GUERRA et al., 2018) or pelletized after washing, drying and grinding (ABEROUMAND, 2009; BLONSKI et al., 2007; CHUPARINA; AISUEVA, 2011; DEMIR et al., 2010; KHRAMOVA et al., 2012; KHUDER et al., 2009; NAYAK et al., 2011).

In view of the analytical capabilities of XRF, the present study aims at demonstrating the feasibility and challenges of portable XRF to assess the nutritional status of soybean leaves under *vivo* and dried conditions. For this purpose, we used a commercial portable energy dispersive X-ray fluorescence spectrometer. The elements of agricultural importance focused in this study were P, S, K, Ca, Mn, Fe, Cu and Zn.

## **2.2 Materials and Methods**

### **2.2.1 Soybean cultivation**

Four seeds of soybean (*Glycine max* (L.) Merrill), cultivar KWS RK 7214 IPRO, were sown in pots containing sand, watered exclusively with deionized water, for two weeks. After, they were transferred to 2.2 L pots containing Hoagland & Arnon's hydroponic solution (HOAGLAND; ARNON, 1950) at 20% until reaching the V3 phenological stage. The plants were finally transferred to regular Hoagland & Arnon's solution for three days. All cultivation assay was carried out in a light room at 24° C and 50% humidity. After this period, the second leaf (all three leaflets) was selected in each plant for direct analysis by using the pXRF.

For comparative purpose, the same leaves were removed from each plant and oven-dried at 65° C until constant weight (~48 hours) and further analysed by pXRF. Subsequently, the leaves were ground in a MA 048 model knife mill (Marconi, Piracicaba, Brazil) and the elemental concentrations were determined by ICP OES after microwave-assisted acidic digestion as described in detail below.

Afterwards, the same sample plants were placed in a Hoagland & Arnon's hydroponic solution at a concentration 10 times higher than a normal one for 3 days. Then, a subsequent superior leaf (third one) was analysed following the same procedures as above mentioned.

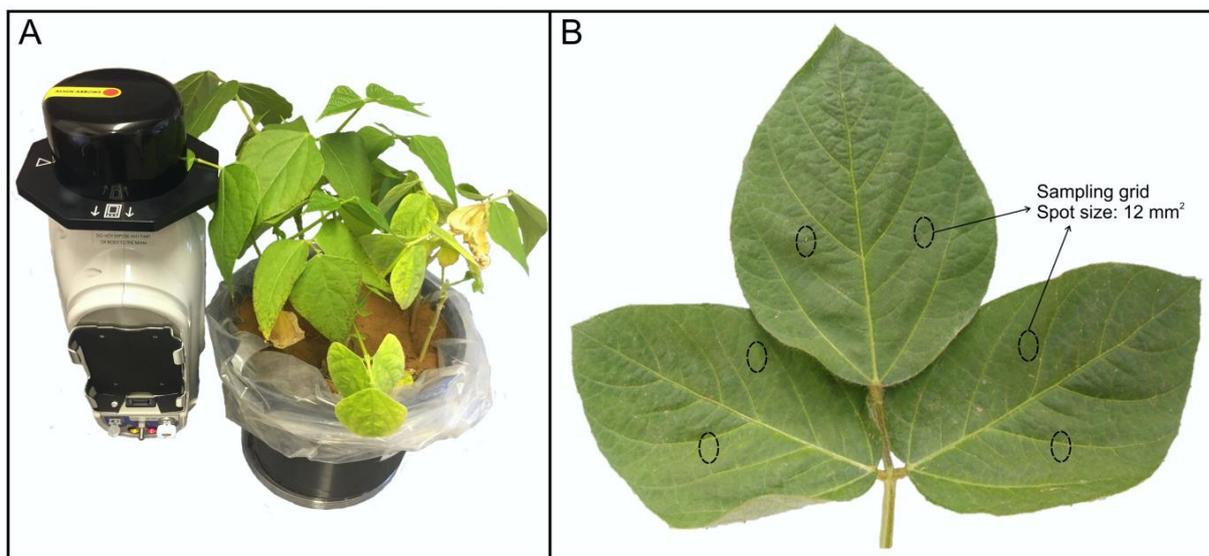
### **2.2.2 Portable XRF analysis**

The measurements were carried out using a portable X-ray fluorescence spectrometer, Tracer III – SD model (Bruker, USA), equipped with a 4 W Rh X-ray tube and 10 mm<sup>2</sup> X-Flash® Peltier-cooled Silicon Drift detector (SDD), operated at 30 µA and 40 kV.

The development of a pXRF method for multi-elemental analysis involved the optimization of dwell time (30, 60, 120, 150, 180 and 300 s), number of spots per leaf (2 to 6), analysis under vacuum or air and the use of primary filters (305 µm Al/25.4 µm Ti and 25.4 µm Ti). These filters are the ones readily available for the portable Bruker Tracer III-SD equipment. These parameters were optimized to yield higher signal-to-noise ratio and lower coefficient of variation (CV) for P, S, K, Ca, Mn, Fe, Cu and Zn.

A schematic overview of the proposed protocol analysis of soybean leaves is shown in Figure 1. It is important to highlight that the leaves were not detached from the petiole, as shown in Figure 1A. The abaxial face of each trefoil was directly analysed (n=2) at one spot per side of the blade by using the pXRF spectrometer (Figure 1B).

Figure 1. (A) *In vivo* analysis of a plant by pXRF equipment; (B) Sampling grid showing the 6 spots for the direct analysis of soybean leaves



All data were acquired using the software Bruker S1PXRF<sup>®</sup>. The data were obtained through the deconvolution process using the Artax<sup>®</sup>. For each plant the same spot was analysed in triplicate.

### 2.2.3 Elemental Sensitivity

The elemental sensitivities were determined separately using a set of thin film calibration standards purchased from Micromatter Co (Vancouver, BC, Canada), ranging from silicon ( $Z=14$ ) to strontium ( $Z=38$ ). It is shown in Table 1 the superficial concentration ( $\mu\text{g cm}^{-2}$ ) of Si, P, S, K, Ca, Sc, Ti, Mn, Fe, Cu, Zn, Se and Sr thin film standards.

Table 1. Concentration of thin film X-ray calibration standard (MicroMatter, Canada)

Serial number	Composition	Element	Superficial concentration ( $\mu\text{g cm}^{-2}$ )
6321	SiO	Si	32.18
6322	GaP	P	14.00
6323	CuS <sub>x</sub>	S	12.00
6324	KCl	K	26.69
6325	CaF <sub>2</sub>	Ca	30.90
6326	ScF <sub>3</sub>	Sc	21.87
6327	Ti	Ti	43.30
30111	Mn	Mn	60.00
6329	Fe	Fe	49.40
6323	CuS <sub>x</sub>	Cu	42.30
6330	ZnTe	Zn	16.16
30112	Se	Se	49.50
30113	SrF <sub>2</sub>	Sr	32.70

#### 2.2.4 Signal-to-noise ratio

For the pXRF method optimization, the signal-to-noise ratios were evaluated. It was determined by dividing the characteristic X-ray net intensities by the background square root (GUERRA et al., 2014; BACHIEGA et al., 2019).

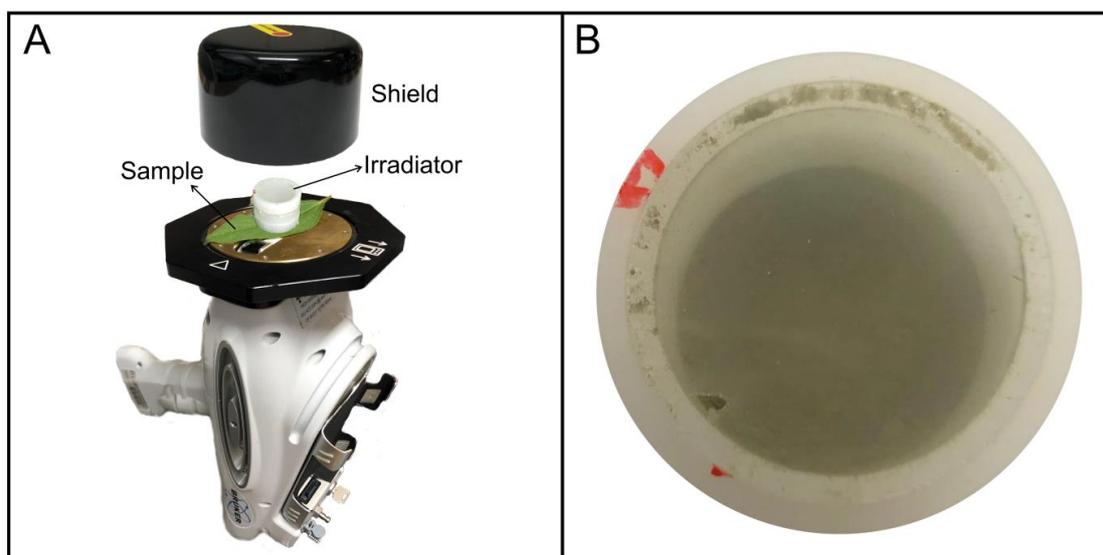
#### 2.2.5 Emission-transmission (ET) method

For quantitative determination of elements, the ET method was chosen (BLONSKI et al., 2007; LEROUX; MAHMUD, 1966). An irradiator was prepared using a set of P.A. salts containing macro (P, S, K and Ca) and micronutrients (Mn, Fe, Cu and Zn). The salts were dried at 60°C for 24 h and ground in an agate mortar. Subsequently, 2 g of the irradiator was added in X-ray cup (Chemplex, n°. 1530), sealed with a 5  $\mu\text{m}$ -polypropylene thin film (Spex, n°. 3520) on both sides of the sample cup. The irradiator was manually pressed with an acrylic piston. The irradiator composition is shown in Table 2 and the ET method scheme and irradiator cup in Figure 2.

Table 2. Composition (%) of the irradiator used in emission-transmission quantitative method

Salt	Concentration (%)
$\text{Na}_3\text{PO}_4$	22.70
$\text{Zn}(\text{PO}_4)_2$	9.10
$\text{K}_3\text{PO}_4$	18.20
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	9.10
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	13.60
$\text{FeSO}_4$	9.10
$\text{CaNO}_3$	18.20

Figure 2. (A) Emission-transmission method scheme; (B) Irradiator sample cup



Equation 1 was used to determine the element concentration in wet basis ( $C_w$ ):

$$C_w = \frac{I^{sam}}{A \cdot S \cdot d} \quad \text{Equation 1}$$

where:

$C_w$ : concentration of the element in the wet basis ( $\text{mg kg}^{-1}$ )

$I^{sam}$ : characteristic X-ray net intensity of the element  $i$  in the sample (cps)

$A$ : absorption factor (unitless)

$S$ : sensitivity ( $\text{cps} \cdot \text{cm}^2 \cdot \mu\text{g}^{-1}$ )

$d$ : surface density of the sample ( $\text{g cm}^{-2}$ )

The sensitivity (S) was calculated using Equation 2 and the values are presented in Figure 3:

$$S = \frac{I^{elem}}{C_s} \quad \text{Equation 2}$$

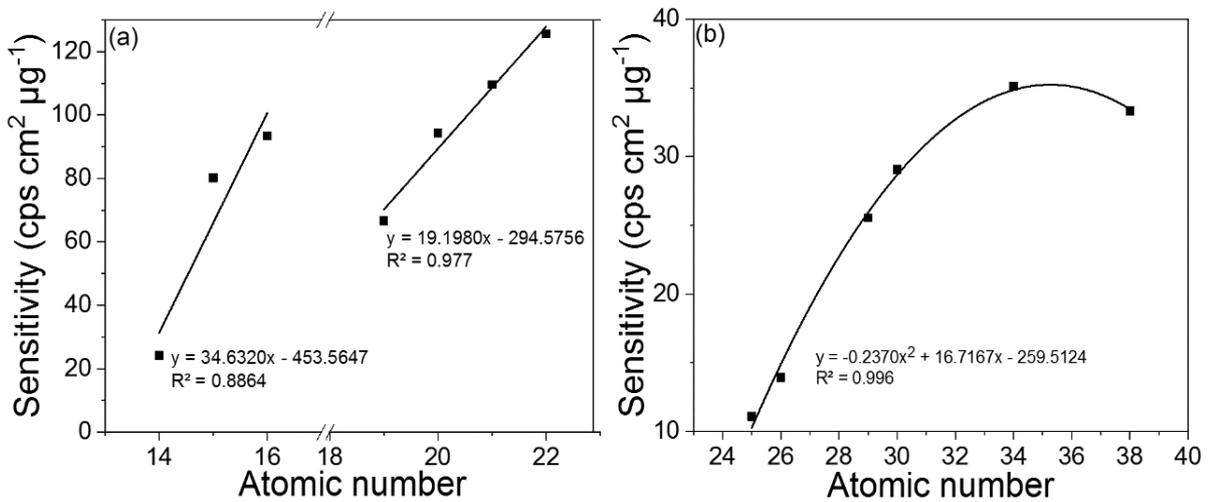
where:

S: sensitivity ( $\text{cps}\cdot\text{cm}^2 \mu\text{g}^{-1}$ )

$I^{elem}$ : characteristic X-ray net intensity of the element i in the thin film calibration standards from Micromatter Co (cps)

$C_s$ : superficial concentration of the element i in the thin film calibration standards from Micromatter Co ( $\mu\text{g cm}^{-2}$ )

Figure 3. Dependence of elemental sensitivity ( $\text{cps cm}^2 \mu\text{g}^{-1}$ ) on atomic number. (a) from 14 to 22. Conditions of analysis: 60 seconds, under vacuum and no filter; (b) from 25 to 38. Conditions of analysis: 180 seconds, under air and filter composed by  $305 \mu\text{m Al}/25.4 \mu\text{m Ti}$



For intermediate thickness samples, the dimensionless absorption factor (A) was determined through the Equation 3:

$$A = \frac{1-H}{-\ln(H)} \quad \text{Equation 3}$$

with:

$$H = \left( \frac{I_{\text{irrad+sam}} - I_{\text{sam}}}{I_{\text{irrad}}} \right)$$

where:

$I^{\text{irrad}}$ : characteristic X-ray intensity of the element  $i$  in the irradiator (cps)

$I^{\text{irrad+sam}}$ : characteristic X-ray intensities of the element of interest  $i$  in the irradiator transmitted by the sample plus the element  $i$  in the sample (cps)

$I^{\text{sam}}$ : characteristic X-ray intensity of the element  $i$  in the sample (cps)

To convert the concentration found in wet basis to the most used dry basis ( $C_d$ ), Equation 4 was used:

$$C_d = \frac{C_w TM}{DM} \quad \text{Equation 4}$$

where:

$C_d$ : concentration of the element  $i$  in the dry basis ( $\text{mg kg}^{-1}$ )

$C_w$ : concentration of the element  $i$  in the wet basis ( $\text{mg kg}^{-1}$ )

TM: total (wet) mass of the sample (g)

DM: dry mass of the sample (g)

### 2.2.6 pXRF validation

For validation of the proposed pXRF method, a pellet of NIST SRM 1547 (Peach leaves) standard reference material (SRM) was prepared by transferring 50 mg of the powdered material to a 15 mm die set and applying  $8.0 \text{ t cm}^{-2}$  for 5 minutes.

### 2.2.7 Microprobe XRF analysis

The dried leaves, previously analysed by the pXRF, were analysed by a microprobe XRF spectrometer (Orbis PC EDAX, USA). In this device, the excitation X-ray beam was provided by a Rh anode operating at 45 kV and 900  $\mu\text{A}$ , using a 1 mm collimator and dwell time of 5 s. The X-ray fluorescence photons were detected by a 30  $\text{mm}^2$  silicon drift detector (SDD) with a dead time smaller than 7 %. To improve the sensitivity towards light elements the measurements were carried out under vacuum. The maps were collected using a matrix of 32 x 25 points.

### 2.2.8 Comparative method (ICP OES)

After XRF analysis, the dried leaves were ground in a knife mill and weighed (n=3 leaflets per plant). The samples were submitted to microwave-assisted acidic digested in closed TFM<sup>®</sup> vessels (Provecto Analitica DGT 100 plus) according to the following procedure: 70 to 100 mg were accurately weighed in the TFM<sup>®</sup> vessels and then 6.0 mL of 20% v v<sup>-1</sup> HNO<sub>3</sub> and 2.0 mL of 30% w w<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> were added. The microwave heating program consisted of 4 steps: a) 7 minutes at 400 W; b) 15 minutes at 850 W; c) 7 minutes at 320 W and; d) 2 minutes at 0 W. After cooling, the residual solutions were transferred to 25 ml volumetric flasks, and the volume made up with high-purity de-ionized water (resistivity < 18.2 MΩ cm). The resulting solutions were analysed by ICP OES, with a dual view (iCap 7400 Duo optical emission spectrometer, Thermo Scientific, Waltham, MA, USA). The analysis conditions of the ICP OES are presented in Table 3. SRM NIST1547 (Peach leaves) was used for quality control in the ICP OES analysis.

Table 3. Characteristics and operational conditions of the analysis carried out in ICP OES (iCap 7400 Duo optical emission spectrometer, Thermo Scientific, USA)

Characteristics	Operational Conditions
View	Radial
Detector	Wavelength range: 166-847 nm
<i>Operational parameters</i>	
Exposure time	10 s
RF power	1.2kW
Plasma gas-flow rate	12 L min <sup>-1</sup>
Auxiliary gas-flow rate	0.5 L min <sup>-1</sup>
Nebulizer gas-flow rate	0.6 L min <sup>-1</sup>
Emission lines	Ca 422.673 nm Cu 324.754 nm Fe 259.940 nm K 766.490 nm Mn 257.610 nm Zn 213.856 nm

### 2.2.9 Limits of Detection

The limits of detection (LODs) for ICP OES analysis were estimated based on the IUPAC recommendation (CURRIE, 1968):

$$LOD = \frac{3.3s}{b}$$

where:

s: estimated standard deviation of the blank signal measurement

b: slope of the calibration curve

LODs for XRF analysis were estimate as:

$$LOD = \frac{3\sqrt{(BG/t)}}{A \cdot S \cdot d}$$

where:

BG: Element background (cps)

t: acquisition time (s)

A: absorption factor (unitless)

S: sensitivity (cps·cm<sup>2</sup> μg<sup>-1</sup>)

d: surface density of the sample (g cm<sup>-2</sup>)

Background (BG) data for K, Ca, Mn, Fe, Cu and Zn K $\alpha$  net intensities were calculated from the spectra obtained by 4 randomly chosen measurements of a leaf. This procedure yielded coefficients of variation below 5% for the elements.

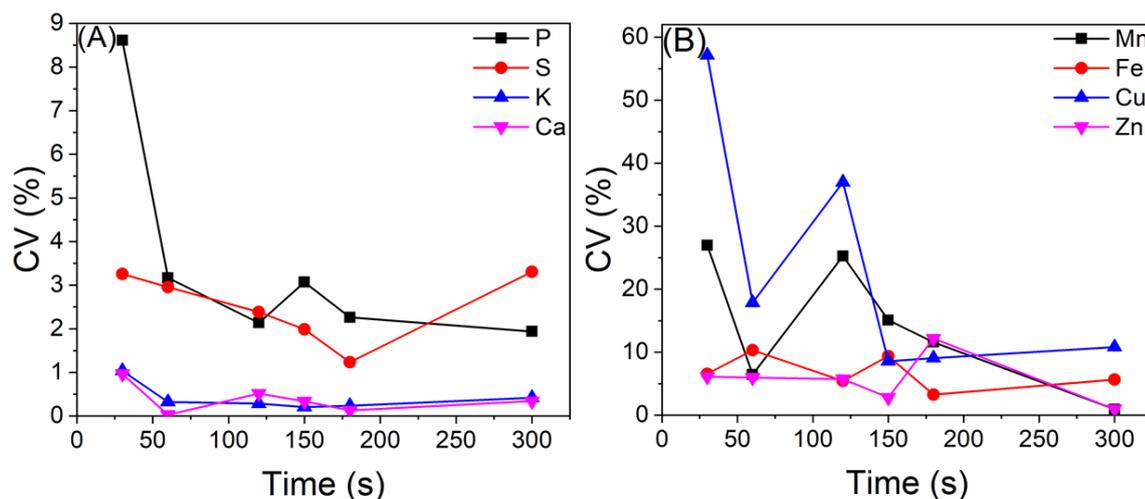
## 2.3 Results and Discussion

### 2.3.1 Optimization of experimental parameters

XRF operational parameters, such as time of analysis, number of spots, measurements under vacuum or air and the use of filters were defined by univariate optimization using a fresh leaf. For this purpose, the voltage and current were fixed at 40 kV and 30  $\mu$ A, respectively. The improvement of results was based on the values of CV for 3 measurements and signal-to-noise ratios (SNRs).

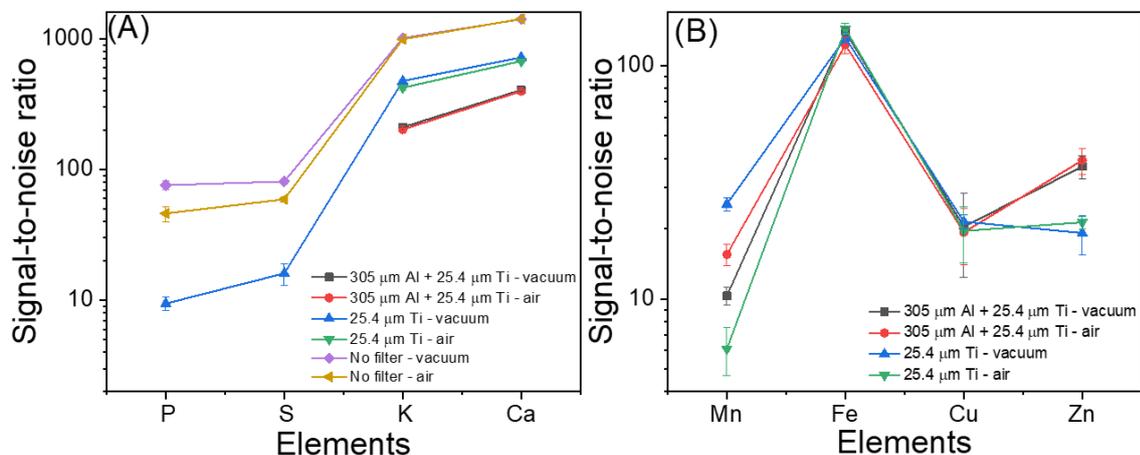
Dwell times from 30 to 300 s were evaluated and the CVs varied from 0.03 to 8.60% for macronutrients and from 0.95 to 57.00% for micronutrients (Figure 4). The shortest dwell times that resulted in the lowest CVs, for most elements, were 60 and 180 seconds for macro (< 5%) and micronutrients (< 15%), respectively.

Figure 4. Evaluation of coefficient of variation (CV) as function of time for macro and micronutrients in soybean leaves. (A): macronutrients; (B): micronutrients



The effects of vacuum and filters on the signal-to-noise ratios (SNRs) for macronutrients (P, S, K and Ca) are shown in Figure 5A and for micronutrients (Mn, Fe, Cu and Zn) in Figure 5B. For macronutrients, the vacuum improved the SNR for phosphorus and sulphur; on the other hand, its effect on the results for K and Ca was negligible. This happens because the air attenuation of low energy characteristics X-rays emitted by P and S are higher than those for K and Ca. In Figure 5B, the higher value for Fe can be explained by its high concentration in plant tissue.

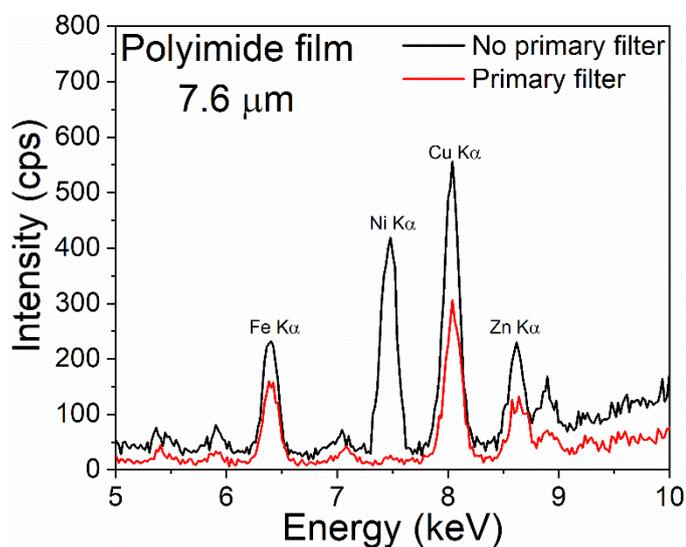
Figure 5. Evaluation of signal-to-noise ratio (SNR) for macronutrients (A) and micronutrients (B) using different filters (no filter, 305  $\mu\text{m}$  Al/25.4  $\mu\text{m}$  Ti and 25.4  $\mu\text{m}$  Ti) and under vacuum and air condition. Time of analysis: 300 s



The filters strongly absorbed the Rh  $L_{\alpha 1}$  (2.697 keV) and Rh  $L_{\beta 1}$  (2.834 keV) lines from the X-ray tube. Such lines are highly effective for P and S excitation, whose corresponding absorption K edges are at 2.146 and 2.472 keV. In general, the results indicated that SNRs increased with analysis under vacuum and no filter. This condition was selected for further macronutrient determinations.

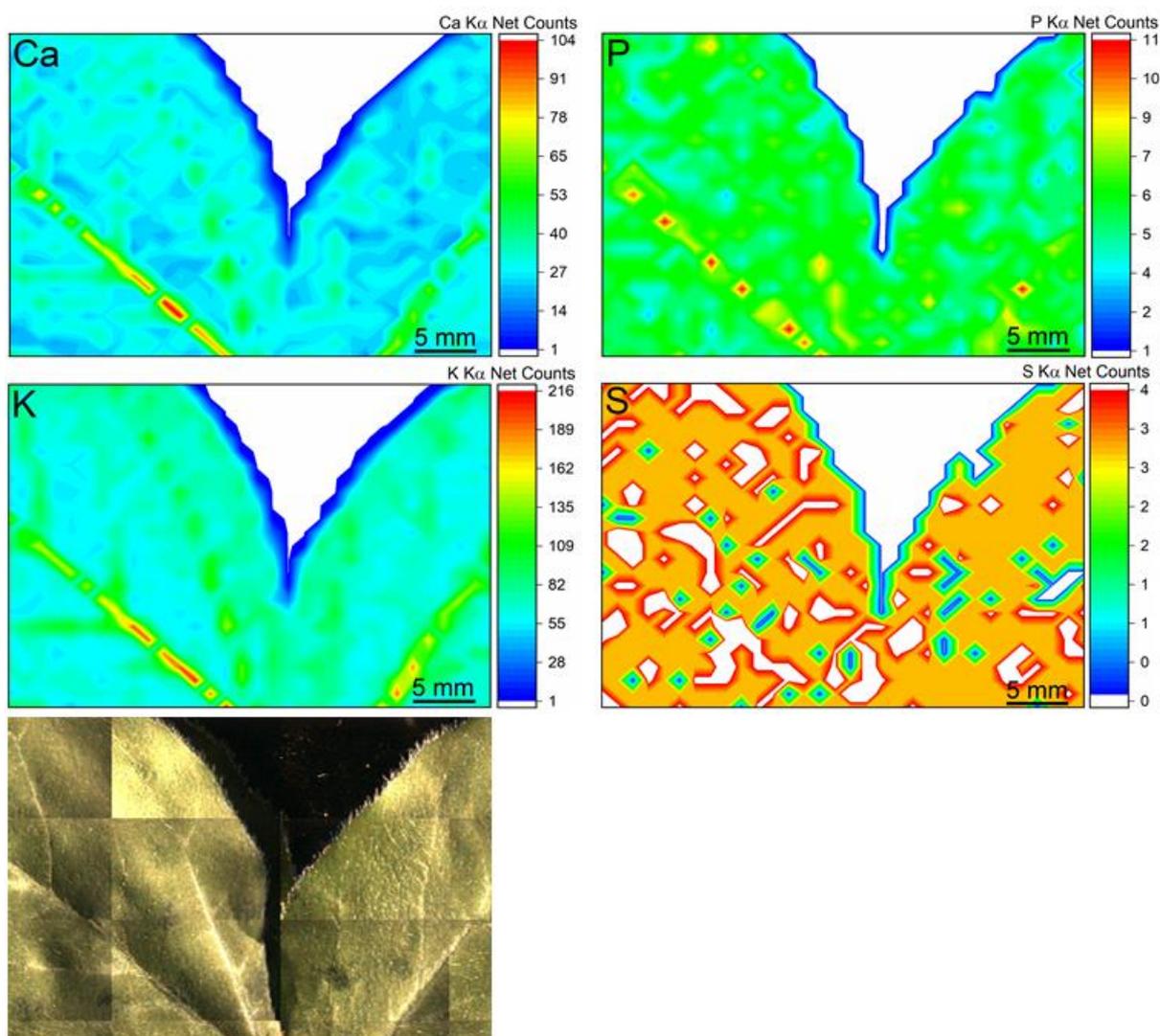
Figure 6 presents the scattered bremsstrahlung produced by the X-ray tube with and without a primary film in the micronutrients X-ray emission energy range. One can notice that the equipment contains spurious signals of Fe, Ni, Cu and Zn (without primary film), but the intensities of these elements are reduced or extinguished (Ni) due to the use of the filter. This background allied to the low content of these micronutrients rendered difficult to perform their quantitative analysis, particularly for Cu. In this case, the filter composed by 305  $\mu\text{m}$  Al/ 25.4  $\mu\text{m}$  Ti (under air) was chosen based on the higher SNRs of Mn and Zn, compared to the 25.4  $\mu\text{m}$  Ti filter. For Fe and Cu, both filters, in the under air condition, had similar SNRs.

Figure 6. Kapton film analysed by the portable equipment. XRF analysis without primary filter



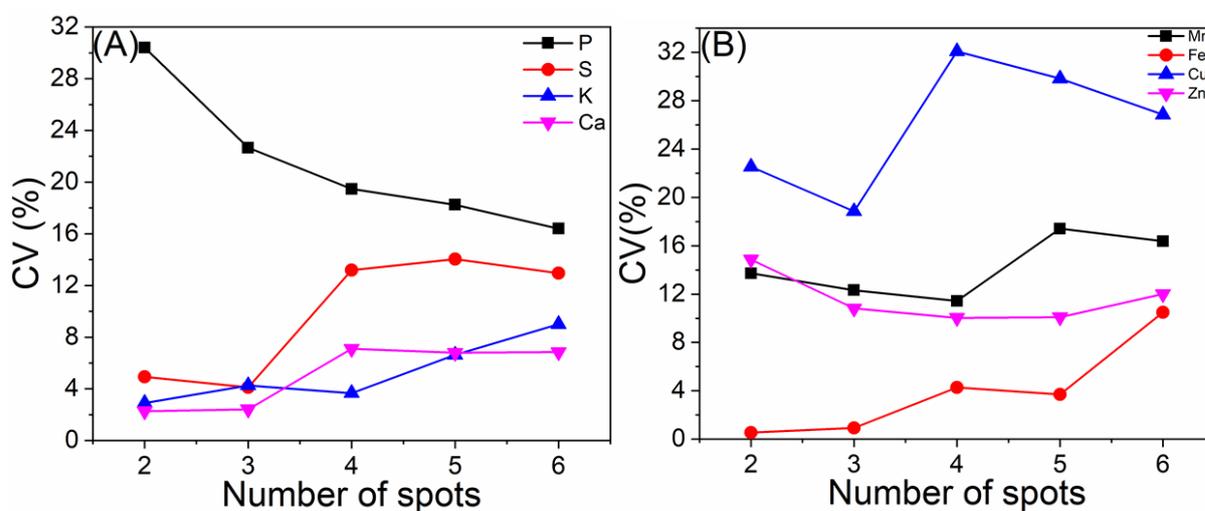
In order to define the minimum number of spots that represent the whole leaf, the influence of number of spots on the CV (%) was verified. The sampling representativeness was based on the micro chemical maps of P, S, K and Ca in 25 mm x 32 mm leaf fragment area for assessing the spatial distribution of the analytes (Figure 7). A heterogeneous elemental distribution over the diagnostic leaf is observed.

Figure 7. Distribution of P, S, K and Ca on soybean leaf revealed by microprobe X-ray fluorescence



The influence of number of spots for macronutrients determination is shown in Figure 8A. The CV (%) decreased for P and increased for K, as we increased the number of spots analysed. S and Ca had a similar pattern in which the variance increased from 2 to 4 spots, and then the variance remained stable. For micronutrients, Zn was the least influenced by the number of points, while the variance for Mn and Fe increased from 4 to 6 spots (Figure 8B). The disparity in the coefficient of variation of elements can be attributed to the heterogeneity of the sample and/or to the low concentration of the elements. The latter feature presented was critical for Mn, Fe, Cu and Zn.

Figure 8. Evaluation of number of spots in leaves of soybean for the determination of (A) macronutrients and (B) micronutrients. Spot size: 12 mm<sup>2</sup>

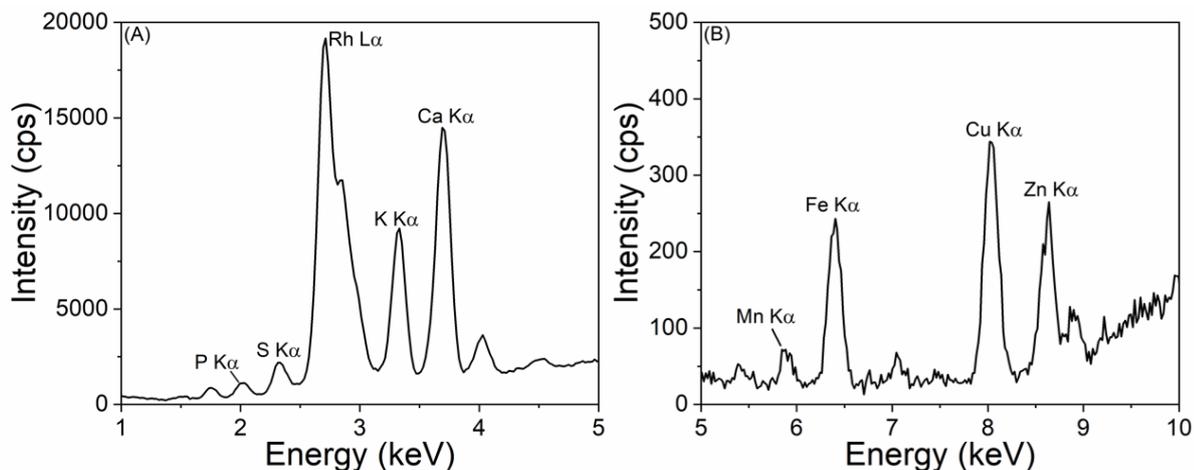


The optimization experiments suggested that two distinct instrumental conditions were necessary to determine macro and micronutrients. Aiming at the simultaneous determination of macronutrients (P, S, K and Ca) in soybean leaves, further experiments were carried out with 60 s dwell time, under vacuum and without primary filter. For micronutrients determination (Mn, Fe, Cu and Zn) samples were irradiated for 180 s, under air and using the 305  $\mu\text{m}$  Al/25.4  $\mu\text{m}$  Ti filter. These experimental conditions were employed in the quantitative assessment of the elements in the soybean leaves.

### 2.3.2 Determination of elements in soybean leaves

Figure 9 presents typical XRF spectra recorded at soybean leaves during the study. It was possible to identify the following X-ray emission lines in the spectrum obtained in the diagnostic leaf: P K $\alpha$  2.01 keV, S K $\alpha$  2.31 keV, K K $\alpha$  3.31 keV, Ca K $\alpha$  3.69 keV, Mn K $\alpha$  5.90 keV, Fe K $\alpha$  6.40 keV, Cu K $\alpha$  8.04 keV and Zn K $\alpha$  8.63 keV.

Figure 9. Fragments of pXRF spectra obtained from the fresh soybean leaf. (A) Sample analysed for 60 seconds, under vacuum, no filter; (B) Sample analysed for 180 seconds, under air and filter composed by 305  $\mu\text{m}$  Al/25.4  $\mu\text{m}$  Ti



Due to the low energy of P and S characteristic X-rays, the emission from the irradiator was more than 90% attenuated by the leaf itself, i.e. the leaf is considered infinitely thick for these two elements. Therefore, the emission-transmission method was applicable only to K, Ca, Mn, Fe, Cu and Zn.

The trueness checking of the pXRF ET method and conventional ICP OES were accomplished through the analysis of the NIST SRM 1547. No significant differences between Mn, Fe, and Zn certified concentrations and the results obtained by pXRF were found by applying the Student's t-test at 95% confidence level. For pXRF, it was not possible to check the method for K and Ca because the pellet, even using a mass of just 50 mg, is considered as infinitely thick sample for these two elements.

Figure 10 compares the quantitative results obtained by pXRF against those from ICP OES. Figure 10A shows the pXRF measured concentrations for leaves under *vivo* conditions and 10B for dried leaves of plants fed with regular nutrient solution. Figure 10C presents the data for leaves under *vivo* conditions and 10D for dried leaves treated with 10-fold strengthen nutrient solution. Except to Figure 10D, the highest correlation coefficients were found for macronutrients.

Figure 10. Comparison of the K, Ca, Mn, Fe, Cu and Zn content in soybean leaves by portable XRF equipment and ICP OES. (A) Fresh leaves analysed directly by portable XRF under regular concentration of Hoagland's nutritive solution. (B) Dried leaves analysed directly by portable XRF under regular concentration of Hoagland's nutritive solution. (C) Fresh leaves analysed directly by portable XRF under 10-fold strengthen concentration of Hoagland's nutritive solution. (D) Dried leaves analysed directly by portable XRF under 10-fold strengthen concentration of Hoagland's nutritive solution

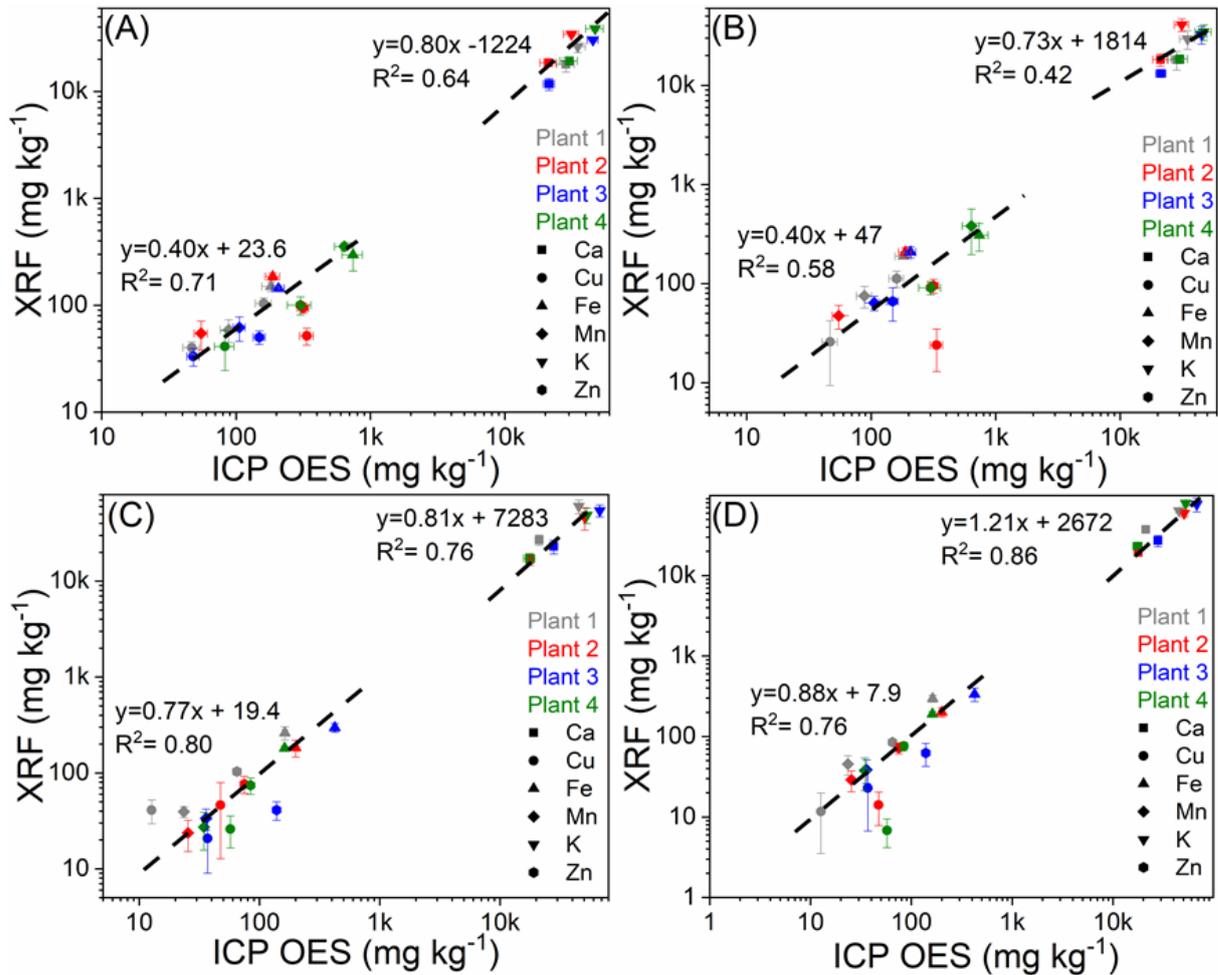


Figure 10 also shows that plants exposed to the 10-fold strengthen Hoagland solution presented higher correlation coefficients between concentration determined by pXRF and ICP OES. The higher concentration of the elements in the plant tissue facilitated the detection by pXRF and improved the correlation with ICP OES.

Another observed trend regards the curve slopes presented in Figure 10. Since the slopes are below 1, the method developed for pXRF underestimated the values of the elements compared to ICP OES. Nevertheless, the values are in the same order of magnitude. This offset might have been caused by the heterogeneity in the distribution of the elements along the leaves. Figure 7 showed that nutrient concentration was not homogenous. The highest spots were found in the leaf veins, especially in the central one. The pXRF sampling (shown in Figure 1B) did not probe these hotspots. On the other hand, the concentration determined by the ICP OES represents an average of the whole trefoil. For the sake of clarity, the data used to plot Figure 10 are also presented in Tables 4-7.

Table 4. Concentration of K, Ca, Mn, Fe, Cu and Zn in soybean leaves by pXRF equipment and ICP OES. Soybean plants cultivated under normal concentration of Hoagland's nutritive solution. Fresh leaves analysed directly by the pXRF

Sample	Technique	Element					
		K	Ca	Mn	Fe	Cu	Zn
		g kg <sup>-1</sup>		mg kg <sup>-1</sup>			
Plant 1	pXRF	26 ± 3	18 ± 3	59 ± 14	150 ± 15	40 ± 5	104 ± 13
	ICP OES	35 ± 5	28 ± 4	88 ± 12	180 ± 30	47 ± 7	160 ± 20
Plant 2	pXRF	34.3 ± 1.6	18.6 ± 1.6	55 ± 16	186 ± 14	52 ± 9	95 ± 10
	ICP OES	31 ± 4	21 ± 3	55 ± 6	190 ± 20	340 ± 40	316 ± 30
Plant 3	pXRF	30.4 ± 1.4	11.7 ± 1.5	62 ± 16	144 ± 8	33 ± 6	50 ± 7
	ICP OES	45 ± 4	21 ± 2	106 ± 11	210 ± 20	48 ± 5	149 ± 15
Plant 4	pXRF	38.7 ± 1.8	19.3 ± 1.7	350 ± 30	300 ± 90	41 ± 17	101 ± 20
	ICP OES	47 ± 7	30 ± 4	640 ± 100	740 ± 130	83 ± 14	301 ± 60

Table 5. Concentration of K, Ca, Mn, Fe, Cu and Zn in soybean leaves by pXRF equipment and ICP OES. Soybean plants cultivated under normal concentration of Hoagland's nutritive solution. Dried leaves analysed directly by the pXRF

Sample	Technique	Element					
		K	Ca	Mn	Fe	Cu	Zn
		g kg <sup>-1</sup>		mg kg <sup>-1</sup>			
Plant 1	pXRF	29 ± 6	18 ± 4	75 ± 19	189 ± 13	26 ± 16	110 ± 20
	ICP OES	35 ± 5	28 ± 4	88 ± 12	180 ± 30	47 ± 7	160 ± 20
Plant 2	pXRF	41 ± 6	18 ± 3	47 ± 13	210 ± 20	24 ± 11	95 ± 16
	ICP OES	31 ± 4	21 ± 3	55 ± 6	190 ± 20	340 ± 40	320 ± 30
Plant 3	pXRF	33 ± 7	13.2 ± 0.7	64 ± 11	210 ± 30	-----	70 ± 20
	ICP OES	45 ± 4	21 ± 2	106 ± 11	210 ± 20	48 ± 5	149 ± 15
Plant 4	pXRF	35 ± 6	18.5 ± 1.8	380 ± 180	310 ± 100	-----	90 ± 14
	ICP OES	47 ± 7	30 ± 4	640 ± 100	740 ± 130	83 ± 14	300 ± 60

Table 6. Concentration of K, Ca, Mn, Fe, Cu and Zn in soybean leaves by pXRF equipment and ICP OES. Soybean plants cultivated under a concentration 10x higher of Hoagland's nutritive solution. Fresh leaves analysed directly by the pXRF

Sample	Technique	Element					
		K	Ca	Mn	Fe	Cu	Zn
		g kg <sup>-1</sup>		mg kg <sup>-1</sup>			
Plant 1	pXRF	60 ± 10	27 ± 4	39 ± 5	260 ± 40	41 ± 11	104 ± 9
	ICP OES	45.2 ± 0.3	21.17 ± 0.08	23.5 ± 0.5	163 ± 9	12.7 ± 0.6	65.1 ± 0.8
Plant 2	pXRF	46 ± 12	17 ± 2	24 ± 9	180 ± 40	50 ± 30	77 ± 16
	ICP OES	50.8 ± 0.8	17.8 ± 0.2	25.5 ± 0.7	200 ± 9	47.4 ± 1.1	74.9 ± 1.2
Plant 3	pXRF	55 ± 8	23 ± 4	34 ± 8	300 ± 30	21 ± 12	41 ± 9
	ICP OES	67.6 ± 0.5	27.93 ± 0.09	36.0 ± 0.5	423 ± 10	37.0 ± 0.6	138.9 ± 0.9
Plant 4	pXRF	49 ± 9	17 ± 2	27 ± 12	181 ± 9	26 ± 9	74 ± 14
	ICP OES	53.82 ± 0.19	18 ± 2	34.6 ± 0.5	162 ± 6	57.4 ± 0.6	84.1 ± 0.8

Table 7. Concentration of K, Ca, Mn, Fe, Cu and Zn in soybean leaves by pXRF equipment and ICP OES. Soybean plants cultivated under a concentration 10x higher of Hoagland's nutritive solution. Dried leaves analysed directly by the pXRF

Sample	Technique	Element					
		K	Ca	Mn	Fe	Cu	Zn
		g kg <sup>-1</sup>		mg kg <sup>-1</sup>			
Plant 1	pXRF	64 ± 4	37 ± 3	45 ± 12	300 ± 30	12 ± 8	85 ± 8
	ICP OES	45.3 ± 0.3	21.17 ± 0.08	23.5 ± 0.5	163 ± 9	12.7 ± 0.6	65.1 ± 0.7
Plant 2	pXRF	59 ± 6	19.4 ± 1.1	29 ± 8	200 ± 30	14 ± 6	72 ± 11
	ICP OES	50.8 ± 0.8	17.8 ± 0.2	25.5 ± 0.7	200 ± 9	47.4 ± 1.1	74.9 ± 1.2
Plant 3	pXRF	77 ± 15	27 ± 4	39 ± 13	340 ± 60	23 ± 16	62 ± 20
	ICP OES	67.6 ± 0.5	27.93 ± 0.09	36.0 ± 0.5	423 ± 10	37.0 ± 0.6	138.9 ± 0.9
Plant 4	pXRF	79 ± 6	23 ± 2	38 ± 17	191 ± 15	7 ± 3	76 ± 10
	ICP OES	52.83 ± 0.19	18 ± 2	34.6 ± 0.5	162 ± 6	57.4 ± 0.6	84.1 ± 0.8

The challenges imposed by the leaf heterogeneity were faced by Guerra et al. (2015) during the analysis of dried sugar cane leaves. In this study the authors have concluded that it is necessary a minimum number of 15 leaf fragments (37.5% of the whole leaf) to make it representative to the whole sample. Likewise, Santos Junior et al. (2012) presented a review regarding the use of LIBS dedicated to analyse plant materials in the form of pressed pellets and they state that even after sampling washing, grinding and homogenizing, it might present differences in the results duo to the heterogeneity of the samples. Such elemental heterogeneity, which is compensated in case of pressed pellets, also explains the standard deviation for the quantitative XRF data expressed in Tables 4-7.

Table 8 presents the limits of detection (LOD) and quantification (LOQ) for ICP OES and portable XRF measurements. As expected, the values found for portable XRF were higher than those for ICP OES. On the other hand, the optimized pXRF instrumental conditions reported in the present study yielded better limits of detection than those previously reported elsewhere (GUERRA et al., 2014). One may notice that the LOD for XRF in fresh leaves was *ca.* 4.5x better than in dried leaves. It is important to highlight that the LOD, considering just the superficial concentration ( $\mu\text{g cm}^{-2}$ ), is better in dried leaves, but when we divide it by the density of the sample, which is *ca.* 7x higher in fresh leaves, the LOD in these samples becomes lower.

Table 8. Limits of detection and quantification by ICP OES and pXRF equipment.

Element	Unit	ICP OES		XRF Fresh leaves		XRF Dried leaves	
		LOD	LOQ	LOD	LOQ	LOD	LOQ
K	g kg <sup>-1</sup>	0.02	0.07	0.02	0.08	0.08	0.27
Ca		0.01	0.0138	0.02	0.05	0.06	0.21
Mn	mg kg <sup>-1</sup>	0.03	0.08	3.5	12	17	56
Fe		1.1	3.3	2.7	9.0	14	46
Cu		0.14	0.42	1.7	5.7	8.4	28
Zn		0.42	1.3	1.8	6.0	9.1	30

Fertilization recommendation is carried out based on tables that state if the vegetal tissue presents low, adequate or high concentration of a nutrient (Table 9). For example, both pXRF and ICP OES show that the plants demonstrated in Figure 10A and Table 4 present high K, Ca, Fe Cu and Zn and adequate Mn. Therefore, from the fertilization standpoint both techniques are suitable to carrying out this task.

Table 9. Nutrient concentration in soybean leaves adopted in the interpretation of foliar diagnosis results classified as low, intermediate or high (EMBRAPA, 2013)

Level	Element					
	K	Ca	Mn	Fe	Cu	Zn
	g kg <sup>-1</sup>		mg kg <sup>-1</sup>			
<b>Low</b>	< 17	< 6.8	< 28	< 59	< 6	< 31
<b>Adequate</b>	17-26	6.8-11.8	28-75	59-120	6-11	31-58
<b>High</b>	> 26	> 11.8	> 75	> 120	> 11	> 58

Finally, the overall sample preparation and instrumental time required to access the concentration of K, Ca, Mn, Fe, Cu, and Zn in one plant by pXRF were approximately 3 h and 51 h for fresh and dried leaves, respectively. The time spent in the procedure for ICP OES was about 51 h. The relative short analysis time offered by pXRF represents a step forward to its use in agriculture. However, both time of analysis, LOD and correlation with established techniques must be improved to allow boarding pXRF equipment in agricultural machinery. Additionally, complementary pXRF strategies must be developed to quantify P and S, this issue might be overcome using fundamental parameters or external calibration curves and will be tested in a further study.

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### 3 FOLIAR UPTAKE OF NANO COPPER OXIDE IN SOYBEAN PLANTS

#### Abstract

Copper (Cu) is an essential element for soybean development. Among the different sources of copper supplementation, the Cu nanoparticles seem to be an innovative approach with a wide range of plant benefits that varies from plant defense until lignin development. Herein, we did a chemical, physiological and morphological study in order to evaluate if Cu nanoparticles can present an improvement in copper uptake compared to a soluble source, such as  $\text{CuSO}_4$ . At first, we characterized the Cu nanoparticles that showed spherical and irregular forms. Nano CuO is absorbed by soybean leaves and redistributed to other leaves, although, how the particles penetrate the cells is still unknown. Shoot dry matter of plants was positively affected by the treatments, especially by the 40 nm nCuO. The copper speciation showed that the CuO applied is transformed from  $\text{Cu}^{1+}$  to  $\text{Cu}^{2+}$  14 days after application. When deposited on the soybean trefoil limbo it was observed that the soluble sources can promote damage to the leaf surface. On the other hand, Cu NPs, can cause epicuticular wax dissolution.

**Keywords:** nanomaterials, copper oxide, mineral nutrition, foliar uptake, epicuticular wax.

#### 3.1 Introduction

Fertilizers correspond to approximately 28% of the total cost production of soybean culture in Brazil (CONAB, 2016). In 2016, it is estimated that the fertilizer industry profited US\$ 1.5 billion, corresponding to 0.4% of Brazilian agribusiness. Foliar fertilizers correspond to 70% of this total amount (ABISOLO, 2017).

Of the total copper applied to the plants  $10 \text{ g ton}^{-1}$  are exported to the grains (EMBRAPA, 2013). Copper content in soybean leaves ranges from 10 to  $30 \text{ mg kg}^{-1}$  (VITTI; TREVISAN, 2000).

Copper plays important roles in higher plants. It participates in the structure of proteins, constituent of enzymes, participates in physiological processes such as electron flux in the light phase and the activation of ribulose diphosphate carboxylase in the dark phase of photosynthesis, respiration, hormonal regulation, indirect effect of biological nitrogen fixation and metabolism of secondary compounds. Besides these functions, copper has an important role in disease resistance, pollen grain structure and uniform grain maturation (MALAVOLTA, 2006; BROADLEY et al., 2011).

Usually, the main sources of micronutrients are made from inorganic products (oxides, carbonates and metallic salts as sulphates, chlorides and nitrates), chelates, fritted trace elements (FTE) and organic complexes (MORTVEDT, 2001; LOPES, 1999). According to Malavolta et al. (2000), the most common fertilizers containing copper are copper sulphate (13% of copper), copper dichloride (16%), cupric oxide (75%), cuprous oxide (89%), ammonia cupric phosphate (32%), copper nitrate (22%) and copper carbonate (48%). For foliar application, copper sulphate, Na<sub>2</sub>Cu EDTA and NaCu HEDTA are the most used (VITTI; TREVIZAN, 2000).

The adoption of nanomaterials in agricultural science has been increasing with the constantly growing population and the demand for higher agricultural yields is needed (IAVICOLI et al., 2017). At the same time, the use of nanotechnology in agriculture and other areas continue to raise doubts and concern over possible human and environmental health problems (KAH, 2015). As possible beneficial uses can be highlighted nanofertilizers, nanopesticides, sensing devices, water and soil remediation and crop coating (IAVICOLI et al., 2017). On the contrary, nanoparticles impact organisms because of their potential to transfer across environmental species and bioaccumulate in species at the top of food chains (NASROLLAHZADEH; SAJADI, 2019).

According to Wang et al. (2016) nanoparticles entrance in plant tissues occurs through either the root tissues or the aboveground organs and tissues (e.g., cuticles, trichomes, stomata, stigma, and hydathodes), including through wounds and root junctions. Even with the occurrence size exclusion limits of a series of barriers (e.g. cell walls, Casparian strip, plasmodesmata), the entrance of nanoparticles in cells is effective. A possible explanation is that size exclusion limits seem to be dynamic and influenced by calcium, silicon, proteins, virus, environmental stresses and by the nanoparticles themselves.

The use of copper nanoparticles in plants has already been reported for seeds (ADHKARI et al., 2012; ATHA et al., 2012; DURAN et al., 2017; DA COSTA; SHARMA, 2016; NAIR; CHUNG, 2014a; 2014b; 2015; PERREAULT; POPOVIC; DEWEZ, 2014; RAJPUT et al., 2018) and roots (EBBS et al., 2016). Giannousi, Avramidis and Dendrinou-Samara (2013) has applied nano CuO in leaves of tomatoes but testing it as an agrochemical against *Phytophthora infestans*.

Adhkari et al. (2012) relate that with increasing concentration of NPs, the elongation of the roots was severely inhibited as compared to that in control and in many cases root necrosis occurred. Duran et al. (2017) state that the nanoparticles did not affect seed germination, but seedling weight gain was promoted by 100 mg Cu L<sup>-1</sup> and inhibited by 1,000 mg Cu L<sup>-1</sup>

of 25 nm CuO and CuSO<sub>4</sub>. Similar results were obtained by Nair and Chung (2014a) and the authors report that application of 500 mg L<sup>-1</sup> of CuO NPs significantly reduced the shoot growth, weight, and total chlorophyll content. However, the root length and fresh weights were significantly reduced at all concentrations of CuO NPs application on soybean.

Alike the cited authors, Rajput et al. (2018) concluded that the CuO NPs inhibited barley growth by affecting the germination rate, root and shoot lengths, affected stomatal aperture and root morphology. Furthermore, the Cu content of roots and leaves of CuO NPs treated plants was 5.7 and 6.4-folds higher than the control (without CuO NPs), respectively. In contrast, Giannousi, Avramidis and Dendrinou-Samara (2013) concluded that CuO NPs are more effective than the trade agrochemicals. Moreover, assessments regarding phytotoxicity indicated that they can be used without any deleterious effect on plants.

In view of all the discussion and uncertainties regarding the use of nano CuO as fertilizers, the aim of this study is to demonstrate the possible benefits of copper nanomaterials as foliar fertilizer.

## 3.2 Materials and Methods

**3.2.1 Nanomaterials characterization. Purity.** 25 and 40 nm CuO nanoparticles were acquired from US Nanomaterials Research Inc (USA). The powder samples (200 mg) were weighted in a 6.3 mm aperture X-ray sample cup (no. 3577 - Spex Ind. Inc., USA) and sealed with a 5 µm thick polypropylene film (no. 3520 - Spex Ind. Inc., USA). The analysis were conducted under vacuum, in triplicate, using a rhodium (Rh) X-ray tube at 50 kV and auto-tunable current adjusted for a detector deadtime below 30% and a 3-mm collimator. The X-ray spectrum of the sample was acquired utilizing a Si (Li) detector for 300 s and through the fundamental parameters method it was possible to determinate contaminants content present in these nanomaterials.

**3.2.2 Structural characterization.** X-ray diffraction (XRD) was used to confirm the majority composition of the CuO nanoparticles. The X-ray diffraction measurements were obtained in a Bruker D2 Phaser instrument, operating with Cu K $\alpha$  ( $\lambda=1.5418$  Å) radiation, 30 kV, 10 mA, sample holder rotation of 15 rpm and Lynxeye<sup>®</sup> energy dispersive one-dimensional detector with 192 channels. The diffractograms were acquired under the following conditions: continuous scanning mode in the 2 $\theta$  range from 10 to 90°, step = 0.02° and acquisition time = 0.5 s, obtained from the sample powders.

Through the diffractograms it was possible to estimate the crystallite sizes using the Scherrer equation (Equation 1) for different planes.

$$D(hkl) = K\lambda / \beta \cos\theta \quad (\text{Equation 1})$$

where:

K: Scherrer's constant (0.94 for spherical crystals);

$\lambda$ : wavelength referring to the X-ray source (0.154184 nm for Cu);

$\beta$ : Full width at half maximum of the peak;

$\theta$ : measured angle in degree.

**3.2.3 Physicochemical features of dispersions.** Dispersions in distilled water of the nanomaterials ( $100 \text{ mg L}^{-1}$ ) were prepared to determine the aggregates' sizes by dynamic light scattering (DLS) and its charge by zeta potential using a Zetasizer equipment (Malvern, UK). The pH/conductivity of the dispersions were determined by a handheld pH meter, model SevenGo Duo SG23 (Mettler Toledo, USA).

**3.2.4 Nano CuO morphology.** CuO nanoparticles with different sizes were used. The nanoparticles dispersions were prepared using 5 mg of the nanoparticles diluted in deionized water. Then, the nanoparticles dispersions were sonicated (Fisher Scientific). After the sonication, 20  $\mu\text{l}$  of the dispersion were deposited on the Parafilm<sup>®</sup> film and the copper grids (300 mesh) carbon-coated were deposited on the CuO nanoparticle dispersion for five minutes. The excess of the dispersion was removed with a filter paper. The morphology of the nanoparticles was evaluated by transmission electron microscopy (JEM 1011, JEOL, Japan) in dispersions of  $100 \text{ mg L}^{-1}$ . The images were analysed by the software ImageJ<sup>®</sup>. Approximately 60-70 particles were measured and a histogram of distribution was created in the software Origin<sup>®</sup>.

**3.2.5 Soybean cultivation.** The experiment was conducted in a greenhouse of the Soil Science Department at Luiz de Queiroz Superior School of Agriculture (ESALQ/USP). The soil used in the experiment was a sandy soil, with low content of Cu ( $0.2 \text{ mg dm}^{-3}$ ). The base saturation (BS) was raised for 60% through liming according to the base saturation method. The soil was collected in the municipality of São Pedro/SP, analysed before and after the experiment, and its physicochemical properties are shown in Table 1.

Table 1. Physicochemical properties of the soil collected in the municipality of São Pedro/SP, before and after the assembly of the experiment

Determination	Unit	Before	After
		Value	
Sand		858	858
Silt	$\text{g kg}^{-1}$	17	17
Clay		125	125
pH $\text{CaCl}_2$		4.1	5.4
O.M. - Walkley	$\text{g dm}^{-3}$	10	< 5.0
H + Al		18	12
K	$\text{mmol}_c \text{ dm}^{-3}$	0.2	1.7
Ca		1	9
Mg		1	5
P		2	18
B		0.07	< 0.15
Cu	$\text{mg dm}^{-3}$	0.2	< 0.1
Fe		8	6
Mn		1.3	1.4
Zn		0.3	0.3
SB	$\text{mmol}_c \text{ dm}^{-3}$	2.2	15.7
CEC		20.2	27.7
BS	%	11	59

SB: sum of bases; CEC: cation-exchange capacity; BS: base saturation.

Four seeds of soybean, cultivar KWS RK 7214 IPRO, were sown per pot. The exceeding seedlings were removed, leaving two plants per pot. Each pot of 2.5 L was filled with two kg of soil, daily irrigated with enough deionized water to keep the soil close to its field capacity.

**3.2.6 Copper solutions.** Copper sulphate solutions, in the same concentrations of the nanomaterials were used for comparison. The experiment was assembled under a completely randomized design with three repetitions. Control treatments consisted of three pots with two plants in each, that only deionized water was applied to the leaves, and three pots with two plants in each, that a solution of deionized water + dispersant (5%) was applied to the leaves.

The application of all treatments was made in the first 3 trefoils through aspersion when the plants were in V3 stage. Before application, the soil was covered in order to not be contaminated by droplets of the spray. The dispersant used was the polyethylene glycol (PEG) with a molecular weight = 15,000 – 20,000 g mol<sup>-1</sup> from Sigma-Aldrich®. The nanoparticles were dispersed in deionized water and in aqueous dispersions containing the dispersant (5%). The factors and levels of the treatments are shown in Table 2.

Table 2. Factors and levels of the treatments applied

Factors	Levels			
<b>Particle size</b>	25 nm nCuO	40 nm nCuO	Micrometric CuO	CuSO <sub>4</sub> ·5H <sub>2</sub> O
<b>Dispersant (PEG)</b>	With	Without		
<b>Dose</b>	0 mg plant <sup>-1</sup>	0.75 mg plant <sup>-1</sup>	7.50 mg plant <sup>-1</sup>	

In total there were 54 pots, which come from the following combination of factors and levels:

- 4 (particle size) x 2 (dispersant) x 2 (dose) x 3 (repetitions) + 6 (control)

The doses were chosen based on the commercial recommended dose (0.75 mg plant<sup>-1</sup>) and 10 times higher (7.5 mg plant<sup>-1</sup>), plus control (0 mg plant<sup>-1</sup>).

For the analysis, one plant was collected 21 days (V7/V8 stage) after the application of the treatments and the other plant, 45 days (R4/R5 stage) after the application. The responses studied were: shoot dry mass (trefoils which have not received the treatments), copper concentration and accumulation.

After harvesting, the plants were divided in sets of leaves (until the third trefoil and the fourth trefoil onwards), the material was identified, placed in paper bags and oven-dried at 65° C for 72 hours. Afterwards, each set of leaves was weighed and the samples grinded in Willey type knife mill (1 mm mesh).

**3.2.7 ICP OES analysis.** After grinding, the plant material was weighed (*ca.* 200 mg) and subsequently digested by microwave-assisted acid digestion in closed TFM<sup>®</sup> vessels (Provecto Analytica DGT 100 plus), containing 6.0 mL of 20% v v<sup>-1</sup> HNO<sub>3</sub> and 2.0 mL of 30% w w<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>. The following microwave heating program was selected: a) 7 minutes at 400 W; b) 15 minutes at 850 W; c) 7 minutes at 320 W and; d) 2 minutes at 0 W. The digested material was diluted to 25 ml and analysed using the ICP OES spectrometer (iCap 7400 Duo optical emission spectrometer, Thermo Scientific, Waltham, MA, USA). The analysis conditions of the ICP OES are presented in Table 3. For trueness checking, the standard reference material NIST 1547 Peach leaves was analysed.

Table 3. Characteristics and operational conditions of the analysis carried out in ICP OES (iCap 7400 Duo optical emission spectrometer, Thermo Scientific, Waltham, MA, USA)

Characteristics	Operational Conditions
View	Radial
Detector	Wavelength range: 166-847 nm
<i>Operational parameters</i>	
Exposure time	10 s
RF power	1.2kW
Plasma gas-flow rate	12 L min <sup>-1</sup>
Auxiliary gas-flow rate	0.5 L min <sup>-1</sup>
Nebulizer gas-flow rate	0.6 L min <sup>-1</sup>
Emission line	Cu 324.754 nm

**3.2.8 Chemical speciation analysis.** Another set of vases containing two plants in each vase were cultivated to receive the following particle sizes: 25nm nCuO, micrometric CuO and copper sulphate. The dispersions and solution of these particles' sizes were prepared in the highest concentration (7.50 mg plant<sup>-1</sup>) and without dispersant. Each treatment was applied using 2 repetitions and the dispersions were sprayed on the leaves 1 and 14 days before analysis.

The analyses for determining copper speciation were accomplished using microprobe X-ray absorption near-edge structure ( $\mu$ -XANES) at the D09B-XRF beamline at the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil). At XRF beamline, synchrotron radiation was generated by a bending-magnet and collimated by slits. The monochromatic beam was produced by a Si (111) crystal and a KB mirror system was used to focus it to the 20  $\mu$ m diameter spot size. The  $\mu$ -XANES was recorded in fluorescence mode using a silicon drift detector (SDD; AXAS-A, KETEK GmbH, Germany). The energy was calibrated utilizing a reference Cu foil.

**3.2.9 Scanning electron microscopy (SEM).** Soybean leaves were sprayed with different treatments and harvested after 14 days. Then, pictures of the leaves' surfaces were taken using a Hirox digital (Hirox, Japan) microscope. Three samples of each treatment were fixed in a Karnovsky solution adjusted to pH 7.2, using phosphate buffer (KARNOVSKY, 1965), dehydrated using a graded cetonic series (10, 30, 50, 70, 90, and 100%), critical point-dried using CO<sub>2</sub> (HORRIDGE; TAMM, 1969), mounted on aluminum stubs using double-sided carbon tape and coated with a 30–40 nm gold film and examined with a scanning electron microscope (Jeol - JSM IT 300) at 15 kV and digitally recorded at a working distance of 15 mm.

**3.2.10 Experimental design and statistical analysis.** The greenhouse experiment was set under a totally randomized design in a 4 x 2 x 3 + 1 factorial scheme (4 particle sizes, 2 dispersant and 3 concentrations), plus a universal control treatment.

All the experimental analyses were conducted at SAS 9.4. Prior to ANOVA the data was tested to check the variance homogeneity (Levene's test) and normality of residues (Shapiro-Wilk test).

For the ANOVA, the data was analyzed by a factorial scheme 5 x 2, being five sources of copper (control, copper sulphate, 25nm, 40 nm and micrometric copper oxide) and with or without dispersant. Least squares means comparison test adopted for the effects was the Tukey-Kramer Test, with 5% significance.

### 3.3 Results and Discussion

**3.3.1 Nanomaterials characterization.** Purity of the nanomaterials is shown in Table 4. Even though 40 nm nCuO presents chromium in its composition (less than 0.05%), both nanomaterials present high purity.

Table 4. Purity and contaminant on the nanomaterials used in the experiment

Nanomaterial	Manufacturer	Purity (%)	Contaminant (mg kg <sup>-1</sup> )
			Cr
25 nm nCuO	US Nanomaterials Research Inc.	100%	-
40 nm nCuO	US Nanomaterials Research Inc.	99.958%	418

The XRD diffractograms are shown in Figure 1 and one can observe that the diffraction pattern is different in some peaks for 25 and 40 nm and this characteristic is emphasized by the estimated values (Table 5) for the crystallite sizes of three different planes: 25 nm nCuO (-111, 200/111 e -202) and 40 nm nCuO (110, 002 e 200). For both sizes of the nCuO, different values were estimated in the three planes, indicating that these materials may have different shapes and composition. Data indicates that 40 nm nanoparticles are CuO while 25 nm nanoparticles are a mix of CuO and metallic Cu.

Figure 1. XRD diffractograms of 25 and 40 nm nCuO

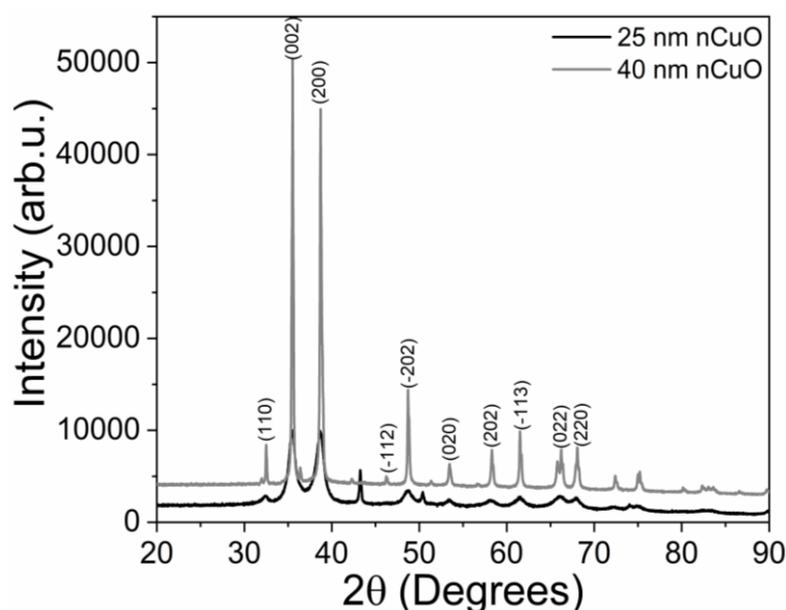
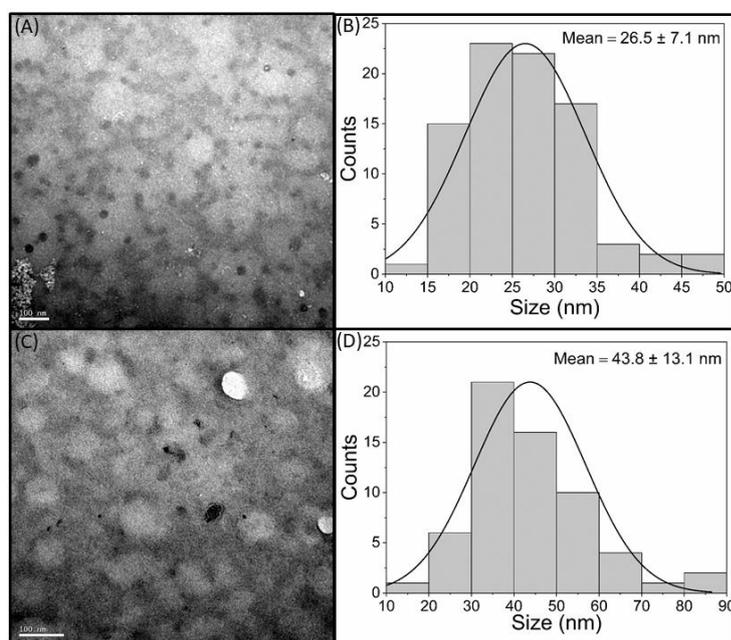


Table 5. Estimated crystallite sizes by Scherrer's equation for nano CuO (25 and 40nm) in different planes

Plane	$D_{(hkl)}$ (nm)	
	25nm nCuO	40nm nCuO
-111	6.0	-----
200/111	5.9	-----
-202	5.0	-----
110	-----	46.0
002	-----	55.4
200	-----	36.6

This information about the nanomaterials' shapes could be confirmed by the images (Figure 2) obtained by transmission electron microscopy (TEM). For 25nm nCuO (Figure 2A) is observed a spherical aspect, while for the 40nm nCuO (Figure 2C) a more elongated aspect is seen. One can notice that both particles present a normal size distribution, as shown in the histograms for 25 nm nCuO (Figure 2B) and 40 nm nCuO (Figure 2D).

Figure 2. TEM images of (A) 25nm nCuO and (C) 40nm nCuO and their respective histograms (B and D). Scale bar: 100 nm



Regarding the DLS values and Zeta potential (Table 6), 25 nm nCuO presents 2 peaks, with a main aggregates' size of 428 nm and positive charge while the 40 nm nCuO presents aggregates of 180 nm and negative charge.

Table 6. Zeta potential and Dynamic Light Scattering of CuO nanoparticles

Nanomaterial	Zeta Potential (mV)	Dynamic Light Scattering (Cluster diameter – nm)	
		Peak 1	Peak 2
25nm nCuO	75.6 ± 5.14	428 (98 %)	5477 (2%)
40 nm nCuO	-7.07 ± 6.22	180 (100 %)	-----

The pH values and conductivity of the dispersions used in the experiment are presented in Table 7. For pH, the data ranged from acid values (4.56) to alkaline values (7.50). Except to the copper sulphate, the treatments in which the dispersant was applied presented a higher value of pH when compared to the ones without dispersant. The same situation is observed for the conductivity. Treatments with dispersant had higher values than treatments without dispersant, in exception to the solutions of copper sulphate.

Table 7. Ph and conductivity of the treatments used in the experiment

Particle Size	Dispersant	Concentration (mg plant <sup>-1</sup> )	pH	Conductivity (μS cm <sup>-1</sup> )
25 nm	With	0.75	7.31	95.3
	With	7.5	7.50	93.6
	Without	0.75	6.59	27.8
	Without	7.5	6.54	31.0
40 nm	With	0.75	7.20	88.3
	With	7.5	7.16	89.5
	Without	0.75	6.59	30.8
	Without	7.5	6.47	26.3
Micrometric CuO	With	0.75	7.20	88.4
	With	7.5	7.17	91.1
	Without	0.75	6.45	30.4
	Without	7.5	6.51	18.23
Copper sulphate	With	0.75	5.20	173.0
	With	7.5	4.56	102.1
	Without	0.75	5.61	163.1
	Without	7.5	5.18	1155
Control	With	0	7.22	85.0
	Without	0	6.94	18.82

**3.3.2 Effect of CuO nanoparticles on the soybean leaf.** Figure 3 shows the digital microscopy analysis of the control leaf (Figures 3A and 3B) and the foliar injuries caused by the application of 25 nm nCuO (Figures 3C and 3D), micrometric CuO (Figures 3E and 3F), copper sulphate (Figures 3G and 3H). Comparing the treatments, we observed that all of them accumulate mainly on the vein surface (Figure 3 C-F and 3G). The nCuO forms a dark precipitate (Figure 3C), while the micrometric CuO does not show any color difference (Figure 3E) and in the CuSO<sub>4</sub> treated leaves it was possible to observe that the veins are black and, in some regions, there is whitish appearance (arrows in Figure 3G). Leaves treated with CuSO<sub>4</sub> presented curly symptom, that differs from the other treatments (Figure 3H) and can present whitish appearance between the leaf veins (arrow in 3H).

Figure 3. Digital microscopy photos of *Glycine max* (L.) Merrill plants exposed to different CuO dispersions and CuSO<sub>4</sub>: A, C, E, G: 2D images. B, D, F, H: 3D images. Injuries caused by foliar application of 25 nm nCuO (C and D), micrometric CuO (E and F), copper sulphate (G and H), against the control leaf (A and B) seen on a Hirox KH-8700 model digital microscope. Photos taken 14 days after foliar application.

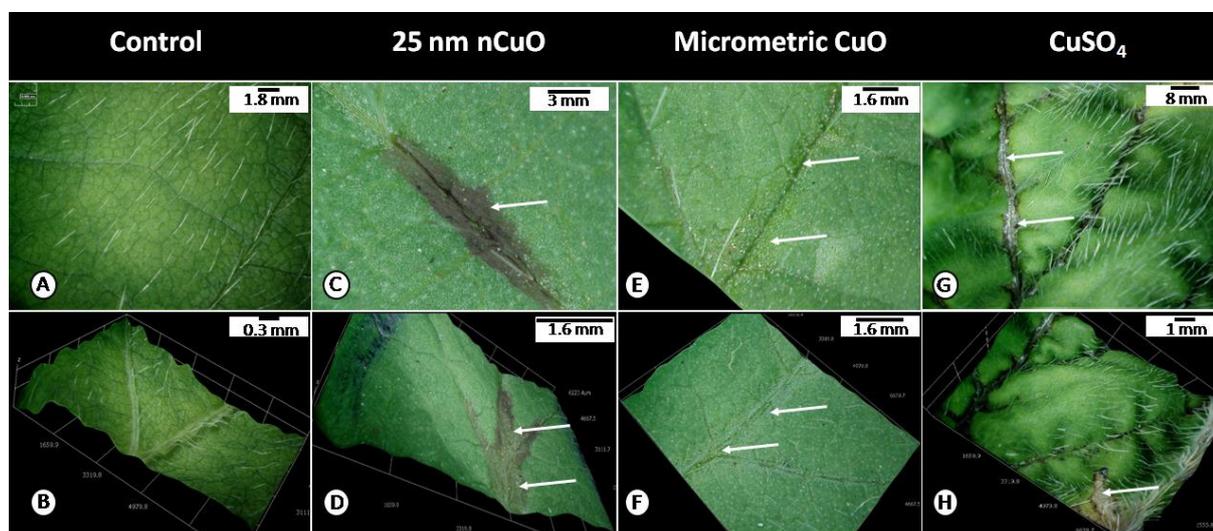
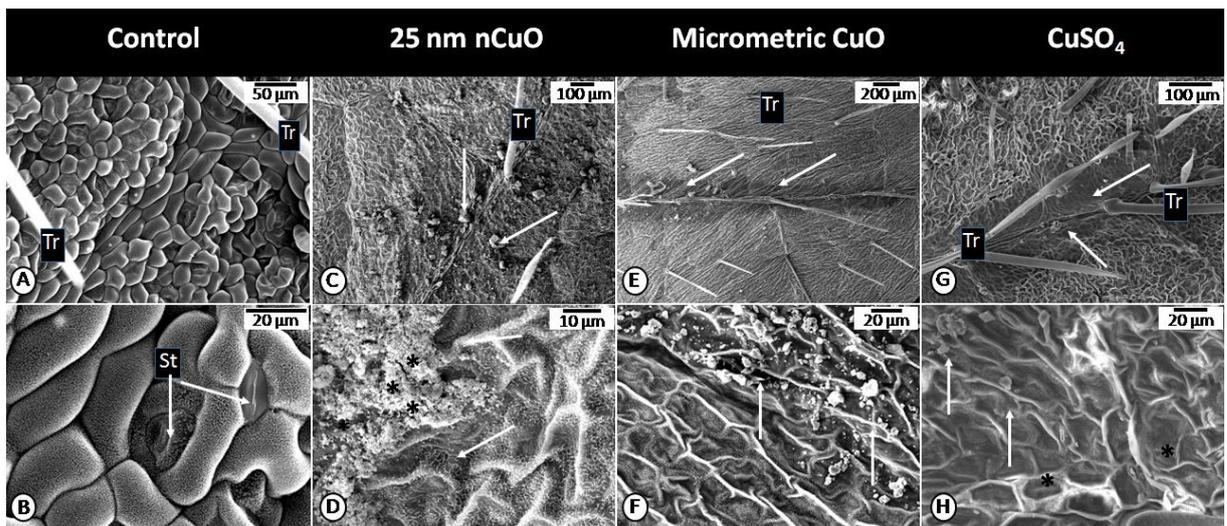


Figure 4 shows the ultrastructural details of the leaves presented in Figure 3. In the control plants, the soybean leaf epidermis is composed by turgid cells covered by epicuticular wax platelets (Figure 4A and 4B). This cuticle pattern agrees with previous works (FENGSHAN et al., 2004; HAGEDORN et al., 2017). Under the scanning electron microscopy, we observed that CuO nanoparticles can form aggregates (Figure 4C and 4D).

On the other hand, no aggregate was observed with micrometric CuO (Figure 4E and 4F) and CuSO<sub>4</sub> (Figure 4G and 4H) treatments. When observed in detail, we noticed that all treatments showed absence or scarce presence of platelets crystals in the region in contact with the CuO nano- and microparticles. It seems that the wax crystals melt down when in contact with Cu NPs.

Figure 4. Scanning electron micrographies of *Glycine max* (L.) Merrill plants exposed to different CuO dispersions and CuSO<sub>4</sub>. A, C, E, G: Overview. B, D, F, H: 3D details. A/B: Control. C/D: Lesions induced by 25 nm nCuO dispersion. Note the deposition of nCuO(\*) and the absence of epicuticular wax crystals (arrows). E: Changes on the vein region (arrows). F: CuO crystals (arrows). G/H: CuSO<sub>4</sub> induces severe damage on the vein (arrows in G) and the absence of epicuticular wax (\*). Tr: Trichomes; St: Stomata. Images taken 14 days after foliar application.



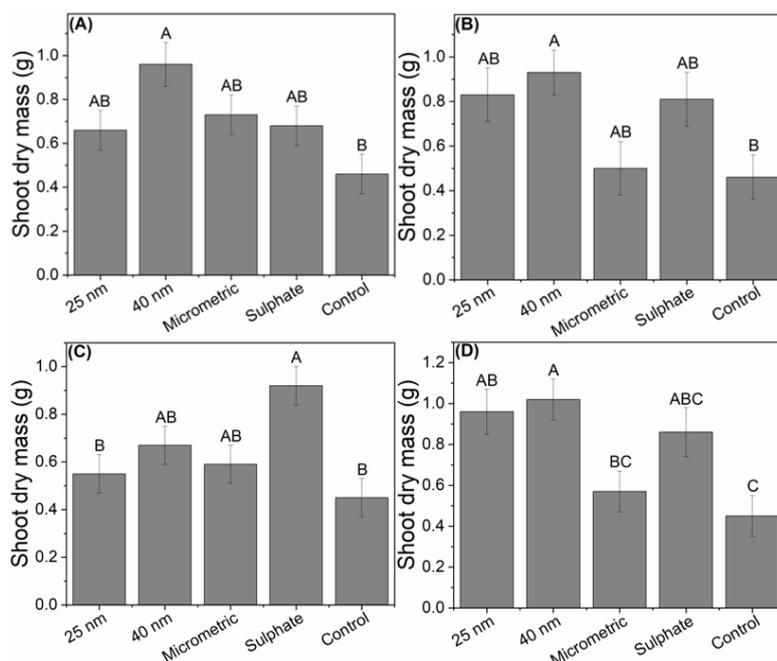
Dappe et al. (2019) have reported the effects of CuO NPs in cabbage. Seeds were germinated in trays and kept in greenhouse for 2 months. One mg of CuO NPs per plant was deposited on the adaxial surface of the 3 higher leaves and plants were harvested 5, 10 and 15 days after application. Dappe et al. (2019), Rajput et al. (2018) and Xiong et al. (2017) results agree to our study. Alike what was observed and shown in Figure 4, the authors report that the nanoparticles cause structural changes in the stomata and reduction of the epicuticular waxes.

**3.3.3 Effects of nCuO on plant biometrics and Cu translocation.** Washing the leaves is a common practice prior to quantification of nutrient absorption or chemical speciation (XIE et al., 2019; LARUE et al., 2014a). This procedure aims at removing the target nanoparticles, or fertilizers, from the leaf surface leaving only the nutrients that were absorbed. However, XRF images (LARUE et al., 2014a) and SEM (LARUE et al., 2014b) showed that even after washing, TiO<sub>2</sub> and Ag nanoparticles remained on top of the epidermis of lettuce. Hence, in the present study, we decided to determine the copper content in the leaves that appeared after the spraying.

The dispersant did not affect the biomass production at 21 days. However, for treatments applied at 7.5 mg plant<sup>-1</sup>, the dispersant increased biomass production at 45 days. This suggests that the dispersant eased the copper uptake.

As presented in Figure 5, the copper increased biomass production at 21 and 45 days.

Figure 5. Shoot dry mass of soybean plants treated with 0.75 (A/C) and 7.5 mg Cu plant<sup>-1</sup> (B/D). The plants were harvested 21 (A/B) and 45 days (C/D) after application.



\*Means with the same letter within each figure do not differ statistically.

At 21 days, for treatments applied at 0.75 mg plant<sup>-1</sup>, the 40 nm CuO yielded the highest biomass production (0.96 g), while the other copper sources were statically equal and the negative control presented the lowest biomass (0.46 g). However, at 45 days copper sulphate presented the highest biomass yield (0.93 g) followed by 40 nm nCuO = micrometric > 25 nm nCuO = negative control.

For treatments applied at  $7.5 \text{ mg plant}^{-1}$  all sources influenced biomass production at 21 and 45 days. In both periods 40 nm nCuO resulted in higher biomass, 0.93 g and 1.02 g for 21 and 45 days, respectively. For the negative control the biomass values were 0.46 g and 0.45 g for 21 and 45 days, respectively. Copper sulphate and micrometric CuO, which are usually employed as fertilizers, produced 0.86 g and 0.57 g at 45 days, respectively.

Some studies agree to the results presented here. Even using lower doses, in 2016, Juarez-Maldonado et al., verified that tomato nCu-chitosan hidrogels-treated presented an improve in height, stem diameter and dry weight of the shoots treated at 15, 30, 60 and  $150 \text{ mg L}^{-1}$ .

In the same sense, Cu nanoparticles were able to increase root and shoot biomass when the seeds were primed with CuO NPs at  $60 \text{ mg L}^{-1}$  and  $100 \text{ mg L}^{-1}$  (ADHIKARI et al., 2012).

There are few studies in which the nanoparticles were foliar sprayed. Hong et al. (2016) have tested the effects of foliar application of CuO NPs in cucumber and similar to our study the authors have tested the effects of nano application over different harvest times. Plants were grown in containers and they were 3 weeks-old (two true leaves) they were sprayed with nano and bulk CuO at 50, 100 and  $200 \text{ mg L}^{-1}$ . Two weeks after treatment one plant was harvested and the other was kept for more 48 days, receiving 250 ml of dispersions every three days. At the highest concentration nano CuO impacted cucumber photosynthetic parameters and reduced fruit firmness, along with the  $50 \text{ mg L}^{-1}$  treatment. In this study the nanoparticles have caused negative effects for the plants.

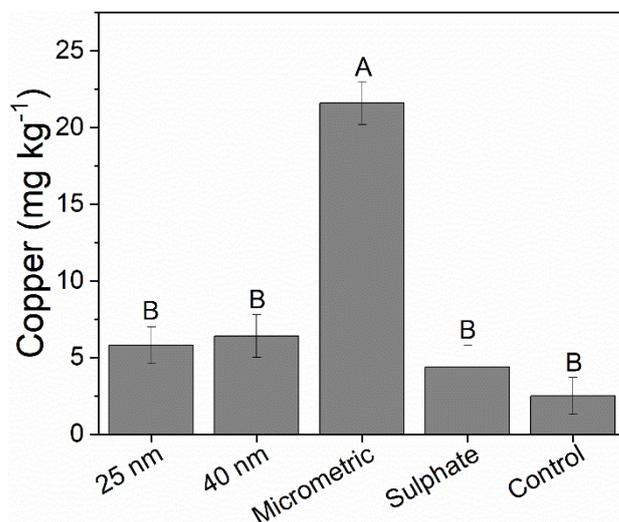
The impacts of CuO NPs in lettuce and cabbage were investigated by Xiong et al. (2017). Dry CuO NPs were deposited on the leaves of three weeks-old plants at 0, 10 and  $250 \text{ mg plant}^{-1}$ . The plants were analysed 5, 10 and 15 days after application. A high uptake occurred after 15-days application, along with a decrease in plant weight, photosynthesis level, water content and the appearance of necrotic areas near the stomata. Differently from the present study, the authors have applied the nanoparticles using a high concentration, which probably impacted negatively the parameters studied.

At 21 days after application, the dispersant increased copper concentration in plant tissues of plants treated with 0.75 and  $7.5 \text{ mg plant}^{-1}$ . On the other hand, at 45 days the dispersant decreased copper concentration in plants treated with  $0.75 \text{ mg plant}^{-1}$  and did not affect the copper concentration in plants treated with  $7.5 \text{ mg plant}^{-1}$ .

The sources of copper did not affect the copper concentration in the tissue of plants treated with  $0.75 \text{ mg plant}^{-1}$ . The concentration of copper found was equal to the negative control. Conversely, the highest copper concentration in tissues was found for plants treated

with  $7.5 \text{ mg plant}^{-1}$  of the micrometric source ( $21.6 \pm 1.4 \text{ mg kg}^{-1}$ ). All other sources were statistically equal to the negative control (Figure 6).

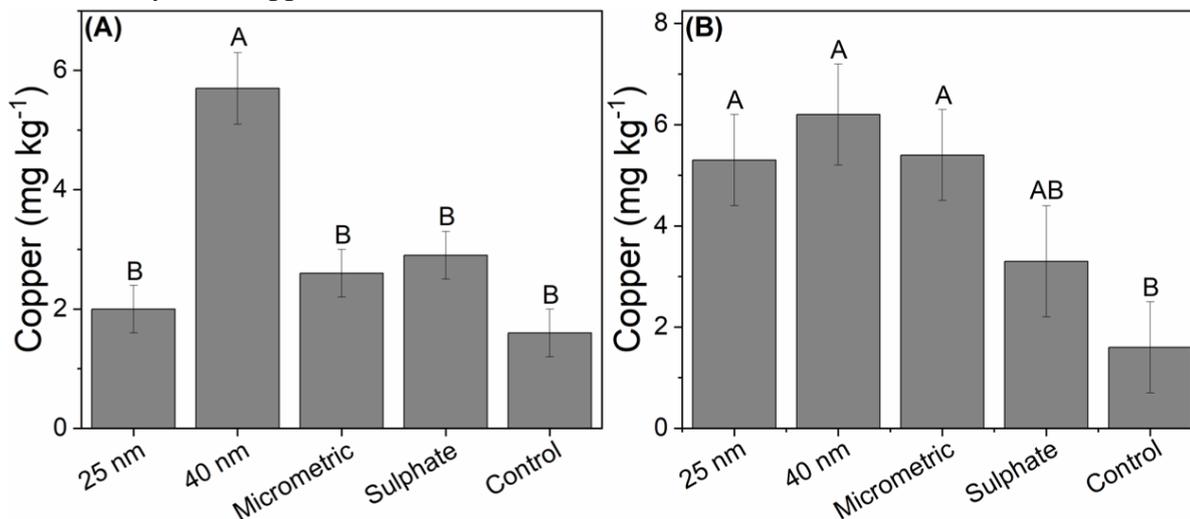
Figure 6. Copper concentration in soybean plants treated with  $7.5 \text{ mg Cu plant}^{-1}$  at 21 days after application.



\*Means with the same letter do not differ statistically.

Figure 7A shows that at 45 days the plants treated with 40 nm at  $0.75 \text{ mg plant}^{-1}$  presented the highest concentration of copper in the tissues ( $5.7 \pm 0.6 \text{ mg kg}^{-1}$ ) and the other sources did not present statistical difference among themselves. The concentration of copper in the tissues of plants treated with  $7.5 \text{ mg plant}^{-1}$  (Figure 7B) depended on the source and it varied accordingly  $40 \text{ nm} = \text{micrometric} = 25 \text{ nm} > \text{sulphate} > \text{control}$ .

Figure 7. Copper concentration in soybean plants treated with  $0.75$  (A) and  $7.5 \text{ mg Cu plant}^{-1}$  (B) at 45 days after application.

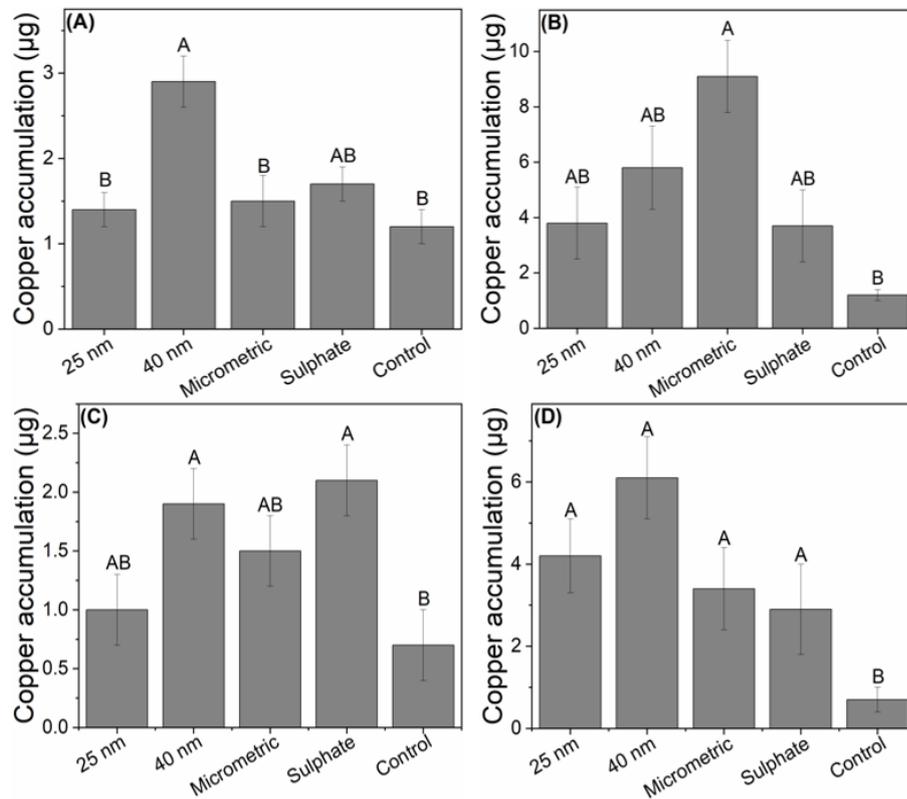


\*Means with the same letter within each figure do not differ statistically.

The data regarding copper accumulation in the leaves is presented in Figure 8. Figure 8A shows that the copper accumulation at 21 days plants treated with 0.75 mg plant<sup>-1</sup> followed the order 40 nm (2.9 µg) > sulphate (1.7 µg) > micrometric (1.5 µg) = 25 nm (1.4 µg) = negative control (1.2 µg). At 21 days the highest copper accumulation (9.1 ± 1.3 µg) was found in plants treated with micrometric CuO at 7.5 mg plant<sup>-1</sup> (Figure 8B). The other sources were statistically equal among themselves but superior to the control. We have to highlight that plants treated with 40 nm nCuO contained 5.8 ± 1.5 µg of copper, while the negative control presented the lowest value (1.20 ± 0.2 µg).

The sources sulphate and 40 nm nCuO at 0.75 mg plant<sup>-1</sup> presented the highest accumulation, 2.1 and 1.9 µg, respectively, at 45 days (Figure 8C). However, at 7.5 mg plant<sup>-1</sup>, copper accumulation was positively affected by all sources (Figure 8D).

Figure 8. Copper accumulation in soybean plants treated with 0.75 (A/C) and 7.5 mg Cu plant<sup>-1</sup> (B/D). The plants were harvested 21 (A/B) and 45 days (C/D) after application



\*Means with the same letter within each figure do not differ statistically.

Laughton et al. (2019) have applied nano CuO and Cu(OH)<sub>2</sub> at a copper concentration of 790 mg L<sup>-1</sup> in leaves of lettuce. The plants were harvested in a 9-days period after application. The authors report that the total copper mass concentration remained the same over the period, however, the time between application and harvest was too short to the plant show any difference. They also report that some leaves were washed and it resulted in the removal of total copper content. Again, in such short time, the products applied did not have enough to penetrate the cuticle of leaves. The results obtained in this study differ from the ones of our study. Plants harvested 21 days after application already showed different values for copper mass concentration (Figure 6), indicating that there was copper penetration and redistribution after this period. Considering what is related by Laughton et al. (2019), the penetration of copper nanoparticles occurs between 9 and 21-days period after application.

The effect of copper foliar application is clearly shown by the treatments supplied at 7.5 mg plant<sup>-1</sup>. At 21-days the 40 nm CuO source produced the highest biomass. It is worth note that the high copper concentration found for micrometric source was a consequence of the low biomass production of this source.

Plants that received copper foliar application at 7.5 mg plant<sup>-1</sup> produced more biomass (Figure 5B and 5D) and accumulated more copper in tissues (Figure 8B and 8D).

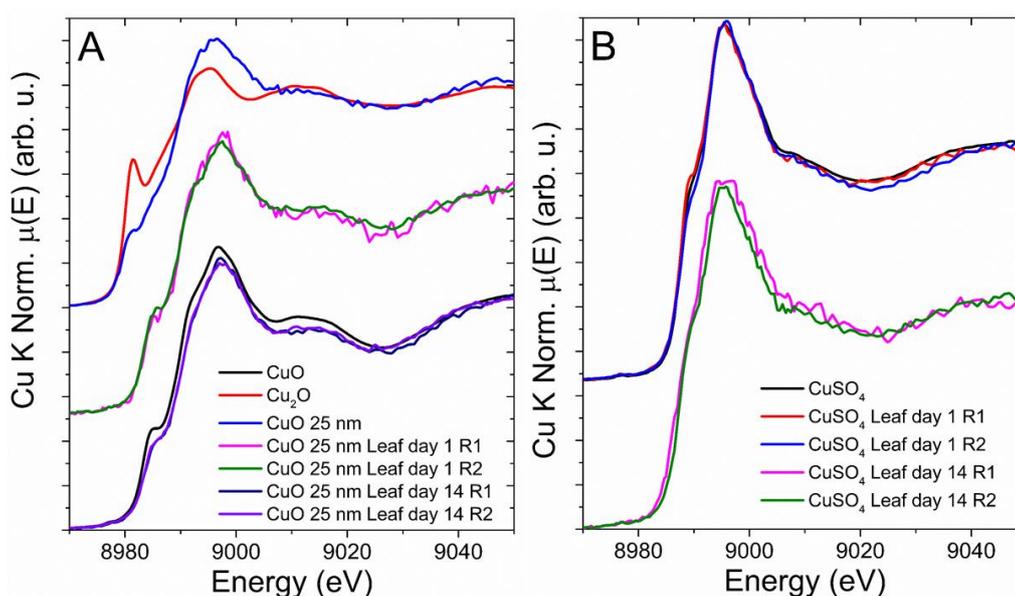
We noticed that past 45 days of treatments application, the copper concentration in the tissues were lower than at 21 days. We believe that it might be related to exportation of copper to grains.

**3.3.4 Characterization of CuO NPs deposited on the leaf surface.** Figure 9 presents the Cu-K edge XANES spectra recorded for leaves exposed to 25 nm CuO and CuSO<sub>4</sub> (aq) applied to leaves as well as pristine materials. In agreement to XRD patterns (Figure 1), XANES spectra for pristine 25 nm CuO suggests that it is a mixed oxide, it showed a small shoulder at 8983 eV which corresponds to 1s to 4p transition in Cu<sup>1+</sup>. After one and 14 days of application, the Cu<sup>1+</sup> fraction was converted to Cu<sup>2+</sup>. However, it is not possible to state whether this took place inside or outside the leaves.

Since we could not guarantee that copper would be completely removed from the leaf surface, we decided to investigate whether it would undergo transformation regardless being inside or outside the leaf. The presence of Cu<sup>2+</sup> in plants treated with 25 nm CuO and CuSO<sub>4</sub> is consistent with previous reports (SHI et al., 2008). The authors reported the presence of Cu<sup>2+</sup> within *Elsholtzia splendens* bound to cell wall and organic species such as histidine,

oxalate, and glutathione. On the other hand, Polette et al. (2000) reported that *Larrea tridentata* accumulated copper as a mixture of  $\text{Cu}^{1+}$  and  $\text{Cu}^{2+}$ .

Figure 9. Cu–K edge XANES spectra recorded in soybean leaves treated with  $630 \text{ mg Cu L}^{-1}$  dispersions. Spectra recorded of the 25 nm nCuO treatment (A) and spectra recorded of the  $\text{CuSO}_4$  treatment (B). R1 stands for repetition 1 and R2 stands for repetition 2



### 3.4 Conclusions

Nanometric copper oxides are capable of supplying this nutrient to plants through foliar spraying. Leaves which were not sprayed with nano CuO presented the nutrient 21 days application application.

The deposition of copper sources on leaves be it copper sulphate or nano CuO, caused damages. All treatments showed an absence or a scarce presence of platelets crystals in the region in contact with theCuO nano- and microparticles.

Shoot dry mass of plants was positively affected by the treatments, especially by the 40 nm nCuO.

Of all the sources studied, the 40 nm nCuO was the one which presented better results of the parameters studied.

The copper speciation showed that the CuO applied is transformed from Cu<sup>1+</sup> to Cu<sup>2+</sup> 14 days after application.

The use of nanoparticles as fertilizers is possible but plant responses to the application vary according to the size of the nanoparticles itself and dose. The use or not of dispersant interfere in the plant responses.

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## 4 FOLIAR UPTAKE OF NANO CERIUM OXIDE IN SOYBEAN PLANTS

### Abstract

The application of the rare earth element cerium in plants has emerged few years ago, although its possible beneficial effects are still controversed. Some studies have shown that in a proper concentration Ce can improve plant growth and development, increase yields and alleviate environmental stresses, while others debate the potential toxicity of these nanoparticles. This study has applied two sizes of cerium oxide nanoparticles, its bulk form and cerium nitrate on soybean plants to try to elucidate whether this element brings beneficial effects. The plants were foliar sprayed with dispersions of the abovementioned particles, using a dispersant or not, in two doses, and harvested 21 and 45 days after application. Both nanomaterials (25 and 70 nm) are similar and show characteristic diffractions for cerium oxide. Nanoparticles of cerium oxide were absorbed by soybean leaves and redistributed to other leaves. The use of cerium nanoparticles, did not interfere in agronomic parameters such as biomass production and the number of pods. Cerium concentration and accumulation presented different values depending on the source and the dose. Among the sources, cerium nitrate was the one which yielded the highest values of both parameters and the dose 10 mg plant<sup>-1</sup> presented higher values than the dose 0.1 mg plant<sup>-1</sup> and the control, which were statistically equal. More detailed studies on physiological and biochemical effects of this rare earth element on crop plants should be carried out for a better understanding of how the interaction Ce-plant occurs.

**Keywords:** nanomaterials, cerium oxide, rare earth element, mineral nutrition, foliar spraying, ICP-MS.

### 4.1 Introduction

Cerium (Ce) is one of the 15 members of the lanthanide series, which along with scandium and yttrium comprise the rare earth elements (REE). Cerium is the most abundant REE in the earth's crust (66 µg g<sup>-1</sup>) and its abundance is not that different compared with Zn and Cu, which are much more involved in environmental investigations than Ce (TYLER, 2004). With a market at US \$ 9 billions (GANGULI; COOK, 2018), it is estimated that the global REE mine production increased from 132,000 metric tons in 2017 to 170,000 in 2018, with ~70% of this value produced by China. Although Ce is not considered

essential to plants, some studies have shown that in a proper concentration Ce can improve plant growth and development, increase yields and alleviate environmental stresses (SALGADO et al., 2019; VILELA et al., 2018; HU et al., 2004; CHAO et al., 2009).

Much attention is being paid to the use of Ce as nano-fertilizers in the agriculture. The majority of Ce is found in the trivalent ( $Ce^{3+}$ ) and tetravalent ( $Ce^{4+}$ ) states, thus  $CeO_2$  is the most common form (CASSEE et al., 2011). In commercial micro-fertilizers, Ce is mostly found as chloride and nitrate forms (SHYAM; AERY, 2012). Different solubility (MUDUNKOTUWA et al., 2011), surface charges (EL BADAWY et al., 2011), chemical reactivity (DREIZIN, 2009) and many other features that can be interesting to the development of new products, including foliar fertilizers.

Cerium nanoparticles are predominantly found as  $CeO_2$  and they have been used in many engineering and biological applications such as catalytic materials (TROVARELLI, 1996), medicines (CELARDO et al., 2011), solar cells (CORMA et al., 2004), gas sensors (JASINSKI; SUZUKI, T.; ANDERSON, 2003) and solid oxide fuel cells (ESPOSITO; TRAVERSA, 2008).  $CeO_2$  nanoparticle is one of the most explored nanomaterial due to its unique property to store and release oxygen (HAILSTONE et al., 2009), and though it is predominantly explored in innovative technologies, the use of Ce and other REE nanoparticles in the agriculture as plant stimulator has recently drawn attention of the research community.

On the other hand, the broadening use of Ce nanoparticles in industrial products have raised concerns about its final disposal in the environment and its potentially toxic interactions with living systems, once Ce nanoparticles can be easily up taken, translocated, accumulated and biotransformed in plant tissues and consequently migrate into the food chains (HUSSAIN et al., 2019; ZHANG et al., 2012). This is a matter of intense debate in the recent literature and the potential toxicity of these nanoparticles and the mechanisms behind it still needs to be deeply investigated.

The Ce impact on plants varies according to the plant species, applied concentration, tested material, particle size and time of exposure. It is compiled in Table 1 a set of studies concerning the effects of foliar application of Ce on plants. In general, the effects occur in a dose-dependent manner. Some of them reported increase in plant height (JAHANI et al., 2019; XIE et al., 2015) and weight (XIE et al., 2019; MA; REN; YAN, 2014) by applying a dose bellow  $100\text{ mg L}^{-1}$ , whereas above this concentration these parameters and photosynthesis rate decreased (JAHANI et al., 2019; HONG et al., 2016). Cerium was also beneficial to inhibit the absorption and accumulation of heavy metals (LI et al., 2013), increase seed yield in drought-stressed plants (DJANAGUIRAMAN et al., 2018), improve photosynthetic parameters and

alleviate UV-B radiation induced stress (LIANG et al., 2006; LIANG; HUANG; ZHOU, 2006). Other effects are associated with enzyme activity and plant mineral, sugar and protein content (LI et al., 2013; XIE et al., 2015; 2019).

Aiming at contributing to the knowledge of how Ce interacts with plants this study investigated the effects of CeO<sub>2</sub> nanoparticles in two different sizes (25 and 70 nm), as well as bulk CeO<sub>2</sub> and Ce(NO<sub>3</sub>)<sub>3</sub> applied as foliar fertilizer in soybean (*Glycine max* (L.) Merrill) plants.

Table 1. Bibliographical survey on the foliar application Ce and their effects on plants. All biometric data presented statistical difference

Plant species	Type and concentration (mg L <sup>-1</sup> )	Application method	Nanoparticle size (nm)	Time of exposure	Main effects	Ref.
<i>Phaseolus vulgaris</i> L.	CeO <sub>2</sub> ; 250, 500, 1000 and 2000	Three-weeks-old plants were sprayed with 200 mL NPs suspension per treatment (n=3) every 48 h during two weeks	10-30	30 days	Most of the applied concentrations decreased stomatal density, anthocyanin, relative water and chlorophyll content, while stomatal length, catalase and electrolyte leakage were increased. Proline and peroxidase were both increased and decreased by the treatments, but most of other the responses were dose-dependent. Aerial height and root length only decreased at 2000 mg L <sup>-1</sup> . NP application caused membrane damage and induced oxidative stress	SALEHI et al., 2018
<i>Spinacia oleracea</i>	CeO <sub>2</sub> ; 10 and 100	Four-week-old plants were sprayed with NPs suspension twice per week for three weeks	~ 4	3 weeks	Both concentrations decreased Ca content in the roots and Zn content in the leaves. 10 mg L <sup>-1</sup> induced more metabolic changes in leaves, while 100 mg L <sup>-1</sup> induced more metabolic changes in roots	ZHANG et al., 2019
<i>Cucumis sativus</i>	CeO <sub>2</sub> ; 50, 100 and 200	Three-weeks-old plants were sprayed with 250 mL NPs suspension per treatment (n=4). The total volume was split in three applications, every four hours	8 ± 1	15 days	Decreased photosynthesis rate at 100 and 200 mg L <sup>-1</sup> , transpiration rate at 100 mg L <sup>-1</sup> , and increased stomatal conductance at 50 mg L <sup>-1</sup>	HONG et al., 2016
<i>Cucumis sativus</i>	CeO <sub>2</sub> ; 40, 80, 160 and 320	Fifteen-days-old hydroponically grown plants were sprayed with a total volume of 100 mL per treatment that was split in three applications, every four hours	8 ± 1	15 days	Some treatments altered dehydroascorbate reductase (DHAR), catalase (CAT) and ascorbate peroxidase (APX) activities in leaves, stems and roots	HONG et al., 2014
<i>Sorghum bicolor</i> (L.) Moench	CeO <sub>2</sub> ; 10	60 days after sowing, each pot was foliar-sprayed with 600 mL, that is, 6 mg of nanoceria for three plants <sup>-1</sup> . After that, water was withheld for 21 days	15 ± 5 nm	21 days	Reduced leaf superoxide radical and hydrogen peroxide levels and decreased cell membrane lipid peroxidation under drought. Increased leaf carbon assimilation rates, pollen germination and seed yield in drought-stressed plants	DJANAGUIRAMAN et al., 2018
<i>Lactuca sativa</i>	CeO <sub>2</sub> ; 50, 100 and 200	Plants were foliar-sprayed once after every other week, in total 3 times	micrometric	30 days	Treatments increased vitamin C and soluble sugar, reduced nitrite content, enhanced activities of superoxide dismutase and peroxidase dismutase, inhibited the absorption of Cd/Pb and decreased content and accumulation of Cd/Pb in shoots and roots	LI et al., 2013

<i>Cyclocarya paliurus</i>	Ce(NO <sub>3</sub> ) <sub>3</sub> ; 17, 86 and 434	Each plant was foliar-sprayed twice with 300 mL of solution, once every 15 days. Drops of Tween20 were added to the solution as a surfactant.	micrometric	30 days	Some treatments increased: seedling height; leaf proteins and sugars; K, P, Mg, Mn, Fe and Cu concentration; concentration of triterpenoids, quercetin and kaempferol; activities of SOD, peroxidase (POX) and phenylalanine ammonia-lyase (PAL) in the leaves. 434 mg L <sup>-1</sup> was only harmful to sugar content	XIE et al., 2015
<i>Calendula officinalis</i> L.	CeO <sub>2</sub> ; 50, 100, 200, 400, 800, 1600 and 3200	Two-week-old seedlings were sprayed with NPs suspension five times during five weeks	10-30	50 days	At 50 and 100 mg L <sup>-1</sup> shoot length, fresh weight and chlorophyll content were increased, while from 200 mg L <sup>-1</sup> these parameters were decreased. By applying more than 200 mg L <sup>-1</sup> the soluble sugar content of leaves and proline were increased. Hydrogen peroxide and membrane electrolyte leakage were respectively increased after 100 and 200 mg L <sup>-1</sup> . The activities of some antioxidant enzymes were induced by high concentrations	JAHANI et al., 2019
<i>Phaseolus vulgaris</i> L.	CeCl <sub>3</sub> (micrometric) and CeO <sub>2</sub> NPs; 40, 80 and 160	100 mL of suspensions and solutions were sprayed at 15 days after transplanting (plants were transplanted 16 days after sowing). The treatments were sprayed on the leaves every other day during 17 days	CeO <sub>2</sub> NPs: 6.9 ± 0.4	64 days	Only CeO <sub>2</sub> at 40 mg L <sup>-1</sup> increased dry weight (51.8% relative to the control). Plants exposed to 80 and 160 mg L <sup>-1</sup> CeCl <sub>3</sub> increased SOD activity and malondialdehyde (MDA) content. Some treatments interfered S, Mn, Mo and Zn content of pods. Chemical speciation analysis pointed out that Ce in treated leaves remained as its pristine form for both CeCl <sub>3</sub> and CeO <sub>2</sub> NPs treatments	XIE et al., 2019
<i>Brassica chinensis</i> L.	CeCl <sub>4</sub> ; 100	Twenty days old plants were foliar fertilized daily during 8 days	micrometric	8 days	Increased plot yield, fresh and dry weight and soluble sugar content. Decreased the titratable acid, vitamin C, Cu, Cd, Ni and Pb contents	MA; REN; YAN, 2014
<i>Brassica juncea</i> L.	CeCl <sub>3</sub> ; 12	Five-week-old seedlings were foliar sprayed once on the leaves until drops began to fall	micrometric	8 days	Mitigated the harmful effect caused by UV-B exposure during the growth of seedlings and increased photosynthetic parameters	LIANG et al., 2006a
<i>Glycine max</i> L.	CeCl <sub>3</sub> ; 20	Seedlings with the third leaf completely developed were foliar sprayed once on the leaves until drops began to fall	micrometric	7 days	Increased photosynthetic parameters and alleviated the inhibition of UV-B radiation on the photosynthesis	LIANG et al., 2006b

## 4.2 Materials and Methods

**4.2.1 Nanomaterials characterization. Purity.** 25 and 70 nm CeO<sub>2</sub> nanoparticles were acquired from MK Impex Corp. (Canada). The nanomaterials were analyzed by an energy dispersive X-ray fluorescence spectrometer (EDX 720, Shimadzu, Kyoto, Japan). The powder samples (200 mg) were weighted in a 6.3 mm aperture X-ray sample cup (no. 3577 - Spex Ind. Inc., USA) and sealed with a 5 µm thick polypropylene film (no. 3520 - Spex Ind. Inc., USA). The analysis were conducted under vacuum, in triplicate, using a rhodium (Rh) X-ray tube at 50 kV and auto-tunable current adjusted for a detector deadtime below 30% and a 3-mm collimator. The X-ray spectrum of the sample was acquired utilizing a Si (Li) detector for 300 s and through the fundamental parameters method it was possible to determinate contaminants content present in these nanomaterials.

**4.2.2 Structural characterization.** X-ray diffraction (XRD) was used to confirm the majority composition of the CeO<sub>2</sub> nanoparticles. The X-ray diffraction measurements were obtained in a Bruker D2 Phaser instrument, operating with Cu K $\alpha$  ( $\lambda=1.5418$  Å) radiation, 30 kV, 10 mA, sample holder rotation of 15 rpm and Lynxeye<sup>®</sup> energy dispersive one-dimensional detector with 192 channels. The diffractograms were acquired under the following conditions: continuous scanning mode in the  $2\theta$  range from 10 to 90°, step = 0.02° and acquisition time = 0.5 s, obtained from the sample powders.

**4.2.3 Physicochemical features of dispersions.** Dispersions in distilled water of the nanomaterials (100 mg L<sup>-1</sup>) were prepared to determinate the aggregates' sizes by dynamic light scattering (DLS) and its charge (Zeta potential) using a Zetasizer equipment (Malvern, UK).

**4.2.4 Nano CeO<sub>2</sub> morphology.** The morphology of the nanoparticles was evaluated by transmission electron microscopy (JEM 1011, JEOL, Japan) in dispersions of 100 mg L<sup>-1</sup>.

**4.2.5 Soybean cultivation.** The experiment was conducted in a greenhouse of the Soil Science Department at Luiz de Queiroz Superior School of Agriculture (ESALQ/USP). The soil used in the experiment was a sandy soil, the base saturation (BS) was raised for 60% through liming according to the base saturation method. Four seeds of soybean, cultivar KWS RK 7214 IPRO, were sown per pot. The exceeding seedlings were removed, leaving 2 plants per pot. Each pot of 2.5 L was filled with 2 kg of soil, daily irrigated with enough deionized water to keep the soil close to its field capacity.

**4.2.6 Cerium solutions.** Cerium based solutions, in the same concentrations of the nanomaterials were used for comparison. The experiment was assembled under a completely randomized design with three repetitions. Control treatments consisted of three pots with two plants in each, that only deionized water was applied to the leaves, and three pots with two plants in each, that a solution of deionized water + dispersant (5 wt.%) was applied to the leaves.

The application of all treatments was made in the first 3 trefoils through aspersion when the plants were in V3 stage. The dispersant used was the polyethylene glycol (PEG) with a molecular weight = 15,000 – 20,000 g mol<sup>-1</sup> from Sigma-Aldrich®. The nanoparticles were dispersed in deionized water and in aqueous dispersions containing the dispersant (5 wt. %). The factors and levels of the treatments are shown in Table 2.

Table 2. Factors and levels of the treatments applied

Factors	Levels			
<b>Particle size</b>	25 nm nCeO <sub>2</sub>	70 nm nCeO <sub>2</sub>	Micrometric CeO <sub>2</sub>	Ce(NO <sub>3</sub> ) <sub>3</sub> ·6H <sub>2</sub> O
<b>Dispersant (PEG)</b>	With	Without		
<b>Dose</b>	0.1 mg plant <sup>-1</sup>	10 mg plant <sup>-1</sup>		

The doses were selected based on a previous cerium effect on plants study of our research group. In total there were 54 pots, which come from the following combination of factors and levels:

- 4 (particle size) x 2 (dispersant) x 2 (dose) x 3 (repetitions) + 6 (control)

For the analysis, one plant was collected 21 days (V7/V8 stage) after the application of the treatments and the other plant, 45 days (R4/R5 stage) after the application. The responses studied were: shoot dry mass, number of pods, cerium concentration and accumulation.

In the days to collect the materials, the plants were divided in sets of leaves, the material identified, placed in paper bags and oven-dried at 65° C for 72 hours. Afterwards, the set of leaves that have not received the treatment was weighed and the samples grinded in Willey type knife mill (1 mm mesh).

**4.2.7 Analytical blank test.** As the contents of cerium to be analysed in the samples were low, a preliminar blank test was performed in order to assure that there was no contamination in the digestion process. Five analytical blanks were prepared in two different laboratories, using the reagents, tubes and microwave ovens of each laboratory.

**4.2.8 ICP-MS analysis.** Samples and standard reference materials were microwave-assisted acid digested in triplicate. A closed vessel microwave oven (ETHOS 1600, Milestone, Italy) was used according to the following procedure: 200 mg of ground material was accurately weighed in the TFM<sup>®</sup> vessels and 5.0 mL of 20% v v<sup>-1</sup> sub-boiled HNO<sub>3</sub> and 2.0 mL of 30% w w<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> were added. The microwave heating program consisted of the following steps: (1): ramp from room temperature to 120 °C in 5 min; (2): keeping at 120 °C for 2 min; (3): ramp from 120 °C to 160 °C in 3 min; (4): keeping at 160 °C for 2 min; (5): ramp from 160 °C to 220 °C in 5 min; (6): keeping at 220 °C for 20 min; (7): cooling for 20 min. After cooling, the final solutions were transferred to volumetric flasks and diluted up to 20 mL with high-purity de-ionized water (resistivity > 18.2 MΩ cm). The solutions were analysed by ICP-MS spectrometer (X-Series 2, Thermo Scientific, Germany). For trueness checking, the standard reference materials NIST 1547 (Peach leaves), NIST 1573a (Tomato leaves) and NIST 1515 (Apple leaves) were analysed.

**4.2.9 Experimental Design and statistical analysis.** The greenhouse experiment was set under a totally randomized design in a 4 x 2 x 3 + 1 factorial scheme (4 particle sizes, 2 dispersant and 3 concentrations), plus a universal control treatment.

All the experimental analyses were conducted at R. environment version 3.6. Prior to ANOVA the data was tested to check the normality (Shapiro-Wilk test). Logarithmic transformation was used for the data regarding cerium concentration and accumulation for both dates of analysis (21 and 45 days after treatment application).

For the ANOVA, Tukey Test with 5% significance was adopted for means comparison of the effects.

### 4.3 Results and Discussion

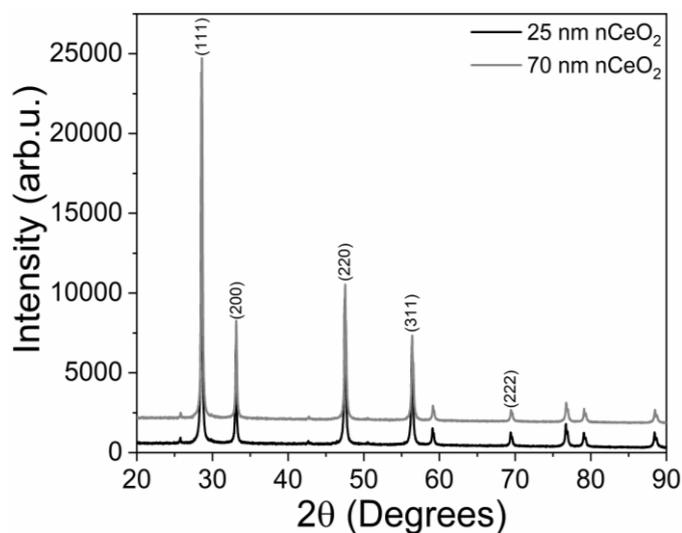
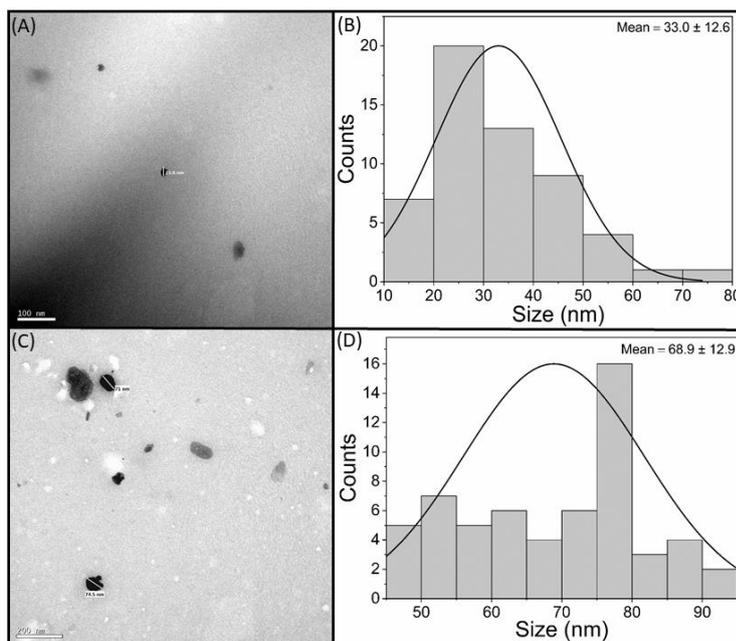
**4.3.1 Nanomaterials characterization.** Purity and contaminants of the nanomaterials used in the experiment are shown in Table 3.

Table 3. Purity and contaminant on the nanomaterials used in the experiment

Nanomaterial	Manufacturer	Purity (%)	Contaminant (mg kg <sup>-1</sup> )		
			Zn	Ni	Zr
25 nm nCeO <sub>2</sub>	MK Impex Corp.	99.88%	474	460	281
70 nm nCeO <sub>2</sub>	MK Impex Corp.	99.87%	674	354	262

The XRD diffractograms are shown in Figure 1. Diffractograms for both nanomaterials (25 and 70 nm) are similar and show characteristic diffractions for cerium oxide, as also described by Masui et al. (2003).

The shape of the nanomaterials could be confirmed by the images (Figure 2) obtained by transmission electron microscopy (TEM). For 25nm nCeO<sub>2</sub> (Figure 2A) is observed a spherical aspect, while for the 70nm nCeO<sub>2</sub> (Figure 2C) a more elongated aspect is seen. The particles present a normal size distribution, as shown in the histograms for 25 nm nCeO<sub>2</sub> (Figure 2B) and 70 nm nCeO<sub>2</sub> (Figure 2D).

Figure 1. XRD diffractograms of 25 and 70 nm nCeO<sub>2</sub>Figure 2. TEM images of (A) 25nm nCeO<sub>2</sub> and (C) 70nm nCeO<sub>2</sub> and their respective histograms (B and D). Scale bar: 100 nm

Regarding the dynamic light scattering and zeta potential values (Table 4), both particles present 2 peaks, with a main aggregates' size (more than 98%) of 237 nm (25 nm nCeO<sub>2</sub>) and 297 nm (70 nm nCeO<sub>2</sub>). The aggregates have a negative charge ranging from -8.9 to 15.7 mV.

Table 4. Zeta potential and Dynamic Light Scattering of CeO<sub>2</sub> nanoparticles

Nanomaterial	Zeta Potential (mV)	Dynamic Light Scattering (Cluster diameter – nm)	
		Peak 1	Peak 2
25nm nCeO <sub>2</sub>	-15.7 ± 6.6	237 (98.4 %)	5284 (1.6%)
70 nm nCeO <sub>2</sub>	-8.9 ± 4.6	297 (98.1 %)	5560 (1.1%)

Although the putative individual particle size of the nanoparticles is 25 and 70 nm, in aqueous dispersion the nanoparticles were aggregated. This occurs due to the Van der Waals forces and was also reported by Marucci et al. (2019).

**4.3.2 Analytical blank test.** As shown in Table 5, the five blanks prepared in Laboratory 2 presented cerium contamination during the process. This contamination came from the tubes used in the digestion process. Previously to this analysis, another set of digestions were carried out but the tubes were not properly decontaminated.

Table 5. Cerium content ( $\mu\text{g L}^{-1}$ ) in analytical blank determined by ICP-MS prepared in different laboratories

Laboratory	Reagents	Blank 1	Blank 2	Blank 3	Blank 4	Blank 5
1	HNO <sub>3</sub> 20%	0.006	< LOD	< LOD	< LOD	< LOD
	HNO <sub>3</sub> 20% + H <sub>2</sub> O <sub>2</sub> 30%	< LOD	0.009	< LOD	< LOD	< LOD
2	HNO <sub>3</sub> 20%	7.232	6.308	6.73	0.229	0.214
	HNO <sub>3</sub> 20% + H <sub>2</sub> O <sub>2</sub> 30%	28.64	471.8	17.4	19.87	19.73

\*LOD= 0.006151  $\mu\text{g L}^{-1}$

**4.3.3 Effects of nCeO<sub>2</sub> on plant biometrics and Ce translocation.** The dispersant by itself did not affect any of the parameters studied (biomass production, number of pods, cerium concentration and accumulation). In the same sense, the source or dose by themselves did not affect the biomass (21 or 45 days after treatment application) and the number of pods.

In literature few studies report effects of foliar-spraying cerium NPs on agronomic characteristics of crop plants. Rodrigues et al. (2019) have also tested the effects of bulk cerium on soybean and the authors report that the treatments did not affect plant biometrics such as plant height, number of leaves and number of pods.

No differences in aerial part of bean (*Phaseolus vulgaris* L.) were observed by Salehi et al. (2018) but the authors report that spraying lowered stomatal density, increased stomatal length and altered photosynthesis. They also report that spraying have caused damage to the leaves, which was also visually observed during the experiment. Most part of leaves that have received the treatments fell before the end of the experiment.

According to Xie et al. (2019) none of the treatments significantly affected plant biometrics (height, number of leaves, leaf length, and dry matter percentage) compared to the control.

Differently from our results, in which particle size did not affect agronomic parameters, different cerium particle sizes impacted cucumber fruit firmness (HONG et al., 2016). Different sizes are likely to cause different responses, including oxidative stress (MATTIELLO et al., 2015) due to their size feature and surface area (HUSSAIN et al., 2009).

Shoot dry mass at 45 days after treatment application was affected by the interaction source x dose (Table 6). The highest biomass production was obtained with the treatment 70 nm CuO in the dose 0.1 mg plant<sup>-1</sup> (1.10 ± 0.24 g), followed by micrometric CuO in the dose 10 mg plant<sup>-1</sup> (0.87 ± 0.26 g) = 25 nm CuO in the dose 0.1 mg plant<sup>-1</sup> (0.78 ± 0.30 g) = nitrate in the dose 0.1 mg plant<sup>-1</sup> (0.68 ± 0.27 g). The remaining treatments produced less biomass than the treatments above mentioned and were statistically equal between themselves and to the control.

Table 6. Shoot dry mass (g) of soybean plants harvested 45 days after treatment application

Source	Dose (mg plant <sup>-1</sup> )	Shoot dry mass (g)
<b>25 nm CuO</b>	<b>0.1</b>	0.78 ± 0.30 ab
	<b>10</b>	0.65 ± 0.18 b
<b>70 nm CuO</b>	<b>0.1</b>	1.10 ± 0.24 a
	<b>10</b>	0.63 ± 0.23 b
<b>Micrometric CuO</b>	<b>0.1</b>	0.62 ± 0.18 b
	<b>10</b>	0.87 ± 0.26 ab
<b>Nitrate</b>	<b>0.1</b>	0.68 ± 0.27 ab
	<b>10</b>	0.57 ± 0.24 b
<b>Control</b>	<b>0</b>	0.65 ± 0.18 b

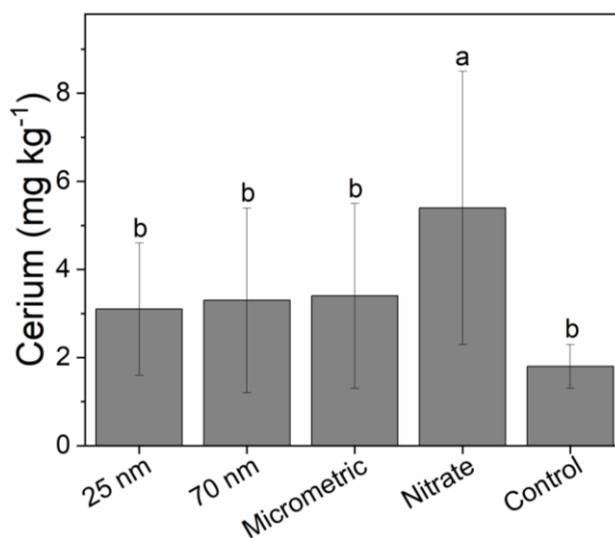
\*Means with the same letter do not differ statistically.

The results for cerium concentration and accumulation in leaves are presented below. Cerium was determined 21 and 45 days after the application of treatments. It is important to highlight that the analyzed tissues did not receive the treatments themselves, thus, in principle, the data show cerium translocation.

The external surface of leaves of higher plants comprises a cuticular layer covered by waxy deposits. Even though the wax contains a wide range of organic compounds that can trap theNPs, cerium was absorbed and redistributed to other leaves as demonstrated next.

Figure 3 shows the cerium concentration in plants 21 days after treatment application. The source cerium nitrate presented the highest concentration ( $5.4 \pm 3.1 \text{ mg kg}^{-1}$ ). All the other sources did not differ from the control. The source by itself presented no statistical difference for cerium concentration at 45 days after treatment application. Thus, the result observed at 21 days is due to the solubility of the nitrate and the assimilation of the element by the leaves is faster than the other sources.

Figure 3. Cerium concentration as function of sources in soybean plants harvested 21 days after treatment application as function of source



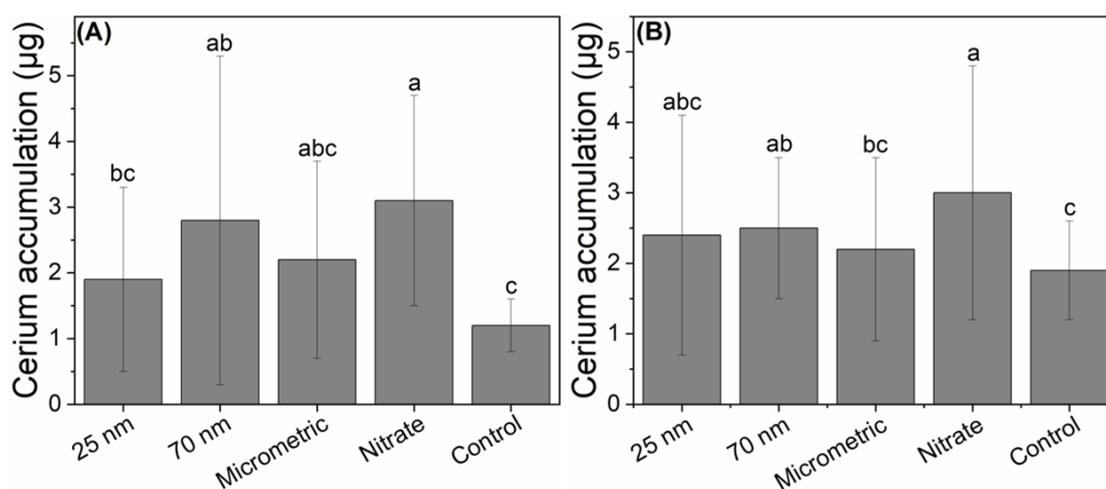
\*Means with the same letter do not differ statistically.

At both 21 and 45 days after treatment application, even control plants present cerium, when it should not. We hypothesize that contamination might have occurred during the grinding process.

Cerium accumulation in leaves is presented in Figure 4. At 21 days after treatment application (Figure 4A), the nitrate presented the highest value ( $3.1 \pm 1.6 \mu\text{g}$ ), followed by 70 nm CuO ( $2.8 \pm 2.5 \mu\text{g}$ ), micrometric CuO ( $2.2 \pm 1.5 \mu\text{g}$ ) and 25 nm CuO ( $1.9 \pm 1.4 \mu\text{g}$ ). All the sources statistically accumulated more cerium than the control ( $1.2 \pm 0.4 \mu\text{g}$ ).

Similar results were observed at 45 days after treatment application (Figure 4B). The nitrate presented the highest value for cerium accumulation ( $3.0 \pm 1.8 \mu\text{g}$ ), followed by 70 nm CuO ( $2.5 \pm 1.0 \mu\text{g}$ ), 25 nm CuO ( $2.4 \pm 1.7 \mu\text{g}$ ), micrometric CuO ( $2.2 \pm 1.3 \mu\text{g}$ ). The control presented the value  $1.9 \pm 0.7 \mu\text{g}$ , statistically less than all the sources.

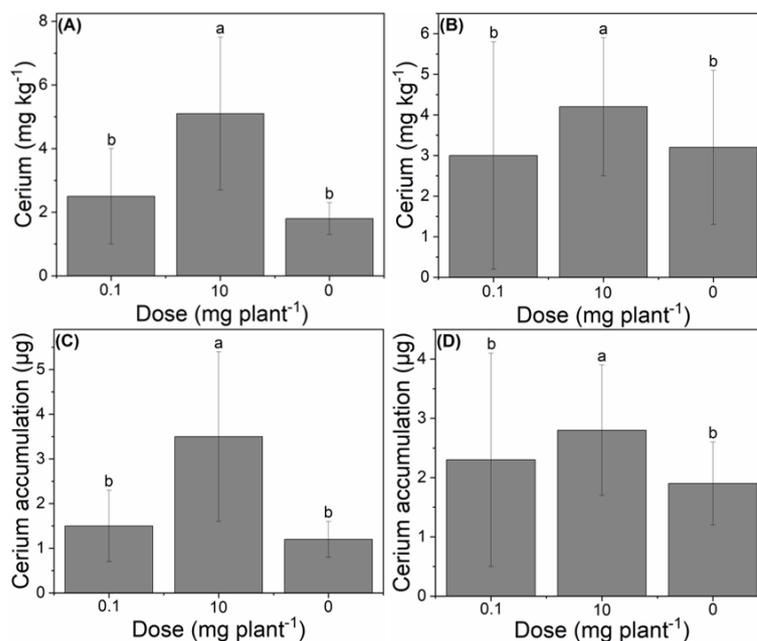
Figure 4. Cerium accumulation as function of sources in soybean plants harvested 21 (A) and 45 (B) days after treatment application



\*Means with the same letter do not differ statistically.

The effects of doses on cerium concentration and accumulation are shown in Figure 5. The results were similar for both parameters at 21 and 45 days after treatment application. The dose  $10 \text{ mg plant}^{-1}$  presented higher values than the dose  $0.1 \text{ mg plant}^{-1}$  and the control, which were statistically equal.

Figure 5. Cerium concentration (A/B) and accumulation (C/D) as function of doses in soybean plants harvested 21 (A/C) and 45 (B/D) days after treatment application



\*Means with the same letter do not differ statistically.

Salehi et al. (2018) declare that Ce concentration in the leaves showed a dose dependent absorption, uptake and translocation of Ce, like the results obtained in our experiment. In their study, cerium uptake occurred by roots or through leaves. When applied through leaves, the element concentration was much higher than soil application. For the authors, this difference could be related to bioavailability, as a result of adsorption-desorption and mobility processes, as well as to limited root uptake.

Hong et al. (2014) have found similar results for cerium concentration and different harvesting time. In their study, Ce concentration in leaves treated with higher concentration was significantly higher than the concentration found in leaves treated with the lower concentration. And alike in this study, the uptake did not increase with time. For them, this happened because the number of NPs on the surface saturated the routes of entrance through the leaves avoiding penetration.

A difference from their study and ours is that they have tested foliar application of NPs as powder and dispersion, and both ways promoted cerium redistribution in the plants.

The effects of the interaction between source x dispersant on cerium concentration and accumulation are presented on Table 7. Cerium concentration at 45 days after treatment application was not statistically significant.

At 21 days after treatment application, the treatments that have presented the highest values of concentration ( $\text{mg kg}^{-1}$ ) follow the order: nitrate without dispersant ( $6.8 \pm 3.5$ ) > micrometric CuO with dispersant ( $4.6 \pm 2.2$ ) = nitrate with dispersant ( $4.1 \pm 2.3$ ) = 70 nm CuO with dispersant ( $3.9 \pm 2.9$ ). All the sources, with or without dispersant, presented higher values of concentration than the control treatment.

Results for cerium accumulation at 21 days after treatment application followed a similar order as the concentration, with nitrate without dispersant presenting the highest value ( $3.90 \pm 1.51 \mu\text{g}$ ), followed by 70 nm CuO with dispersant ( $3.85 \pm 3.26 \mu\text{g}$ ). The control without dispersant produced the least accumulation value ( $1.07 \pm 0.06 \mu\text{g}$ ).

The results of cerium accumulation at 45 days after treatment application did not range as much as at 21 days but once again nitrate without dispersant presented the highest value ( $3.80 \pm 2.31 \mu\text{g}$ ). Micrometric CuO without dispersant ( $3.80 \pm 2.31 \mu\text{g}$ ) = 25 nm CuO with dispersant ( $1.47 \pm 1.06 \mu\text{g}$ ) = Control with dispersant ( $1.33 \pm 0.46 \mu\text{g}$ ) were statistically equal and presented the least accumulation values.

Table 7. Cerium concentration ( $\text{mg kg}^{-1}$ ) and accumulation ( $\mu\text{g}$ ) of soybean plants harvested 21 and 45 days after treatment application

Source	Dispersant	21 DAA		45 DAA
		Concentration ( $\text{mg kg}^{-1}$ )	Accumulation ( $\mu\text{g}$ )	Accumulation ( $\mu\text{g}$ )
25 nm CuO	With	$2.9 \pm 1.4$ b	$1.38 \pm 0.78$ bc	$1.47 \pm 1.06$ bc
	Without	$3.2 \pm 1.6$ b	$2.40 \pm 1.70$ abc	$3.30 \pm 1.77$ ab
70 nm CuO	With	$3.9 \pm 2.9$ ab	$3.85 \pm 3.26$ ab	$2.70 \pm 1.35$ ab
	Without	$2.8 \pm 0.9$ b	$1.82 \pm 0.48$ abc	$2.25 \pm 0.68$ ab
Micrometric CuO	With	$4.6 \pm 2.2$ ab	$2.77 \pm 1.56$ abc	$2.35 \pm 0.68$ ab
	Without	$2.2 \pm 1.0$ b	$1.70 \pm 1.34$ bc	$2.08 \pm 1.77$ bc
Nitrate	With	$4.1 \pm 2.3$ ab	$2.32 \pm 1.33$ abc	$2.13 \pm 0.45$ ab
	Without	$6.8 \pm 3.5$ a	$3.90 \pm 1.51$ a	$3.80 \pm 2.31$ a
Control	With	$1.7 \pm 0.4$ bc	$1.30 \pm 0.56$ bc	$1.33 \pm 0.46$ bc
	Without	$1.9 \pm 0.7$ bc	$1.07 \pm 0.06$ c	$2.50 \pm 0.27$ ab

\*Means with the same letter do not differ statistically.

\*\*DAA = days after application

The foliar uptake of cerium is also reported in literature (SALEHI et al., 2018; HONG et al., 2014; XIE et al., 2019), however, differently from nutrients such as potassium and magnesium, which are redistributed by the floem, the redistribution of cerium is still unknown.

Cerium speciation was not performed in this experiment but it is reported in literature that plants treated with  $\text{CeO}_2$  nanoparticles remain in the same form (WANG; TARAFDAR; PRATIM, 2013; HERNANDEZ-VIEZCAS et al., 2013).

### 4.3 Conclusions

Nanoparticles of cerium oxide were absorbed by soybean leaves and redistributed to other leaves. Biomass production and the number of pods were not influenced by cerium application.

Cerium nitrate, due to its soluble form, presented the highest values of cerium concentration and accumulation at 21 and 45 days. Both parameters were also dose-dependent.

Cerium can not be considered a benefic element because its effects in plants are still controversial, depending on particle size, dose and application way (soil or spraying). On the other hand, we did not observe toxic effects such as reported by several studies in the literature.

There are few studies regarding foliar application of nano cerium. For a better understanding of the influence of Ce NPs after foliar application, detailed studies on physiological and biochemical effects of this rare earth element on crop plants should be carried out.

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## 5 GENERAL CONCLUSIONS

Agriculture preceded the industrial revolution by many centuries, but research in nanotechnology for industrial application started many years ago, while for agriculture purposes, research is something very recent and challenging. The number of scientific publications in this area really increased in the last decade.

Methods for direct and *in vivo* analyses of plants, such as X-ray fluorescence spectroscopy (XRF), are extremely important in order to reduce time of diagnosis, costs, and diminish the number of steps in the analytical sequence and, then, reduce the generation of chemical waste. Our attempt to create a method like this was not successful to determine copper content due to its low concentration in plants, which is lower than the limit of detection of the equipment, but it was possible to access the nutritional status of other nutrients.

Regarding the established hypothesis, this thesis showed that nanoparticles of different sizes are absorbed by leaves and are redistributed to different parts of the plant, even in low concentrations. The use of nanomaterials has affected agronomic parameters such as shoots dry mass but hasn't influenced the number of pods. Copper and cerium content in the leaves were altered by particles' size, use of dispersant and dose.

Concerning the nanoparticles characteristics, despite their nominal size indicated by the manufacturers, when in contact with a solvent, agglomerates are formed, greatly increasing particle size. This happens due to Van der Waals forces and it was observed through dynamic light scattering analyses. Particles with a nominal size of 25-70 nm can form aggregates of hundreds of nanometers. Purity tests have shown that there is a minimal contamination in the nanoparticles (less than 1%), that may not interfere in the use of these nanomaterials.

Transmission electron microscopy (TEM) images have shown that the nanoparticles present a spherical or a more elongated shape and their estimated size is very to the one informed by the manufacturer.

X-ray diffraction data indicates that nano CuO 40 nm is copper oxide while 25 nm nanoparticles are a mix of CuO and metallic Cu. Both nano CeO<sub>2</sub> are constituted of cerium dioxide.

Digital microscopy analysis on the deposition of nano CuO on the leaves indicated that all of them accumulate mainly on the vein surface. The nCuO formed a dark precipitate, while the micrometric CuO did not show any color difference and in the CuSO<sub>4</sub> treated leaves it was possible to observe that the veins were black and, in some regions, there was whitish appearance, which differed from the other treatments.

X-ray absorption near edge structure (XANES) spectra for pristine 25 nm CuO suggested that it is a mixed oxide corresponding to 1s to 4p transition in Cu<sup>1+</sup> and after 14 days of application, the Cu<sup>1+</sup> fraction was converted to Cu<sup>2+</sup>.

Nano copper oxide dispersions can be used as sources of the element but plant responses are different according to the particle size and concentration.

Nanoparticles of cerium oxide were absorbed by soybean leaves and redistributed to other leaves. The use of cerium nanoparticles did not interfere in the agronomic parameters such as biomass production and number of pods.

Cerium concentration and accumulation on the leaves that haven't received foliar spray presented different values depending on the source and dose.

No toxic effects on plants were observed in both studies. This probably happened due to the low doses applied to the plants. Our idea from the beginning was to provide to the plants doses that were environmentally relevant.

Lastly, further studies are required to integrate the results here presented. The possible use of nanoparticles in agriculture is tremendous and research in this field is less than a decade old. The adoption of new technologies is paramount to achieve higher productivity and sustentable agriculture and food industry.

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