University of São Paulo
“Luiz de Queiroz” College of Agriculture

Uptake of zinc by soybean leaves using sources with different solubilities

Anita Beltrame

Dissertation presented to obtain the degree of Master in Science. Area: Soil and Plant Nutrition

Piracicaba
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versão revisada de acordo com a Resolução CoPGr 6018 de 2011

Advisor:
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1. Solubilidade, Soja, Absorção de folhas I. Título

Dedicatória

À minha avó, Geny Zambão Beltrame, que sempre viu o estudo como uma das principais conquistas dessa vida. À ela, que mostrou que o mundo deve ser destemidamente bem vivido todos os dias e em todas as experiências.

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SUMMARY

RESUMO

Absorção de zinco pelas folhas de soja usando fontes com diferentes solubilidades

O zinco é um micronutriente essencial para a soja, e a absorção nas plantas é afetada pela solubilidade das fontes de zinco no solo. Neste estudo, investigamos a absorção de zinco pelas folhas de soja usando fontes com diferentes solubilidades por aplicação foliar. Usamos três fontes diferentes de zinco: óxido de zinco, sulfato de zinco e zinco EDTA, que têm solubilidades variadas e os experimentos foram instalados em quatro áreas de cultivo de soja, Santa Carmem/MT, Itaara/RS, Itai/SP e Piracicaba/SP. A dose de Zn utilizada foi de 400mg de Zn por hectare, com objetivo de avaliar se o desempenho da soja quando adubada com fontes pouco solúveis podem se equiparar às fontes solúveis. Essa dose foi dividida em duas aplicações, em V4 e V8 e as coletas foliares foram feitas em V6, R2 e R4. Também foram coletados trifólios para análise de enzimas antioxidativas, análise do micronutriente nos grãos, concentração do micronutriente nas folhas e avaliações de produtividade e peso de 1000 grãos. Neste estudo não foi possível relacionar diferentes rendimentos da soja quando comparadas fontes de baixa solubilidade com fontes de alta solubilidade.

Palavras-chave: Solubilidade, Soja, Absorção de folhas

ABSTRACT

Uptake of zinc by soybean leaves using sources with different solubilities

Zinc is an essential micronutrient for soybeans, and its absorption in plants is affected by the solubility of zinc sources in the soil. In this study, we investigated the zinc uptake by soybean leaves using foliar application of different zinc sources with varying solubilities. We used three different zinc sources: zinc oxide, zinc sulfate, and zinc EDTA, each with different solubilities. The experiments were conducted in four soybean cultivation areas: Santa Carmem/MT, Itaara/RS, Itai/SP, and Piracicaba/SP. The applied dose of zinc was 400 mg per hectare, with the objective of evaluating whether soybean performance when fertilized with less soluble sources could match that of soluble sources. This dose was split into two applications, at growth stages V4 and V8, and leaf samples were collected at stages V6, R2, and R4. Trifoliate leaves were also collected for the analysis of antioxidative enzymes, assessment of micronutrient content in the grains and leaves, as well as productivity and 1000-grain weight evaluations. However, in this study, it was not possible to establish a clear relationship between different soybean yields when comparing low-solubility sources with highly soluble ones.

Keywords: Solubility, Soybean, Leaves uptake

1. INTRODUCTION

The soybean crop (*Glycine max* (L.) Merrill.) is widely used as a source of food for humans and animals, as well as for biodiesel production, and has a world production of 363 million tons (EMBRAPA, 2020). According to the same organization, 79% of world soybean production is destined for animal feed and the remaining (18%) is for oil production. The use of soybean by the chemical, pharmaceutical, and agro-industry industries has shown accelerated growth in grain yield and oil production (Freitas, 2011). Currently, from and economic and food perspective, soybean is the most important oleaginous plant cultivated in the world (EMBRAPA, 2006). In the current scenario, with increased demand for food and scarcity of areas for agricultural advancement, it is necessary to develop technologies able to promote increased productivity in agricultural areas already explored (GONÇALVES; JÚNIOR et al., 2010).

Earlier in Brazil, soil acidity and low contents of available nutrients were the main constraints for crop growth in most regions of Brazil, especially in the Cerrado region (LOPES, GUILHERME, 2016). Those constraints were significantly reduced by the continuous usage of lime products (to ameliorate soil acidity-related problems) and heavy application of synthetic fertilizers over the years to improve soil nutrient contents. However, currently, micronutrients are one of the most limiting factors for obtaining adequate yields in many agricultural systems of Brazil (CERETTA et al., 2005). Micronutrients deficiencies occurs very often in sandy soils as those of the Brazilian Cerrado, and in areas under conventional agricultural management that experiences low levels of available zinc (Zn), less than 1 mg dm^{-3} (GALRÃO, 2004).

Sandy soils present low contents of micronutrients due to the small portion of igneous rocks (basalt and gneiss) in the parent material; the low content of organic matter in the soil and the consequent leaching of zinc during soil formation, and the non-replacement of micronutrients according to the extraction by crops cultivation (GALRÃO, 2004). Brazilian Cerrado soils have low zinc availability and, although this micronutrient is required in low amounts by soybean (61 g t^{-1} grains), there is still a need for good nutrition with zinc in order to achieve adequate plant development (RESENDE, 2004).

The low adoption of micronutrients in fertilization programs in the past, the improvement in yield potential promoted by best management practices and improved cultivars, as well as the expansion of Brazilian agricultural into sandy soils with low micronutrients contents have intensified the need of zinc application for profitable yields (VENDRAME et al., 2007). However, zinc addition must be carried out in a balanced way, protecting the culture against the imbalance between nutrients in the system (SOUZA et al., 2010). Intensification of lime usage for ameliorating soil acidity has increased soil pH in cultivated areas (CAIRES et al, 2001) and, as a consequence, has caused a reduction of cationic micronutrients availability due to the precipitation into unavailable forms (BRADY; WEILL, 2007). This is an example how management practices can interact negatively, requiring a good understanding of soil management practices in the mechanisms affecting the availability of plant nutrients for adequate crop growth.

The importance of zinc for the plant is due to its role in several physiological processes, being an enzymatic activator of oxidative cycles (MARSCHNER, 2012). Zinc deficiency causes shortening of internodes, and emission of small, chlorotic, and lanceolate leaves in soybean (SFREDO, 2008).

In addition to the need for a better understanding of the time to apply Zn in soybean, it is still essential to evaluate the best strategy to supply Zn to soybean, as well as the sources of Zn to be used (GARCIA et al., 2009. Another important issue in foliar fertilization is the source of Zn used, since it is directly associated with the velocity of uptake, amount of nutrient absorbed, as well as the process of Zn translocation within plant tissues. For foliar fertilization, soluble sources were preferred due to their facility in penetrating leaf epiderma through cuticles pores, trichomes, or stomata (Thomas Eicherdt, 2008).

However, more recently, poorly soluble sources have been produced by micronizing oxides or carbonates until reaching micron scale, resulting in products with high density and more concentrated than the usual soluble sources. However, the capacity of leaves in absorbing, metabolizing and translocating such particles is still a matter of controversy. Macedo et al. (2021) found Zn and B uptake from concentrated suspension in coffee plants grown for 240 days after foliar fertilization under controlled conditions. In opposite, Migliavacca (2021), in a short-term study, found that poorly soluble sources of Mn were not able to revert deficiency symptoms by soybean whereas the soluble sources reverted such symptoms.

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2 UPTAKE OF ZINC BY SOYBEAN LEAVES USING SOURCES WITH DIFFERENT SOLUBILITIES

Abstract

O zinco é um micronutriente essencial para a soja, e a absorção nas plantas é afetada pela solubilidade das fontes de zinco no solo. Neste estudo, investigamos a absorção de zinco pelas folhas de soja usando fontes com diferentes solubilidades por aplicação foliar. Usamos três fontes diferentes de zinco: óxido de zinco, sulfato de zinco e zinco EDTA, que têm solubilidades variadas e os experimentos foram instalados em quatro áreas de cultivo de soja, Santa Carmem/MT, Itaara/RS, Itai/SP e Piracicaba/SP. A dose de Zn utilizada foi de 400mg de Zn por hectare, com objetivo de avaliar se o desempenho da soja quando adubada com fontes pouco solúveis podem se equiparar às fontes solúveis. Essa dose foi dividida em duas aplicações, em V4 e V8 e as coletas foliares foram feitas em V6, R2 e R4. Também foram coletados trifólios para análise de enzimas antioxidativas, análise do micronutriente nos grãos, concentração do micronutriente nas folhas e avaliações de produtividade e peso de 1000 grãos. Neste estudo não foi possível relacionar diferentes rendimentos da soja quando comparadas fontes de baixa solubilidade com fontes de alta solubilidade.

Keywords: ZnO, Nano-fertilizers, soybean

2.1 Introduction

Foliar sprays are most used to prevent or correct symptoms of Zn deficiency and may be effective in reversing micronutrient deficiency. However, after foliar application, Zn contents may be low in the leaves, due to the low rate of remobilization to other parts of the plant, due to its low mobility in the phloem (MARSCHNER, 2012). The greatest interest in foliar spraying is to increase the rate of photosynthesis in the leaves and stimulate the absorption of nutrients by the root of the plant, in addition to providing micronutrients in soils that have low levels; correct deficiencies situations where it is impossible to apply fertilizers via the soil; increase the use of fertilizers; increase the speed of plant growth and quickly correct the nutritional balance (MARSCHNER, 2012; MOCELLIN, 2004).

Nano-materials are materials that are smaller than 100nm. Nano-technologies have been found in the field and have already been applied to fertilizer crops (Scott and Chen, 2003; Wiesner et al., 2006). Nano-fertilizers are materials developed to deliver nutrients to plants in a targeted manner and controlled, improving their efficiency and reducing the environmental impact of conventional fertilizers (Naderi et al., 2011).

Using nano-fertilizers has advantages and one of them is that application can be done in small quantity compared with usual products (Subramanian et al., 2015) and can release nutrients gradually. This means the plants can absorb the nutrients more effectively, resulting in improved growth and yield. In addition, nano-fertilizers can also reduce the risk of leaching and runoff, which can cause soil pollution and other environmental problems (Seleimam et al, 2020).

Most of the micronutrient's sources used in agriculture comes from oxide compounds that serves as raw materials, that undergoes different processes of treatment, resulting in products varying in solubility, particles sizes, and presence of organic ligands (such as in chelates). New Zn fertilizers have been developed to explore their capacity, modifying the size, solubility, reactivity and surface area (Subramanian and Sharmila Rahale, 2012). Some studies reported positive effects on vegetable seed germination of bulk form of nano-ZnO (Singh et al., 2013), in peanuts reported leaf chlorophyll content, seed germination, and root and stem growth (Prasad et al., 2012).

This study tested the hypothesis that concentrated suspension products have a potential to improve nutritional status and yield of soybean when such products are sprayed under real field conditions. Our objective here was to compare foliar fertilization of soybean with sources of Zn with different solubility on the nutritional status of the plant, enzymatic activity and yield of soybean under different field conditions of Brazilian agriculture.

2.2 Material and Methods

2.2.1 Field experiments

2.2.1.1 Locations

The experiment was installed in four locations, under representative areas of soybean cultivation in Brazil (Figure 1), Itai (São Paulo), Piracicaba (São Paulo), Itaara (Rio Grande do Sul) and Santa Carmem (Mato Grosso). In Itai (SP), in the 2021-2022 soybean grown season, the experiment was installed at Fazenda 9 de Julho with geographic coordinates 23°18'31.3"S, 49°01'51.8"W and 654m altitude, with significant rainfall throughout the year, even in the driest months such as June and August. According to Köppen and Geiger, this climate is classified as Cfa. The average annual temperature in Itaí is 20.7 °C and the average annual rainfall is 1377 mm.

In Piracicaba (SP), the experiment was carried out at the Experimental Station from the Genetics Department (ESALQ/USP), with geographic coordinates 22° 45' – 22° 50' S, 48° 00' – 48° 05' W and 460m of altitude. This area has a tropical rainfall climate with a large difference between summer and winter, approximately 200 mm. The climate classification is Aw according to Köppen and Geiger. The average annual temperature is 23 °C and the average rainfall is 1464 mm.

The experiment was also carried out at the Experimental Station of the Phytus Institute, Itaara / RS, with geographic coordinates S29°35'32.5” O53°49'19.2” and an altitude of 460 m. The average annual temperature is 19 °C and average annual precipitation fluctuates around 1500 mm. And finally, in Santa Carmem (MT), the experiment was installed at Fazenda Triângulo, with geographic coordinates 11° 52' S, 55° 00' W, and 340 meters of altitude. The climate is classified as Aw (tropical climate with a dry winter season), according to the Köppen - Geiger classification.

Prior to field establishment, soil samples were collected by mean of collecting five subsamples in each block, at the depths of 0-20 and 20-40 cm, ensuring low levels of Zn in all fields. Soil chemical analysis were performed according to Raij et al. (2001) while physical soil analysis was performed according to Lima et al (2013), (Table 1).

Piracicaba -22.725, -47.6476 and 460m of altitude
Itai 23.414 49.0927and

Figure 1. Field experiments locations

Table 1. Soil chemical-physical analysis in each experimental area, in the 2021/2022 grown season[1].

Prof	**pH**	**P**	**S**	**K**	**Ca**	**Mg**	**Al**	**H+Al**	**SB**	**CTC**	**V**	**m**	**B**	**Cu**	**Fe**	**Zn**	**Mn**	**MO**	**sand**	**silt**	**clay**
m	$CaCl_2$	– mg dm^{-3} –		——— mmol$_c$ dm^{-3} ———							— % —		——— mg dm^{-3} ———					g dm^{-3}	——— g kg^{-1} ———		
Experiment 1 – Itai/SP																					
0-0.2	5.15	36.3	12.3	2.93	28.7	5.3	<0.1	25.7	36.9	62.6	59	0	0.74	1.1	28.5	1.1	2.3	20.15	668	56	277
Experiment 2 – Piracicaba/SP																					
0-0.2	4.76	<7	11.1	2.57	15.7	6.3	2.8	25.5	24.6	50.1	49	10	0.20	0.3	51.7	0.4	3.9	16.6	788	59	153
Experiment 3 – Santa Carmen/MT																					
0-0.2	5.70	18.4	6.0	3.56	20	3	<1	29	-	52	44	0	0.13	0.4	60.0	0.8	3.9	21.1	75.5	4.3	20.2
Experiment 4 – Itaara /RS																					
0-0.2	5.50	11.9	53.9	2.93	75	23	<0.1	49	-	99	67	0	-	2.3	-	1.2	-	1.5	150	510	340

[1] pH in calcium chloride ($CaCl_2$) 0.01 mol L^{-1} ; phosphorus (P) extracted by Mehlich (RS and MT) and by resin in SP and determined by colorimetry; Potassium (K) extracted by Mehlich^{-1} and determined on atomic emission spectrophotometer; calcium (Ca) and magnesium (Mg) extracted with 1 mol L^{-1} KCl and determined in an atomic absorption spectrophotometer; sodium (Na) extracted with Mehlich $^{-1}$ and determined by flame photometer; potential acidity (H+Al) extracted with buffered calcium acetate (pH = 7) and determined by titrimetry; aluminum (Al) extracted with 1 mol L^{-1} KCland determined by titrimetry ; SB: Sum of exchangeable bases; CTC: Cation exchange capacity at pH 7; V: Saturation of CTC by soil bases; m: Aluminum saturation; MO: Organic matter by dycromate oxidation and determined by titrimetry; Sand, Silt and Clay determined by densimeter by the method of Buyoucos.

2.2.2 Soil management

Piracicaba/SP		Itai/SP		Santa Carmem/MT		Itaara/RS	
Fertilization		Fertilization		Fertilization		Fertilization	
Dolomitic lemestone (24% CaO, 17% MgO and 70% PRNT)	2.3 to 2.4 t/ha	MAP	170kg/ha				
Phosfate (P2O5)	60kg/ha	NPK (6-30-10)	252kg/ha	NPK	100kg/ha	SSP (0-18-0)	300kg/ha
Potassium (KCl)	90kg/ha	Potassium (KCl)	48kg/ha	Potassium (KCl)	100kg/ha	Potassium (KCl)	100kg/ha
Boron	0.56kg/ha	Boron	1.87kg/ha	Boron	270g/ha		
Nitrogen	12.5k/ha						
Seeds treatments		Seeds treatments		Seeds treatments		Seeds treatments	
Cultivar	NS6700IPRO			Cultivar	TMG 2383 IPRO	Cultivar	BMX ativa
Cropstar (insecticide)	400mL/100kg seeds	Cropstar (insecticide)	10ml/kg seed	Standak (insecticide)	1.5mL/kg	Cruiser OPTI (insecticide)	0.15L/kg
Standak (insecticide)	200ml/100kg seeds	Standak (insecticide)	4ml/kg seed	Maximu XL (fungicide)	1ml/kg	Apron RFC (fungicide)	0.15L/ha
Rizokop (*Bradyrhizobium japonicum*)	211mL/100kg seeds			Cobalt	1g/ha		
Azokop (*Azospirillum brasiliense*)	69mL/40kg seeds			StarFix (fungicide)	2mL/kg		

2.2.3 Treatments and experimental design

The experiment was carried out in a randomized block design, with five treatments and six replications, totaling 30 experimental units in each location (Table 2).

The source Zn sulfate is water soluble and commonly used in foliar fertilization programs with 20% of Zn v/v. The source ZnO + Additive is a concentrated suspension containing ZnO finely ground in addition with adjuvants, emulsifier, and absorption agents, commercial product from Yara^{TM}, 40% of Zn and 1% of nitrogen. The source ZnO microparticulate is a reagent grade ZnO commercial product named Zintrac^{R} from Yara^{TM}, 40% of Zn, with particles smaller than 10 micrometers. Treatment 5 (zinc chelate – SO_4) is a soluble organic product formed by the combination between a chelating agent (EDTA) and the metal (zinc), 15% of Zn, commercial product named Kellus^{R} from ICL^{TM} (Table 2). The rate (400 g ha^{-1} Zn) was chosen because it fits within the range of dosage commonly used in foliar fertilization programs.

Table 2: Treatments used in the 4 locations.

Treatm.	Sources of Zinc	Growth stage V4	Growth stage V8	Total
		g ha^{-1}		
1	Control	0	0	0
2	$ZnSO_4$	200	200	400
3	ZnO + additives	200	200	400
4	ZnO microparticulate	200	200	400
5	Zn chelate	200	200	400

2.2.4 Climatic data

It should be noted that the experiment in Santa Carmem, the only one that showed a statistical difference in productivity, owes rainfall above the average for other locations.

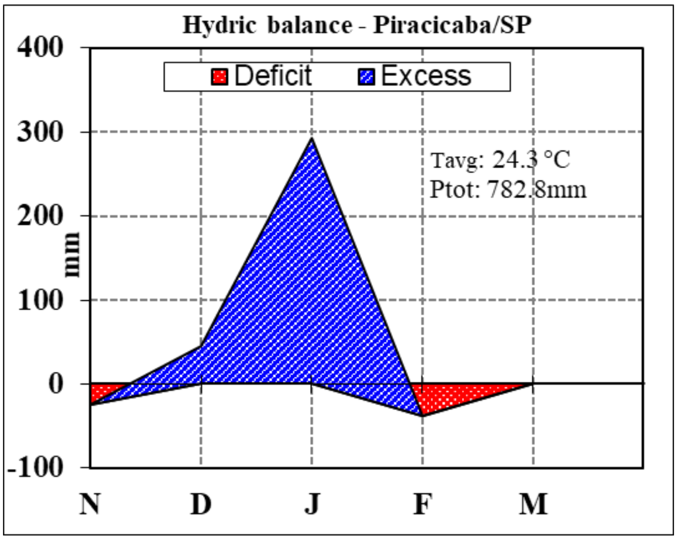

Figure 1: Climate data from Piracicaba/SP. Tavg means average of temperature in degrees Celsius and Ptot means total of precipitation in mm.

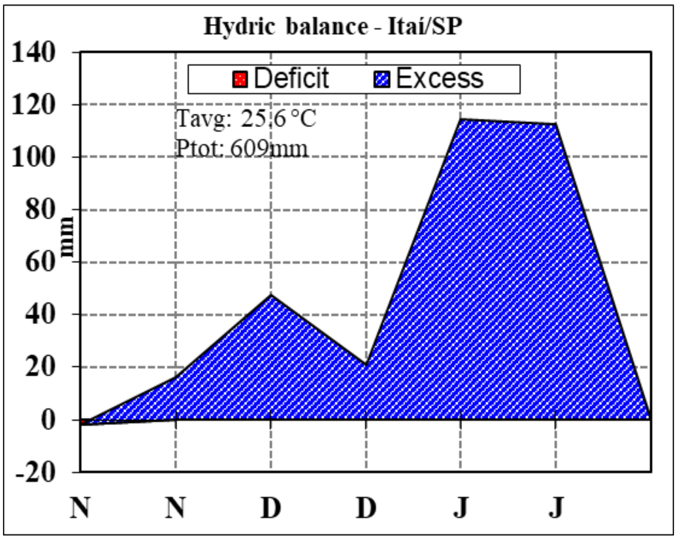

Figure 2: Climate data from Itai/SP. Tavg means average of temperature in degrees Celsius and Ptot means total of precipitation in mm.

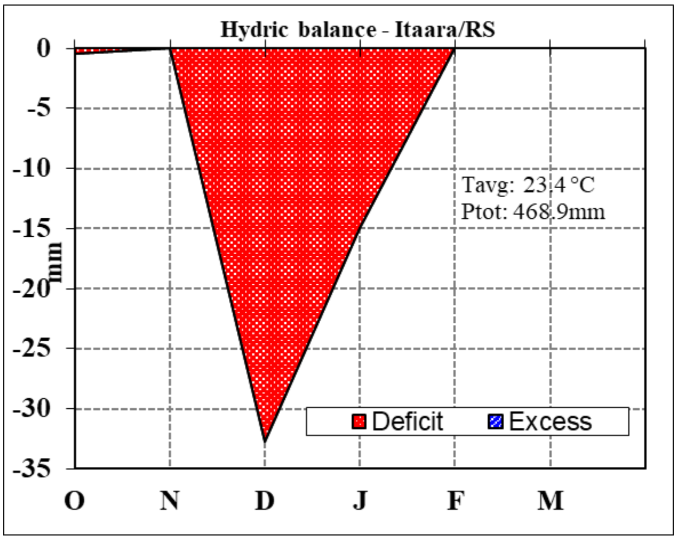

Figure 3: Climate data from Itaara/RS. Tavg means average of temperature in degrees Celsius and Ptot means total of precipitation in mm.

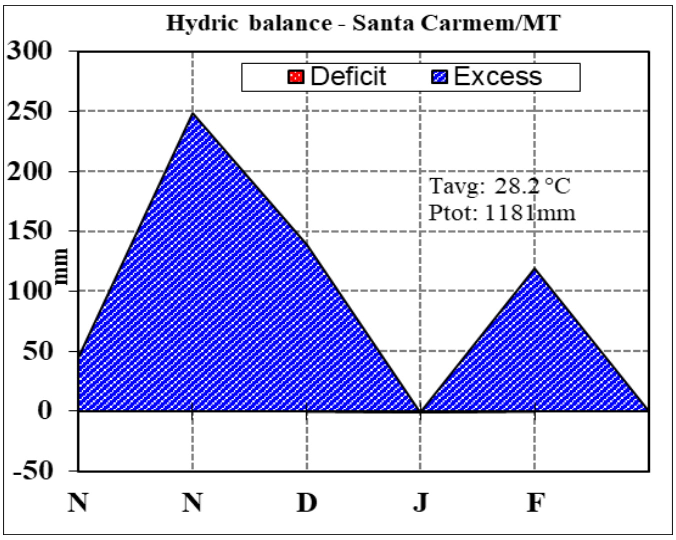

Figure 4: Climate data from Santa Carmem/MT. Tavg means average of temperature in degrees Celsius and Ptot means total of precipitation in mm.

2.2.5 Assessments

Soil analysis samples were collected at depths of 0-20 in the experimental area for chemical and physical characterization of the soil, before the installation of the experiment. The pH in $CaCl_2$; MO – acquired by sodium dichromate, acquired by colorimetry; P and K-pathogenic with ion exchange resin in São Paulo state and by Melich in Rio Grande do Sul and Mato Grosso states; H+Al (potential acidity) – pH SMP; Al, Ca, and Mg – inheritance with KCl 1 mol L-1; S – generated with 0.01 mol L-1 calcium phosphate; Boron - hot water/microwave; Cu, Fe, Mn, and Zn - pediatric with DTPA. (Chemical Analysis for Fertility Assessment of Tropical Soils. Agronomic Institute of Campinas, 2001); Na – tolerated with Mehlich 1 (Manual of Chemical Analysis of Soils, Plants, and Fertilizers. 2nd revised and expanded edition. Embrapa, 2009); Si – protein with calcium chloride 0.01 mol L-1 (Soil, plant, and fertilizer. Federal University of Uberlândia – Institute of Agricultural Sciences – Technical Bulletin, 2004).

In stages, V2 and V4 and at harvest, the stand of plants in the plots was determined, considering their useful area (7 linear meters of the 4 central lines), to know the number of plants that matured per plot. Leaf collections were made at stages V6, R1, and R4 to determine leaf Zn content, collecting the third mature trefoil of 10 central plants, and deducting 1m of borders on all sides. The leaves were washed in running water and then soaked in deionized water. Leaf and grain analyze were carried out according to the book: "Evaluation of the Nutritional Status of Plants" (Malavolta E.; Vitti G.C.; 1989), Nitro-Perchloric digestion and Reading of the element in atomic absorption spectrophotometry.

The productivity of the useful area of each plot was determined by weighing the grains. These were threshed and dried in an oven and yields were determined by correcting the moisture content in the grain to 13%. From these grains, sub-samples were taken to determine the weight of 1000 grains.

The third mature trefoils were collected and immersed in liquid nitrogen, to reduce enzymatic activity, and later these trefoils were stored on dry ice (-78 ºC) while still in the field, to be later stored in ultra-freezers at -80ºC. Material extraction was performed using 0.5 g of leaf samples stored in a -80ºC freezer, macerated in liquid nitrogen and homogenized in 5 mL of 50 mM potassium phosphate buffer. The extractor was prepared by mixing 50 mM KH_2PO_4 and 50 mM K_2HPO_4 adjusting the pH to 7.6. To homogenize the extractor, 0.1 mM of EDTA Triplex-III was added. After extraction, the samples were centrifuged at 15,000 rpm and 4ºC for 30 min, the supernatant was collected and used in the determination of protein concentration and enzymatic activity of the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPOX).

The protein concentration in the leaves was determined using the methodology described by Bradford (1976), in which 20 µL of the extract is homogenized in 1 mL of Bradford's reagent. For determination, the standard curve of bovine serum albumin (BSA) was used at concentrations between 0.1 and 1 mg mL-1. Spectrophotometer readings were taken at 595 nm.

The activity of the enzyme superoxide dismutase (SOD) was quantified using the methodology of Giannopolis and Ries (1977), with modifications. The method is based on the photochemical reduction of NBT (Nitro Blue Tetrazolium) by SOD. For the reaction, 500 µL of 50 mM Na_2CO_3 was used; 500 µl of 12 mM L-methionine; 500 µl of 75 µM NBT; 500 µl of 2 µM riboflavin and 50 µl of enzyme extract. The volume was completed to 5 mL using potassium phosphate buffer (pH 7.6) containing 0.1 mM Na-EDTA. The reaction starts with the addition of riboflavin, the tubes were then placed under the lights of the growth chamber for 8 min. Readings were performed in a spectrophotometer at 560 nm and expressed in U of SOD mg-1 protein. One SOD unit is defined as the SOD activity that results in 50% inhibition of NBT reduction.

The activity of the enzyme guaiacol peroxidase (GPOX) was determined using the methodology described by Matsuno and Uritani (1972). For the analysis, 50 µL of the extract of each sample was used, added to 750 µL of phosphate-citrate buffer at pH 5.0 and 50 µL of guaiacol 0.5%; adding 50 µL of 3% H_2O_2 after sample addition. The tubes were left in a water bath at 30oC for 15 min, followed by an ice bath. The reaction is stopped with the addition of 25 µL of 2% sodium metabisulfite. For the composition of the blank, 50 µL of the extractor was used instead of the sample. Absorbance was obtained by reading the samples in a spectrophotometer at a wavelength of 450 nm. The activity is expressed in mol-1min-1mg-1 of protein, calculated based on the enzyme extinction coefficient.

The activity of the enzyme ascorbate peroxidase (APX) was determined as described by Moldes et al. (2008). For the analyses, 650 µL of potassium phosphate buffer, 100 µL of ascorbate, 100 µL EDTA and 100 µL H_2O_2 were used. For the blank, it was necessary to replace the sample with potassium phosphate buffer. And the readings were taken in a spectrophotometer at 290 nm, in quartz cuvettes, at 30ºC. The reaction was monitored for 1 minute and activity values are expressed in nmol/minute/mg of protein.

2.3 Results and Discussion

2.3.1 Leaf nutrient contents

In the phenological stage V6, Zn foliar application increased leaf content of Zn in all locations (Figure 5). In the phenological stage R2, Zn content showed higher values only at Piracicaba/SP (Figure 6) and, in the R4 stage, the Zn content in the leaves were higher in the Zn treated plants only at Itaara/RS (Figure 7). These results indicate that foliar feeding of plants has a remarkable effect on foliar Zn contents in periods closer to the application time, reducing over time as plants continue growing. Similar findings were observed by Migliavacca et al (2021).

In the phenological stage V6, ZnO + additives showed higher Zn concentration in the leaf compared to other Zn sources in most locations (Figure 6). In the phenological stage R2, differences between Zn content were observed only in Piracicaba/SP site and, at this local, ZnO + additives and Zn chelated promoted higher values of Zn concentration compared to other treatments (Figure 6). In the phenological stage R4, differences for Zn concentration were found only in Itaara/RS site, with Zn microparticulate and Zn chelate presenting higher Zn concentration than Zn sulfate and ZnO + additives (Figure 7).

The insoluble sources of Zn (based on ZnO) increased Zn concentration in the leaves not only in the first period after application (V6), but also in longer periods following application (R2 and R4 stages). In this study, the leaves were washed in running water and then soaked in deionized water prior the analyses, removing any particles of ZnO product that might remain adhered to the cuticle. This is an indication that the ZnO-based fertilizers were able to effectively increase Zn concentration in internal tissues of the plants, showing a promising effect in improving Zn nutrition of soybean crop. This is an important finding considering the controversial aspects of foliar feeding of plants with insoluble sources of nutrients (Silva, R.S et al, 2017; Monteiro, F. A. et al, 2019).

After the second application of 200mg of Zn, the sources matched up and the only location we had a difference between treatments was in Piracicaba. Sohrab Davarpanah, 2016, proved that the foliar application of ZnO was better when applied with Boron in citrus, increasing all fruits parameters and yield producer while the application of ZnO by itself did not increase the fruit production.

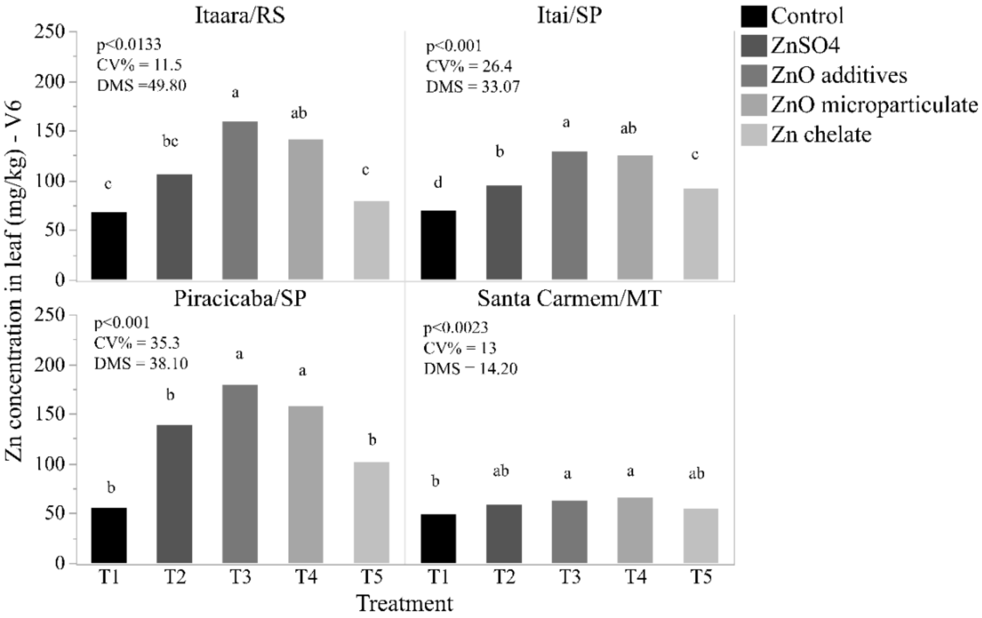

Figure 5: Leaf Zn content at stage V6. Means followed by similar letters do not differ statistically by the Tukeytest (p<0.10). The bars indicate the standard error of the mean. DMS, Minimum difference significance, and CV, coefficient of variation.

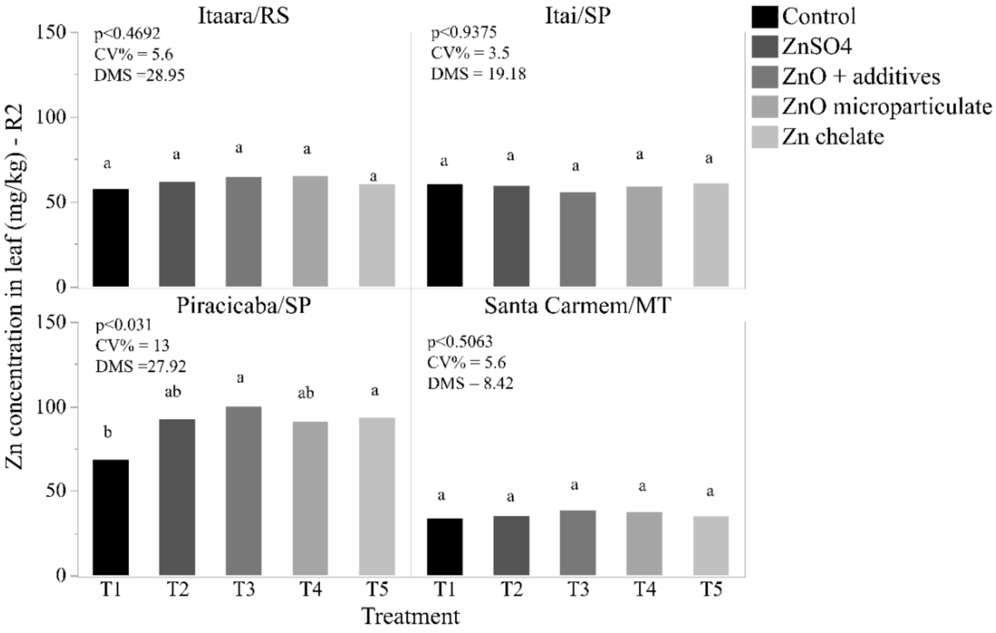

Figure 6: Leaf Zn content at the R2 stage. Means followed by equal letters do not differ statistically from each other by Tukey's a (p<0.10) probability test and the bars indicate the standard error of the mean. Minimum difference significance (DMS). Coefficient of variation (CV%).

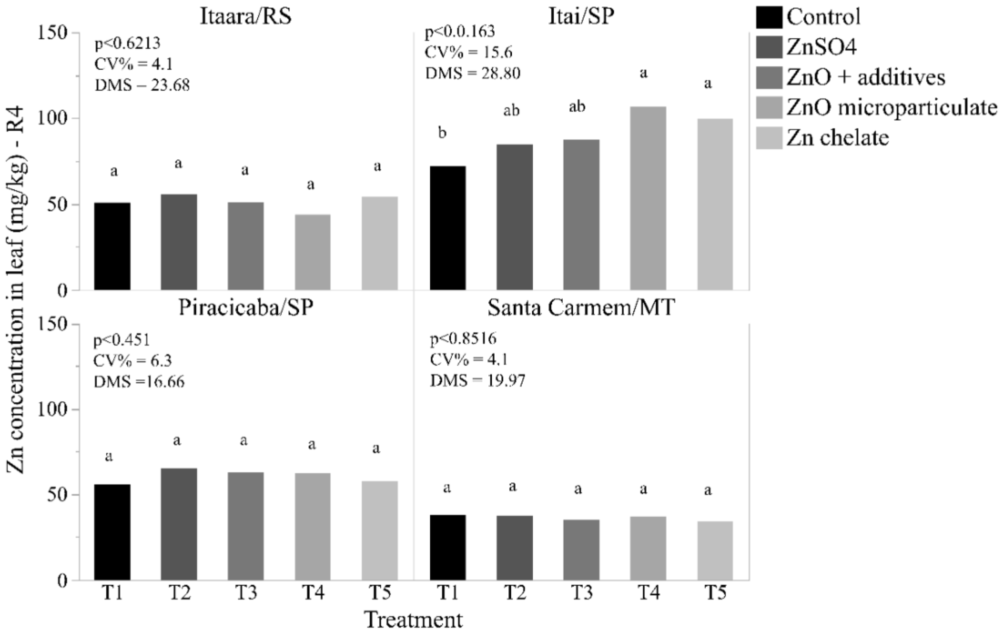

Figure 7: Leaf Zn content at stage R4. Means followed by equal letters do not differ statistically from each other by Tukey's a ($p<0.10$) probability test and the bars indicate the standard error of the mean. Minimum difference significance (DMS). Coefficient of variation (CV%).

2.3.2 Enzimatic analysis

Enzymatic analysis was performed only in the Piracicaba/SP site for operational issues. At this site, variation of enzymatic activity was found over sampling periods but not for Zn foliar treatments (Figures 8, 9, 10). Despite some higher mean values were obtained for Zn foliar treatments in selected treatments and in specific sampling periods, the means were not statistically different from the control treatment. This is an indicator that, under the studied conditions, Zn foliar spray did not increase the activity of such enzymes, even those enzymes being directly associated with Zn supply. Different from our findings, Yusefi-Tanha and Falla (2020) found increased values of SOD, APX and GPOX activity in plants receiving soluble sources of Zn (such as $ZnCl_2$) in higher concentrations.

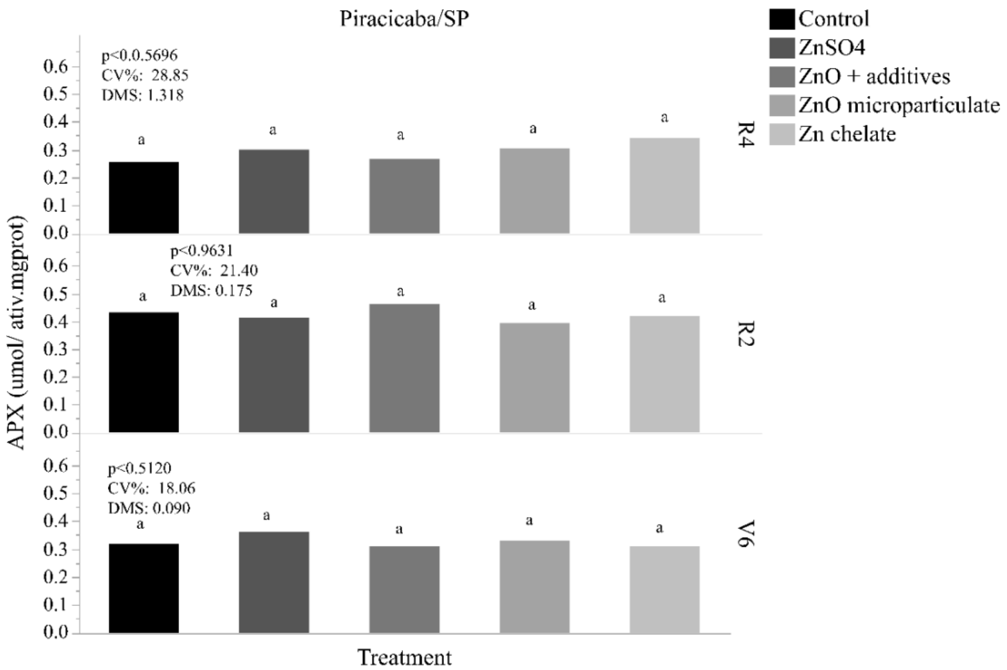

Figure 8: Activity of ascorbate peroxidase enzyme (APX) in Piracicaba-SP. Means followed by similar letters do not differ statistically by the Tukey test (p<0.10). DMS, minimum difference significance; CV, coefficient of variation.

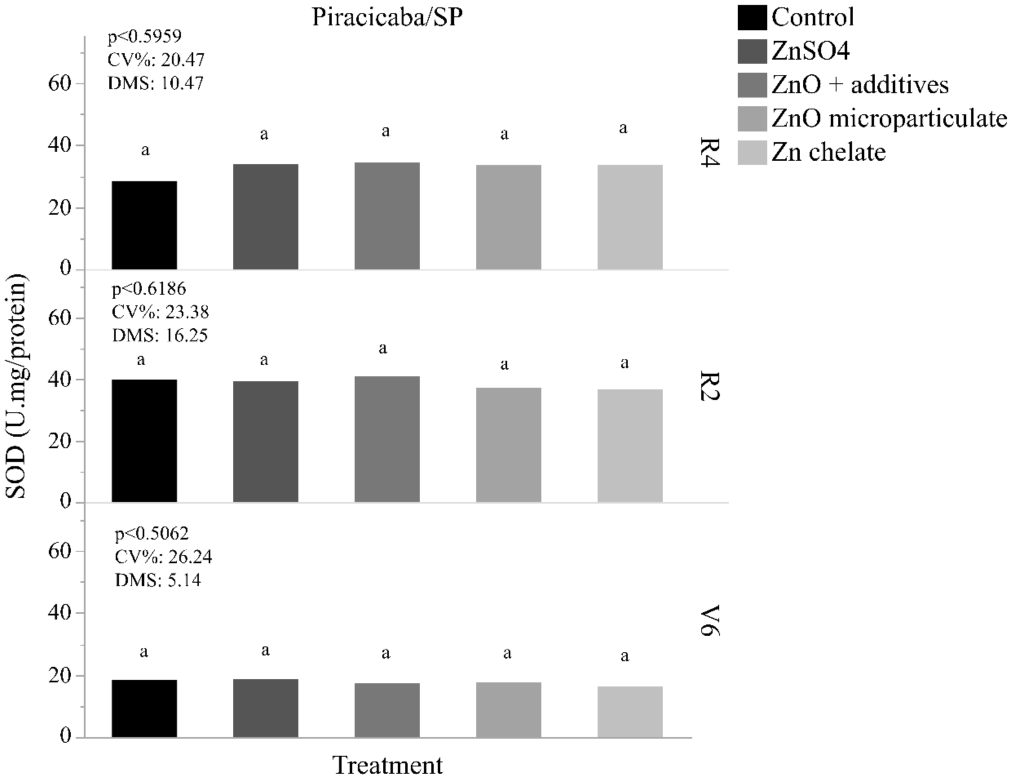

Figure 9: Activity of superoxide dismutase enzyme (SOD) in Piracicaba-SP. Means followed by similar letters do not differ statistically by the Tukey test (p<0.10). DMS, minimum difference significance; CV, coefficient of variation.

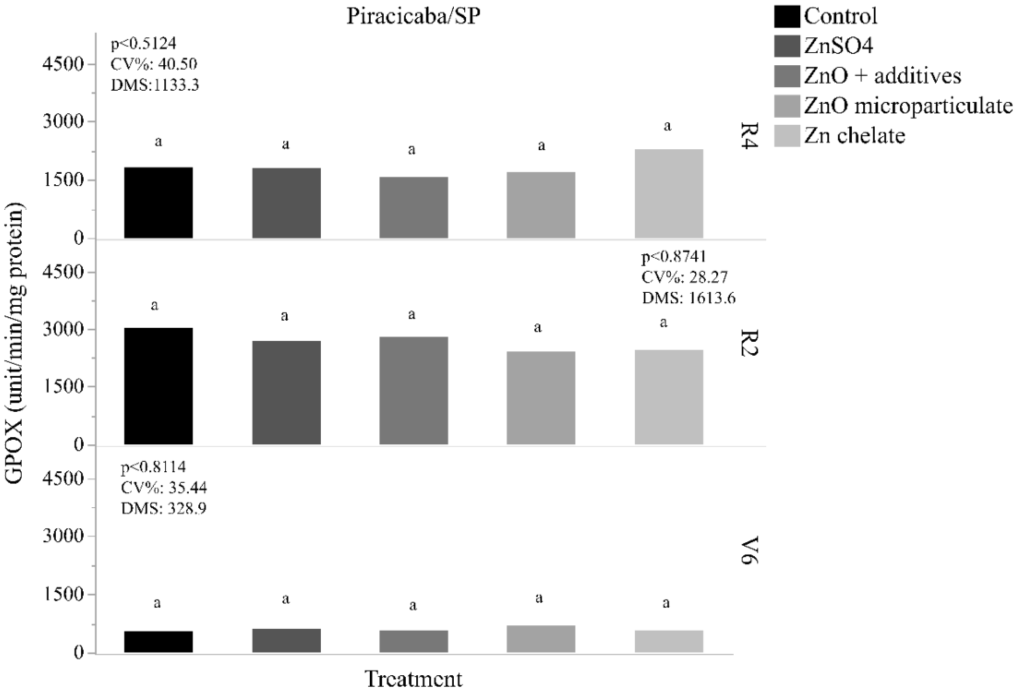

Figure 10: Activity of guaiacol peroxidase enzyme (GPOX) in Piracicaba-SP. Means followed by similar letters do not differ statistically by the Tukey test ($p<0.10$). DMS, minimum difference significance; CV, coefficient of variation.

2.3.3 Soybean yield

The soybean yield ranged from 2489.57kg.ha^{-1} in Piracicaba, 1930.4kg.ha^{-1} in Itaara/RS, 3271.47kg.ha^{-1} in Santa Carmem/MT and 4529.3kg.ha^{-1} in Itai/SP. Those values are within the range of yield levels obtained for soybean cultivation under field conditions, which present a mean yield in Brazil of 3026kg.ha^{-1} (CONAB, 2022). Differences on yield obtained in the studied locals are associated with better weather conditions occurring in Itai/SP and Santa Carmen/MT as compared to the remaining locals.

In Itaara/RS the foliar fertilization with $ZnSO_4$ resulted in higher soybean yield compared to the other treatments (Figure 11). At this site, the yield ranged from 2019.1kg.ha^{-1} for the ZnSO4 to 1850.6kg.ha^{-1} in the control treatment, representing a 168.5kg.ha^{-1} yield gain for the Zn foliar supply. At this local, ZnO microparticulate and Zn chelate presented higher mean values compared to the control, but with not statistical significance. On the remaining locals, there was no difference in soybean yield according to the foliar supply of Zn sources (Figure 11), similar to the findings of Imran (2015), but many of studies proof the efficiency of Zn from ZnO NPs in different crops (Table 3). Eisvand, Hamid Reza et al (2020) also showed that foliar application of ZnO in soybeans showed no difference in soybean yield, but associated the use of Zn foliar spray to improve plant quality under water stress.

2.3.4 Weight of grains

Weight of grains is an important factor for soybean yield. However, in most cases, weight of grains is a characteristic directly associated to the behavior of the cultivar. In other words, different cultivars might present different weight of grains. As expected, weight of grain varied consistently among sites, presenting mean values of 57.4g 1000 grains in Piracicaba/SP, 614.53g 1000 grains in Itai/SP, 163.8g 1000 grains in Itaara/RS, and 109.74g 1000grains in Santa Carmem/MT (Figure 12).

At Santa Carmem/MT, ZnO + additives and ZnO microparticulate presented higher weight of grains compared to the control, demonstrating that foliar spray with Zn has the potential to improve grain filling in the reproductive stage. However, in the remaining sites, Zn foliar spray did not improve weight of grains compared to the control. According to Yusefi-Tanha (2020), the application of Zn at a concentration of 400 mg/kg was the one that had the least increase in grain weight. In opposite, Imran (2015) found that only the supply of Zn trough the soil plus Zn supplied through the leaves were able to increase mass accumulation in the grains. This is an indicator that a good supply of Zn to soybean has a potential to improve the weight of grain by improving grain filling stage, but this can be obtained only under some circumstances of optimal Zn nutrition via soil and foliar supply, and for specific cultivars.

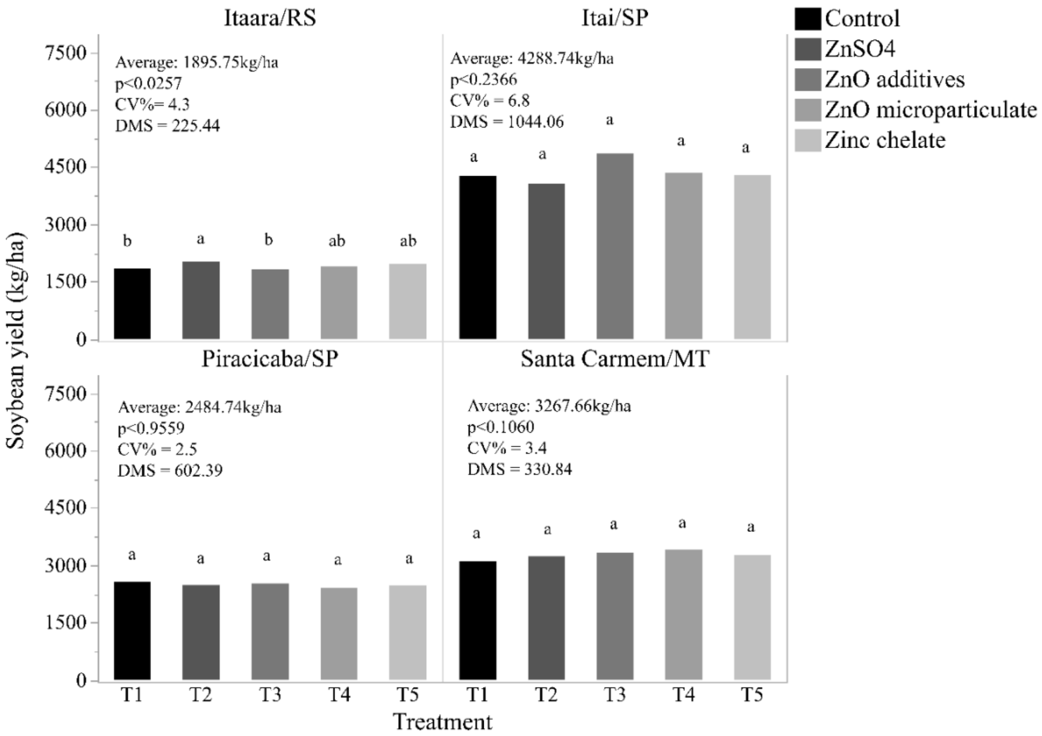

Figure 11: Soybean yield in the four locations. Means followed by similar letters do not differ statistically by the Tukey test (p<0.10). DMS, minimum significative difference; CV, coefficient of variation

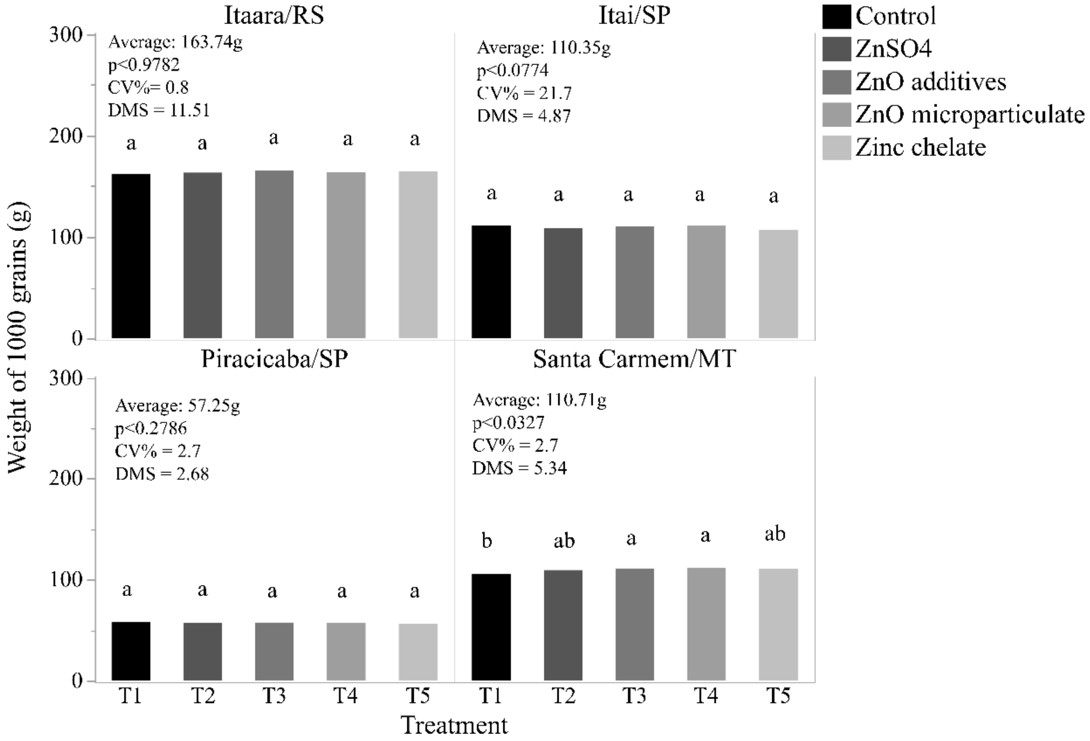

Figure 12: Weight of 1000 grains at four locations. Means followed by similar letters do not differ statistically by the Tukey test (p<0.10). DMS, minimum significant difference; CV, coefficient of variation.

Table 3: Studies that proves the efficiency of ZnO NPs in yield capacity in different crops limited to 10 years.

Article	Reference	Yield capacity
Foliar Application of Zinc Oxide Nanoparticles and Zinc Sulfate Boosts the Content of Bioactive Compounds in Habanero Peppers	GARCÍA-LÓPEZ, Josué I. Et al. Foliar application of zinc oxide nanoparticles and zinc sulfate boosts the content of bioactive compounds in habanero peppers. **Plants**, v. 8, n. 8, p. 254, 2019.	100mg/L of Zn, from ZnO NPs, increase number of fruits and weight of fruits
Impact of Foliar Application of Zinc and Zinc Oxide Nanoparticles on Growth, Yield, Nutrient Uptake and Quality of Tomato	AHMED, Razu et al. Impact of Foliar Application of Zinc and Zinc Oxide Nanoparticles on Growth, Yield, Nutrient Uptake and Quality of Tomato. **Horticulturae**, v. 9, n. 2, p. 162, 2023.	100ppm of Zn, from ZnO NPs, increase number of fruits and weight of fruits
Effect of nanoscale zinc oxide particles on the germination, growth and yield of peanut	T. N. V. K. V. Prasad, P. Sudhakar, Y. Sreenivasulu, P. Latha, V. Munaswamy, K. Raja Reddy, T. S. Sreeprasad, P. R. Sajanlal & T. Pradeep (2012) Effect of nanoscale zinc oxide particles on the germination, growth and yield of peanut, Journal of Plant Nutrition, 35:6, 905-927	400ppm of Zn, from ZnO, Increase the dry weight of dry pods
Foliar Spraying of zno Nanoparticals on Curcuma longa Had Increased Growth, Yield, Expression of Curcuminoid Synthesis Genes, and Curcuminoid Accumulation	Khattab, S.; Alkuwayti, M.A.; Yap, Y.-K.; Meligy, A.M.A.; Bani Ismail, M.; El Sherif, F. Foliar Spraying of zno Nanoparticals on *Curcuma longa* Had Increased Growth, Yield, Expression of Curcuminoid Synthesis Genes, and Curcuminoid Accumulation. *Horticulturae* **2023**, *9*, 355.	10mg/L of Zn, from ZnO NPs, increase the number of rhizomas, weight and diameter
Foliar Applications of zno and Its Nanoparticles Increase Safflower (Carthamus tinctorius L.) Growth and Yield under Water Stress	Ghiyasi, M.; Rezaee Danesh, Y.; Amirnia, R.; Najafi, S.; Mulet, J.M.; Porcel, R. Foliar Applications of zno and Its Nanoparticles Increase Safflower (Carthamus tinctorius L.) Growth and Yield under Water Stress. Agronomy 2023, 13, 192.	10g/L of Zn, from ZnO NPs, increase the number of capitula per plant and biomass yield

Impact of Foliar Application of zno and Fe3O4 Nanoparticles on Seed Yield and Physio-Biochemical Parameters of Cucumber (Cucumis sativus L.) Seed under Open Field and Protected Environment vis a vis during Seed Germination	Gupta, N.; Jain, S.K.; Tomar, B.S.; Anand, A.; Singh, J.; Sagar, V.; Kumar, R.; Singh, V.; Chaubey, T.; Abd-Elsalam, K.A.; Singh, A.K. Impact of Foliar Application of zno and Fe_3O_4 Nanoparticles on Seed Yield and Physio-Biochemical Parameters of Cucumber (*Cucumis sativus* L.) Seed under Open Field and Protected Environment *vis a vis* during Seed Germination. *Plants* **2022**, *11*, 3211.	300mg/L of Zn, from ZnO NPs, increase de yield and the weight of 1000seeds
The impact of nanofertilizer on agro-morphological criteria, yield, and genomic stability of common bean (Phaseolus vulgaris L.)	Salama, D.M., Abd El-Aziz, M.E., Shaaban, E.A. *et al.* The impact of nanofertilizer on agro-morphological criteria, yield, and genomic stability of common bean (*Phaseolus vulgaris* L.). *Sci Rep* **12**, 18552 (2022).	40mg/L of Zn, from ZnO NPs combined with MnO_2 NPs and MoO3 NPs increase the weight of seed and bean yield
Effects of Foliar Application of zno Nanoparticles on Lentil Production, Stress Level and Nutritional Seed Quality under Field Conditions	Kolenčík, M.; Ernst, D.; Komár, M.; Urík, M.; Šebesta, M.; Ďurišová, Ľ.; Bujdoš, M.; Černý, I.; Chlpík, J.; Juriga, M.; Illa, R.; Qian, Y.; Feng, H.; Kratošová, G.; Barabaszová, K.Č.; Ducsay, L.; Aydın, E. Effects of Foliar Application of zno Nanoparticles on Lentil Production, Stress Level and Nutritional Seed Quality under Field Conditions. *Nanomaterials* **2022**, *12*, 310	1mg/L of ZnO increase number of pods, weight of 1000grains and lentails yield
3. Nano-enabled Zn fertilization against conventional Zn analogues in strawberry (*Fragaria × ananassa* Duch.)	Saini, S., Kumar, P., Sharma, N. C., Sharma, N., & Balachandar, D. (2021). Nano-enabled Zn fertilization against conventional Zn analogues in strawberry (Fragaria× ananassa Duch.). Scientia Horticulturae, 282, 110016.	200μg g^{-1} of Zn, from ZnO NPs, increase number of fruits and fruit yield

2.3.5 Zinc concentration in the grains

The measurement of Zn concentration in the grain was only evaluated in Itaara/RS and Santa Carmem/MT (Figure 13). At Itaara/RS site, the $ZnSO_4$ resulted in higher Zn concentration in the grain. $ZnSO_4$ is a soluble source of Zn that might be translocated into other organs of the plants, such as grains, resulting in higher content of Zn in the grains at harvest time. Improvement of Zn content of grains, especially those cultivated for human and animal consumption, has a huge potential to improve Zn nutrition and reduce the negative effects of a Zn deficient dietary (Cakmak and Kutman, 2018; White, P. J., & Broadley, M. R., 2009). However, for the Santa Carmem/MT site, foliar Zn supply was of null effect in improving Zn concentration in soybean grain (Figure 13). Despite the huge variation in weight of grains for those two sites (~160 g 1000 grains in Itaara/RS *versus* ~100 g 1000 grains in Santa Carmem/MT) (Figure 12), those sites presented similar values of Zn concentration in the grains, ranging from 35 to 45 mg kg^{-1}, demonstrating that Zn concentration in the grain is driven by nutritional parameters and has no relationship with weigh of grains. Indeed, the application of foliar zinc (Zn) fertilizers is commonly employed in agricultural practices with the primary objective of enhancing grain quality rather than inducing a significant yield response (MONTALVO et al, 2016).

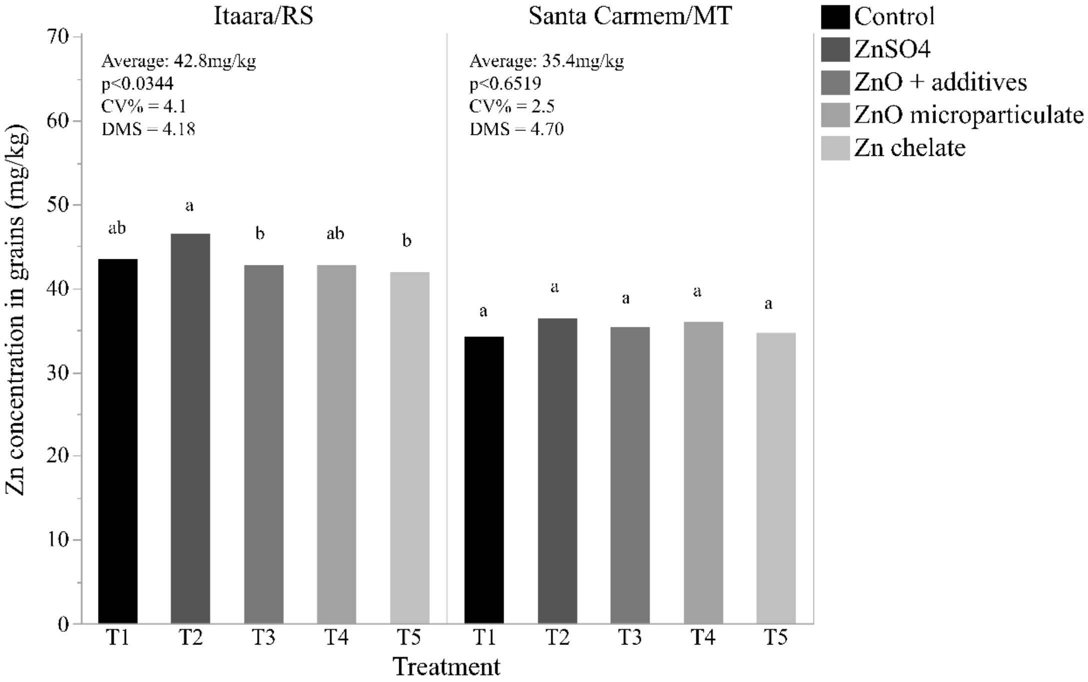

Figure 13: Zn content in grains. Means followed by similar letters do not differ statistically by the Tukey test (p<0.10). DMS, minimum difference significance, VC, coefficient of variation.

2.3.6 Statistical analysis

Statistical analyses were performed using JMP SAS software. Analysis of variance (ANOVA) was performed and when p<0.10 the test of means was Tukey 10%.

2.4 Conclusion

Insoluble sources of Zn showed potential in increasing Zn content in the leaves of soybean in the stages close to the application period, even under washing of the leaves. This is an indicator that insoluble sources of Zn might have a role in soybean Zn nutrition, especially in short periods following foliar application.

Different from common knowledge, Zn foliar spray did not improve activity of enzymes related with Zn supply in the Piracicaba site. This is an indicator that enzyme activity might be increased in some situations, but not in others.

In the remaining sites and for the remaining Zn sources, there was no positive effects of Zn foliar spray on yield. Similarly, only in one site (Santa Carmem/MT) the Zn supply with ZnO + additives and ZnO microparticulate improved the weight of grains of soybean. The foliar supply of $ZnSO_4$ improved the Zn concentration in the grain at Itaara/RS, demonstrating a potential for biofortification.

This study demonstrated the variability of Zn foliar spray response to soybean crop grown under field conditions. The effect of Zn foliar spray was not unanimous in improving Zn foliar content, enzymatic activity, soybean yield, weight of grains, and Zn concentration in the grain. Despite this variability, Zn foliar spray demonstrated potential in improving soybean Zn nutrition, yield, and Zn concentration in the grain in some circumstances, demonstrating a viable technology to supply Zn for crops grown under field conditions. Despite the positive results for insoluble sources on Zn in improving Zn foliar content in early stages of growth, this study demonstrates that soluble sources of Zn ($ZnSO_4$) improved more consistently soybean yield, weight of grains and concentration of Zn, being a superior source of Zn for foliar spray when compared to insoluble sources of Zn.

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3 ^{65}ZINC TRANSLOCATION IN SOURCES WITH DIFFERENT SOLUBILITIES

Abstract

In order to enhance the effectiveness of zinc (Zn) fertilizers, innovative methods are needed to assess their performance. This research utilized the stable isotope ^{65}Zn as a tracer to investigate the uptake and distribution patterns of ZnO nanoparticles (NPs) in comparison to $ZnSO_4$ when applied to young leaves. Soybean plants were cultivated in a nutrient solution containing 10% Zn, and they were subjected to treatments of ZnO and $ZnSO_4$ with ^{65}Zn, applied in V4 and V8. The Zn isotopes present in the roots, stems, leaves that were sprayed with the fertilizers, and leaves that were not sprayed were analyzed using a gamma-ray spectrometer (GRS). Comparing the Zn concentrations derived from the fertilizers, significant disparities were observed in terms of Zn utilization and distribution among the different fertilizers. However, when examining the total Zn content in plant tissues, as typically determined in conventional studies, no noticeable differences were observed. Notably, foliar application of $^{65}ZnSO_4$ led to greater Zn uptake and mobilization in leaves, roots, grains, and stems when compared to ^{65}ZnO NPs.

Keywords: ^{65}Zn, ZnO, Foliar spray

3.1 Introduction

There are different sources of Zn in agriculture, the most regular use in foliar applications are soluble sources such as $ZnSO_4$, and ZnEDTA, but most of them have low concentrations of Zn and can cause some phytoxides and burn plants. Alternative sources for foliar application have been used in agriculture, sources with fewer solubles, more concentrate such as ZnO nanoparticulate, and more sustained release of Zn. The solubility of ZnO is ksp 10−11.2 (Mertens, 2013) and the Zn salts are $ZnSO_4$, ksp 35.7, which may decrease the absorption of Zn (Li, C.; Wang, P. 2019). The labeling technique was really useful to visualize the zinc dynamic in soybean. Radioactive isotope (^{65}Zn) has been used to study the translocation and absorption of Zn in different crops (Cakmak et al., 1998; Erenoglu et al., 1999; Haslett et al., 2001) These studies help understanding how different sources of fertilizer can move in the plant through absorption leaves.

Foliar absorption is the process by which plants absorb nutrients, water, and other substances through their leaves. This process is important for plants, especially when the roots are not able to absorb enough nutrients from the soil. The absorption occurs through the stomata on the leaf surface, which allows gases and water to pass through (He et al., 2018). The substances that are absorbed by the leaves can be either beneficial or harmful to the plant. Nutrients such as phosphorus, potassium and nitrogen are often absorbed through foliar absorption (Fernández et al., 2009), while pollutants such as pesticides can also be absorbed, leading to potential harm. Overall, foliar absorption is an considerable process for the growth and survival of plants, and understanding this process can be useful for agricultural and horticultural practices (Schreiber, 2010; Riederer and Schreiber, 2001).

Traditional fertilizer experiments lack the capacity to distinguish between the zinc (Zn) supplied by the fertilizer and the pre-existing Zn levels in plant tissues. However, employing isotopic techniques can offer a more comprehensive comprehension of Zn transport, accumulation, and uptake within plants. These techniques provide high precision and low detection capabilities, enabling the identification of differences that conventional methods often overlook. This becomes particularly significant when evaluating new fertilizers, as subtle distinctions may remain undetectable through the measurement of total Zn content in plant tissues using conventional approaches.

The objectives of this study were twofold. Firstly, it aimed to evaluate and confront the effectiveness of different treatments in terms of absorption and mobility of zinc. Secondly, the study sought to determine the translocation pattern of zinc within the leaf after it has been absorbed.

3.2 Material and Methods

3.2.1 Experimental design and foliar application

The Nuclear Instrumentation Laboratory of CENA (Center for Nuclear Energy in Agriculture) was responsible for the experiments under controlled conditions employing radioactive labeled zinc sources. The experiment was carried out from July to October 2022, to complement the light irradiation, 800 µmol of photon/m² from artificial sources were used.

The experiment was carried out in a greenhouse of radioactive material located at CENA - Centro de Energia Nuclear na Agricultura (-22.707482193786237, -47.645313301174845). For this, soybean seeds were germinated in germination paper towels and moistened with $CaSO_4$, for 7 days, until they reached an adequate size for transplanting, and these plants were cultivated in nutritive solutions according to (Cakmak, adapted by LIN - laboratory of nuclear instrumentation) in the following composition: 1M KNO_3; 1M $Ca(NO_3)*4H_2O$; 1M $NH_4H_2PO_4$; 1M $MgSO_4*7H_2O$; 25mM KCl, 2.5mM H_3BO_3; 1mM $MnSO_4*7H_2O$; 1mM $ZnSO_4$; 0.25mM of $CuSO_4*5H_2O$ and 0.34mM of $(NH_4)6Mo7O_{24}$ and 50µM of NaFeEDTA, all pots were aerated and the solutions were changed every 5 days. The experiment was installed on August 20th, 2022, and was harvested on November 3rd, 2022.

The treatments were cultivated in hydroponic solution with 10% of the normal Zn concentration, in 3 L pots and had two controls: i) complete nutrient solution, in which plants received only water instead of foliar treatments; and ii) nutrient solution with 10% of the recommended Zn concentration, in which plants received only water instead of foliar treatments. The experiment was carried out with five replications in a randomized block design and had 40 pots, half of which were harvested in V6-V7, while the other half was harvested in R5.3-R5.4.

Foliar applications of formulated ZnO fertilizer and aqueous $ZnSO_4$ solution, both radio-labeled, occurred in two moments. Half of the plants received a single spray at V4 and the other half of the plants received two spraying at stages V4 and V8, each plant received 1.3 ml of treatment, resulting in a concentration of 0.65 mg Zn per plant. The treatments were applied to the 3 oldest trefoils and the monitoring of the fraction of the applied zinc that is translocated to other parts of the plant was carried out at stages V6-V7 and R5.3-R5.5 (Figure 1). Zinc determinations were measured by gamma radiation spectrometry.

The first foliar application with radio-labeled sources was carried out in the V4-V5 stage, with an average relative humidity of 85%. The first harvest occurred 10 days after the first foliar application, stage V8, and the harvests were divided into treated leaves (TL), which received foliar treatment, no treated leaves (NTL), which did not receive foliar applications, roots (RT), stem (ST) and grains (GR). Once harvested, the samples were dried for 72h at 65ºC and ground. At this point, half of the experiment was finished, leaving only 20 pots that received 2 foliar applications.

The second application was made at stage V8, with an average relative humidity of 65%. The second collection was performed at stages R5.3-R5.5 and also had the same classifications and drying procedures. During the application of the treatments of the two experiments, the surfaces of the vessels were covered properly to avoid any contamination of the solution nutritious by foliar applications of Zn.

The radiation levels allowed evaluation of how the Zn resulting from the treatments is partitioned in the tissues.

3.2.2 Preparation of radio-labeled sources

The radioactive-labeled zinc materials were obtained through neutron capture in the nuclear reactor at the Institute for Nuclear Energy Research. Upon irradiation, we obtained two capsules containing 15.7 mg of ZnO, with an activity of 165 kBq and 193kBq, each; and two capsules containing 57 mg of $ZnSO_4$, with an activity of 162 kBq and 164kBq, each. To prepare the zinc oxide fertilizer, the radioactive ^{65}ZnO was mixed with additives supplied by Yara company. The $^{65}ZnSO_4$ was dissolved in water. The final volume of the solution and dispersion were 25ml and 0.125μL, respective, and the concentration of Zn was 0.5mg/mL.

3.2.3 Climate data

Climate data from greenhouse 20/07/2022 up to 18/18/2022 from measured temperature and humidity meter.

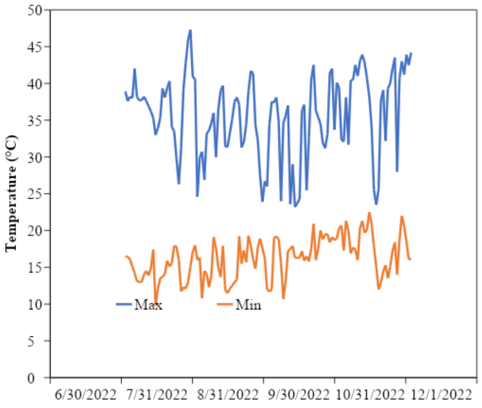

Figure 14: Temperature variation (°C)

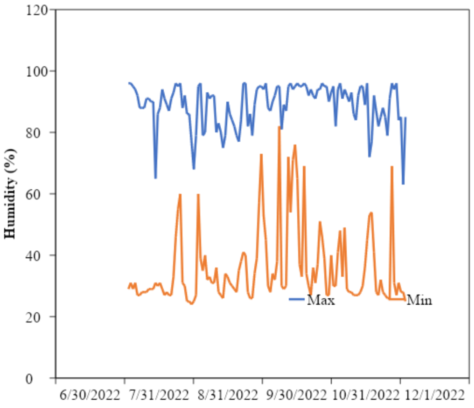

Figure 15: variation in relative humidity (%)

3.2.4 Stomatal density, trichomes, and stem anatomy analyses

For these evaluations, 20 pots were distributed in a randomized block under a bench, in which 10 pots contained soybeans grown in complete nutrient solution and the other 10 pots cultivated soybeans with 10% of the recommended Zn. At the phenological stage V4, the third mature trefoils were collected and analyzed under an ultraviolet light microscope trichomes and stomata of the abaxial and adaxial parts and for counting the ImageJ software was used.

Using the same plants, stem segments were cut and fixed in resin according to Marques & Soares, 2022. All air and water were removed from the samples by adding and increasing the ethanol concentration for 15 minutes and stirring for 15 minutes. This process was repeated 5 times to ensure that no air remained inside the conducting vessels. After removing all air and water, these samples were placed in 100% resin and dried for about 3 hours and then cut into a microtome with a 7µm slice to be analyzed in a light microscope. The slides were stained with toluidine blue and analyzed under a light microscope.

3.2.5 Monitoring of particle size and chemical map

Twelve soybean plants were cultivated in a greenhouse, which would receive a single treatment and a single moment (V5).

The soil used in the pots in the greenhouse was taken from the Anhumas-Piracicaba experimental station. This soil was chosen because it was the same soil in which the soybean in the field experiment of the same project would be grown, and also because it is soil that does not have a zinc content of 1 mg/dm3.

Table 4: Micronutrients in the soil of Anhumas/Piracicaba.

MICRONUTRIENT ANALYSIS - Piracicaba soil				
Cu	Fe	Zn	Mn	B
*****DTPA*************				(Hot water)
mg/dm³				
0.4	26	0.7	2.6	0.24

On October 29th, 4kg of soil was corrected by applying 2.8g of limestone applied per pot, 9.6 g of simple superphosphate (SPS) per pot, and 0.81 g of potassium chloride per pot. After applying correctives and fertilizers, 650 mL of water was added, the ideal amount for this soil to reach its field capacity and thus, for the limestone to be able to react with the soil. It is worth remembering that each pot has 4 kg of soil, that is, the recommendations were made based on this volume of soil and your needs based on the soil analysis

On November 11th, with the soil already ready for cultivation, soybean seeds were inoculated with *Bradyrizhobium japonicum* with a dose of and sowing. The chosen cultivar was NS6700IPRO, which is from a maturation group 7.1. The sowing of 4 seeds per pot was carried out, considering the vigor of the seeds.

In addition, the measurement of humidity and the average temperature in the greenhouse was carried out throughout the cycle, as well as monitoring the need for water by pots, preventing the plants from becoming stressed.

ZintracR-YaraTM product was applied to the soybean leaves. ZintracR is a foliar fertilizer providing Zn for seed treatment, seedlings, and foliar nutrition. In its composition, there is 16 g of N/L and 693g Zn/L. Its package insert recommends 0.5 to 1 l/ha of the product in soybean plants at stage V3 and with a spray volume of 30 to 200 l/ha.

To make the concentrated suspension, 0.030 grams of the product was used for a final volume of 50 mL of a commercial product, which would give a desired concentration of 245 mg of Zn per liter of solution. On the day of application, it was necessary to sonicate the concentration in order to obtain good homogeneity of the product with the water and not interfere with the results. The suspension was sonicated twice, 10 minutes in the laboratory and 10 minutes before application in the greenhouse.

The application was made with a drop of 1uL and 4 drops were made per leaf. Being that in a vase, it will analyze a leaf and the four drops of that leaf. Furthermore, it was important to be careful not to apply the drop too close to the veins in order not to compromise the results. It is essential to mention that the leaf chosen from each vase was the central leaflet of the 4th trefoil of the plant. After this application was made on December 14th, 5 analyzes of the experiment took place. The first analysis was performed (24h after application, the second analysis 1 week after application, the third analysis 2 weeks after application, the fourth analysis 3 weeks after application, and the fifth analysis was performed 4 weeks after application.

To prepare the sample, first, the four drops of the leaf to be analyzed are cut with a slide. These four cut drops are deposited in an aluminum sample holder covered with double-sided carbon adhesive tape. Then, the samples are metalized with carbon by Balzers med 010. After having the samples prepared, they were analyzed by the scanning electron microscope JEOL IT 300, with an analysis condition of 20 kV. In addition, SEM EDX analyses are performed with an Oxford X-ray detector and using Az tech software.

3.2.6 Statistical analysis

Statistical analyses were performed using JMP SAS software. Analysis of variance (ANOVA) was performed and when $p<0.10$ the test of means was Tukey 10%.

3.3 Results and Discussions

3.3.1 Radio-labeled sources

In Figure 18, the results clearly demonstrate that sparingly soluble sources, such as ZnO nanoparticles (NPs), exhibited a significantly lower absorption rate compared to soluble sources. The data reveals that the uptake of Zn from ZnO nanoparticles was approximately 40% less than that from soluble sources and the datas are showing in the tables 3 and 4.

Interestingly, in the initial collection, the untreated leaves (NTL) exhibited the highest accumulation of ^{65}Zn, indicating a notable translocation via the foliar route even with the poorly soluble source. However, the plants treated with zinc sulfate displayed a higher translocation rate, as evidenced by Figure 18. This aligns with the findings of Casey L. Doolette and Thea L. Read (2018), who demonstrated that radioactive zinc sulfate particles can travel distances of up to 25mm from the application site.

Additionally, the investigation conducted by Elham Yusefi-Tanha examined the effects of ZnO particles with sizes ranging from 38-59nm and a concentration of 200mg/kg soil. Their findings demonstrated that these particles enhanced the Zn concentration in the roots and stems compared to soluble Zn sources. However, in the present experiment, the soluble Zn sources displayed greater mobility in soybean plants, aligning with expectations. On the other hand, research conducted on wheat plants indicates that approximately 40% of foliar-applied Zn can translocate from the treated leaf to other plant organs within an 8-day period (Erenoglu, B.; Nikolic, M.; Roemheld, V.; Cakmak, I. 2002).

Overall, these findings emphasize the importance of solubility when considering the absorption and translocation of Zn in different crops. While poorly soluble sources like ZnO nanoparticles show some level of translocation, soluble sources such as zinc sulfate demonstrate more efficient mobility within the plant. These insights contribute to our understanding of the factors influencing Zn uptake and distribution in agricultural systems.

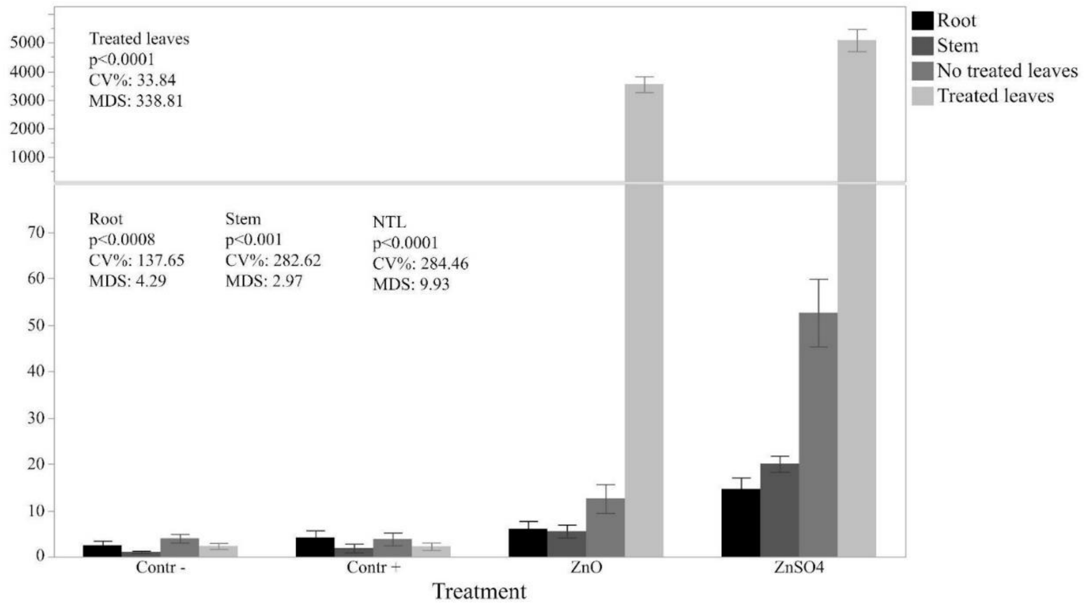

Figure 18: ^{65}Zn activity in the first collection. The bars indicate the standard error of the mean. Minimum difference significance (DMS). Coefficient of variation (CV%).

In the second sampling period, the grains exhibited higher levels of 65Zn, highlighting the significance of nutrient translocation via the plant's drainage system. Consistent with Figure 19, the soluble source of zinc demonstrated superior translocation compared to the poorly soluble source. These findings align with the research conducted by Thea L. Read and Casey L. Doolette in 2011, who demonstrated that zinc sulfate was the most translocated source to wheat grains. Higher levels of zinc were collected 15 days after the application of the radioactive material, indicating the efficient uptake and translocation of zinc sulfate in wheat plants.

Indeed, studies have been conducted on the uptake and translocation of Zn in various plant species, including beans and citrus. Bukovac and Wittwer (1957) as well as Ferrandon and Chamel (1988) conducted research specifically on beans and reported that plants were able to transport approximately 80% of the applied Zn. This suggests a significant capacity for Zn uptake and distribution within the bean plants.

Similarly, studies conducted by Wallihan and Heymann-Herschberg (1956) focused on citrus plants. Their findings also demonstrated that citrus plants were capable of transporting around 80% of the applied Zn. This indicates a similar efficiency in Zn uptake and translocation in citrus crops, further supporting the ability of plants to effectively transport and utilize the nutrient.

On the other hand, with pistachio seedlings studies, Zhang and Brown (1999), showed that around 5% of the absorbed Zn by the leaves was translocated into other parts of seedlings and the results are in good agreement with the results obtained with soybean. These results show that the movement of Zn by treated leaves depends on the crops. For $^{65}ZnSO_4$ translocation in wheat, the plant could transport 26% to 40% of the total absorbed Zn by leaves even under deficiency (B. Erenoglu, M. Nikolic1, V. Römheld1 & I. Cakmak).

In a study conducted by Sohrab Davarpanah in 2016, it was demonstrated that the foliar application of ZnO in combination with Boron (B) yielded better results in citrus crops contrasted to the application of ZnO alone. The combination of ZnO and B led to distinguished improvements in various fruit parameters and increased overall fruit yield. However, when ZnO was applied on its own, it did not show a significant increase in fruit production. These findings highlight the importance of considering the synergistic effects of nutrient combinations in foliar applications to maximize crop productivity and yield in citrus cultivation.

In previous studies, it has been observed that the availability of soluble Zn2+ decreases, resulting in a preference for the absorption of dissolved Zn by the leaves. (Li et al, 2018) and in the present investigation as we show in Table 2, the limited uptake of ZnO-NP can be attributed to their slow particle dissolution rate (Read et. al, 2020).

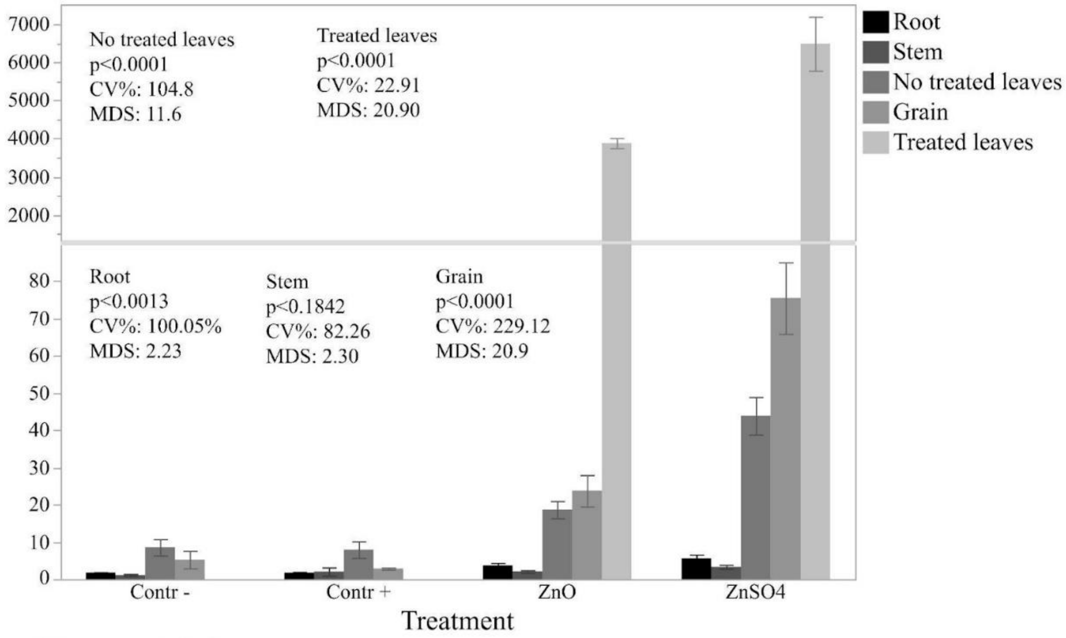

Figure 19: ^{65}Zn activity in the second collection. The bars indicate the standard error of the mean. Minimum difference significance (DMS). Coefficient of variation (CV%).

Table 5: γ-Spectrometry data showing the activity of total applied ^{65}Zn detected in each of the harvested soybeans.

Exposure time	Treat.	Treated leaf (Bq)	New leaf (Bq)	Stem (Bq)	Root (Bq)	Grain (Bq)	Total translocated (%)
1st harvest (12 days after application)							
	ZnO	3668.00	17.30	5.60	6.10	-	0.79
	$ZnSO_4$	5103.00	62.10	20.10	15.30	-	1.91
2nd harvest (70 days after the second application)							
	ZnO	19207.80	122.00	4.50	106.50	30.30	1.37
	$ZnSO_4$	39805.30	266.10	8.50	180.90	95.90	1.39

The low absorption and translocation of Zn may be due to the fact that the plants are kept in a nutrient solution with only 10% Zn. Some articles review the effects of nutrient deficiency on plant absorption and metabolism, highlighting that under deficient conditions, plants can exhibit adaptive mechanisms that lead to a reduction in nutrient uptake. This occurs as an adaptive response of the plant to conserve energy and allocate it to vital processes during nutrient scarcity.

Will et al. (2011) observed that in soybean (Glycine max) plants with boron (B) deficiency, the foliar absorption of B was significantly reduced compared to B-sufficient soybean. This decrease in foliar B absorption was attributed to the effects of B deficiency on leaf surface traits. Studies from Fernández et al. (2014a) reported the phosphorus (P) absorption of leaves applications was less in wheat plants with P deficiency compared to P-sufficient wheat. These findings indicate that nutrient deficiencies, such as B and P deficiencies, can impact the foliar absorption of these nutrients in different crop species.

Likewise, Zn deficiency resulted in a significant decrease of foliar Zn absorption as $ZnSO_4$ in sunflowers, ranging from 50% to 66% compared to zinc-sufficient plants (Cui Li et al, 2018). The same study showed that the primary factors contributing to the reduced Zn absorption under zinc deficiency were the decrease in leaf trichome density and potential alterations in the composition and structure of the adaxial leaf surface, as we could study in this experiment when the plants were conducted in 10% lower Zn the number of trichomes and stomas decrease, explaining why the absorption of label-sources was low.

Table 6: Activity in Bq detect in each soybean part at first harvest.

Treatments	Root	Stem	PAN	PAV
ZnO	12.80	10.42	36.94	3244.79
ZnO	3.11	2.70	11.20	3133.57
ZnO	3.98	3.18	11.05	3282.79
ZnO	5.78	6.68	13.18	3457.34
ZnO	4.84	4.90	14.06	4626.72
$ZnSO_4$	10.14	18.10	64.39	5409.77
$ZnSO_4$	17.02	22.83	72.74	5873.90
$ZnSO_4$	8.05	15.18	43.57	4227.16
$ZnSO_4$	16.60	19.72	83.23	4086.15
$ZnSO_4$	24.73	24.90	46.75	5830.58
Contr +	3.52	1.69	1.91	3.40
Contr +	11.50	6.02	16.52	4.76
Contr +	1.16	0.48	6.27	0.53
Contr +	4.04	2.51	4.57	1.69
Contr +	4.30	0.54	4.12	1.14
Contr -	6.79	1.86	7.23	4.81
Contr -	4.01	1.96	5.04	1.80
Contr -	0.96	0.82	2.70	1.05
Contr -	1.53	1.37	3.85	2.35
Contr -	1.65	0.67	5.59	1.82
média	4.57	2.94	11.12	1569.19
desvio	6.35	8.16	25.72	2334.17
CV%	138.88	277.63	231.22	148.75

Table 7: Activity in Bq detect in each soybean part at second harvest.

Treatments	Root	Stem	PAN	PAV1	PAV2	Grain
ZnO	106.86	3.12	112.38	13756.89	10782.83	28.00
ZnO	152.09	3.13	116.60	21046.39	2889.19	44.27
ZnO	116.04	6.64	138.84	18946.07	4327.99	41.17
ZnO	97.62	4.60	68.05	8737.13	2883.33	20.01
ZnO	59.87	5.14	174.13	6095.93	6572.93	17.91
$ZnSO_4$	229.56	15.53	292.52	35688.32	26023.18	67.90
$ZnSO_4$	67.32	9.13	219.86	9574.83	8069.17	139.80
$ZnSO_4$	216.55	5.26	317.71	27647.94	10646.30	86.10
$ZnSO_4$	235.60	6.29	253.32	35070.37	12828.36	114.96
$ZnSO_4$	155.37	6.24	246.84	24744.80	8734.57	70.57
Contr +	39.37	2.82	13.07	1.61	0.48	0.00
Contr +	18.32	1.38	81.42	7.70	11.56	2.23
Contr +	28.46	1.92	68.88	1.23	1.42	3.18
Contr +	44.58	7.37	39.25	7.41	23.17	4.28
Contr +	63.24	0.64	48.99	6.07	3.96	1.25
Contr -	30.25	1.38	14.97	6.91	3.93	3.23
Contr -	26.93	4.64	52.79	3.27	2.58	13.98
Contr -	36.21	1.75	54.08	1.90	3.56	3.29
Contr -	44.46	1.80	35.54	5.43	3.28	3.31
Contr -	45.09	3.16	113.19	9.67	5.16	25.55
média	61.56	3.88	96.90	3052.80	1453.25	18.96
desvio	71.10	3.46	95.34	12668.53	6693.70	41.02
CV%	115.50	89.18	98.39	414.98	460.60	216.32

3.3.2 Effect of Zn in plant anatomy

We did some anatomical analysis on the leaf and stem in order to verify the role of Zn in the soybean structure. As proven by previous studies, the abaxial face of soybean leaf has a greater density of trichomes and stomata (Figure 1). We presented here the interference of Zn in forming these leaf structures. Zn is directly related to the synthesis of growth hormones and thus directly linked to leaf area and the number of leaf organs, as shown by Parma Nand Sharma (1995). Zn is predominantly transported as free Zn2+ ions through the phloem from the treated leaf to other parts of the plant (Fernandez Victoria; Brown Patrick H, 2013). However, the mechanisms of transport for foliar-applied Zn within the plant after absorption through the leaf surface remain poorly understood (Fernandez Victoria; Brown Patrick H, 2013). According to Tsonko Tsonev (2012), the excess of Zn is also responsible for opening the stomata and determines the rate of K+ influx in these cells (Brennan, 2005), and its excess can cause a reduction in the number of guard cells and decrease size of the stomata.

The stem cross sections indicated that the soybean stems with Zn deficiency have decreased cambium activity and reduced thickness of the secondary xylem (Figure 2). Interestingly, no differences were observed in the phloem. Also, no statistical differences were verified in the diameter of the vessel elements.

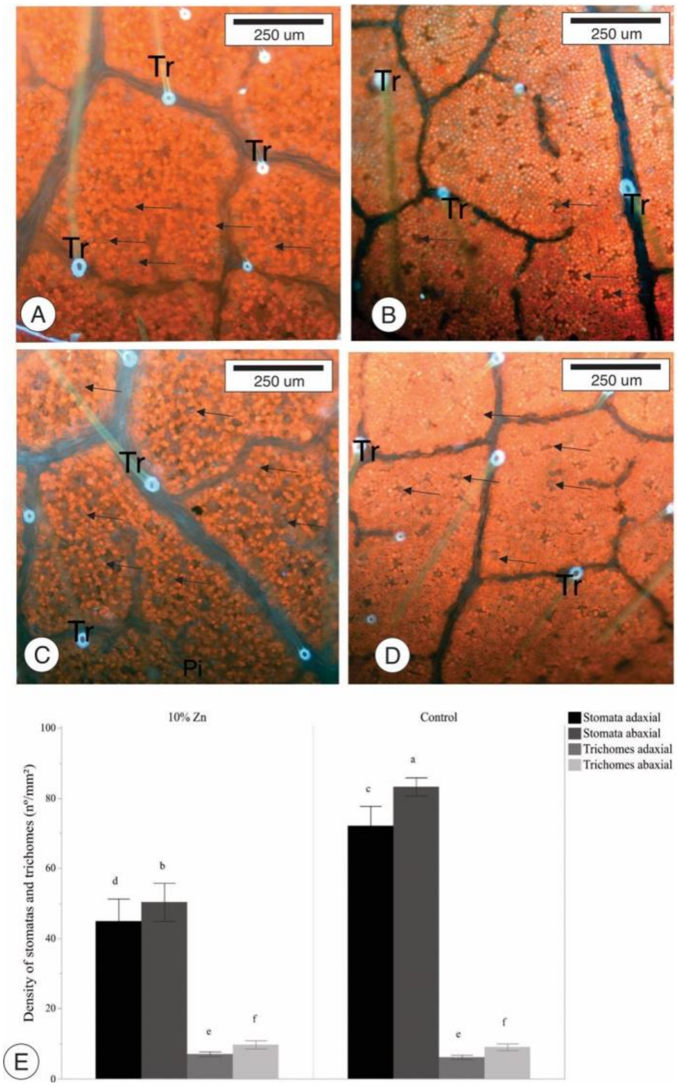

Figure 20: Analysis of the adaxial (A; C) and Abaxial (B;D) of the soybean leaf surface under UV light and analyzed under epifluorescence microscopy the Imagens it is possible to verify the autofluorescence within the guard-cells – stomata (arrows) and the trichome base (Tr). E. Number of stomata and trichomes on soybean leaves. Means followed by equal letters do not differ statistically from each other by Tukey's a ($p<0.10$) probability test and the bars indicate the standard error of the mean.

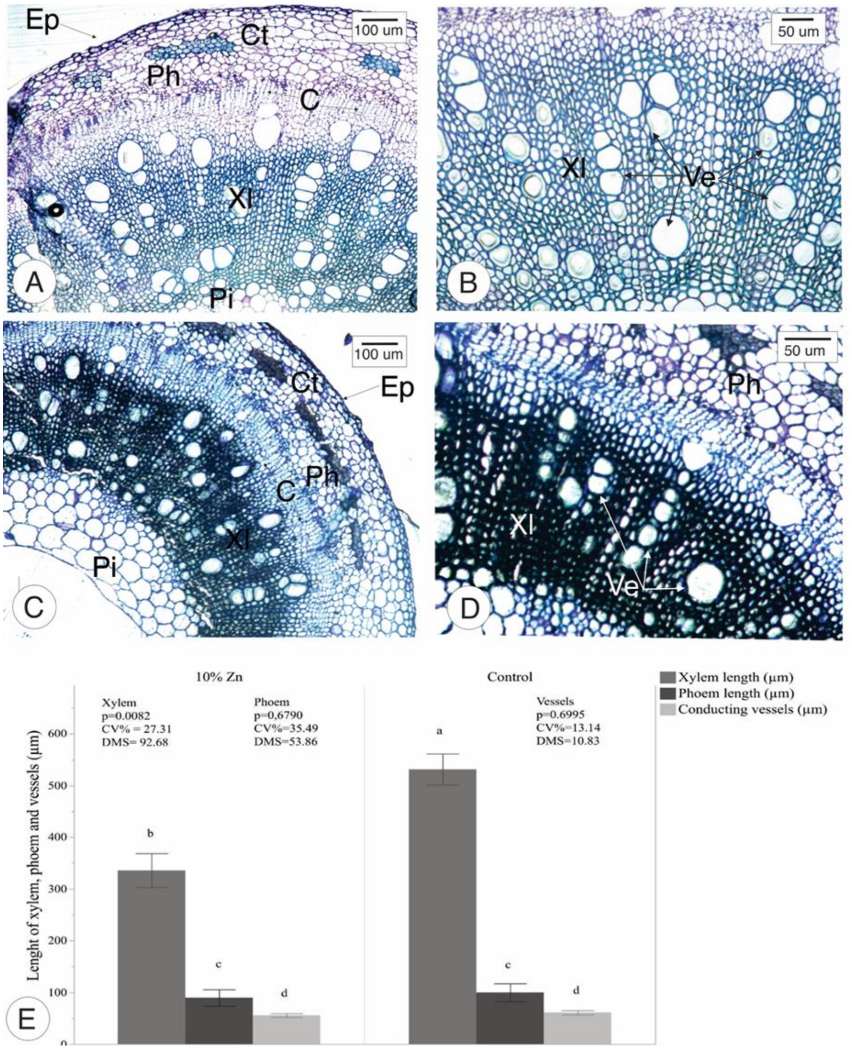

Figure 21: Cross section of the soybean stem (A-D) of the control and treatment plants and the biometric data of the xylem, phloem, and vessel element diameter (E). The soybean stems with the recommended dose of Zn presents more cells of secondary xylem compared to the 10% Zn. Size of xylem, phloem, and conducting vessels in soybean stems. Means followed by equal letters do not differ statistically from each other by Tukey's a (p<0.10) probability test and the bars indicate the standard error of the mean.

After carrying out the horizontal and vertical measurement of 500 particles of the Zintrac product for 4 weeks, it was possible to assemble two histograms (Figures 22 and 23).

When analyzing them, it appears that over time the counted particles had their measurements with higher values, something that was not expected. In addition to being proven by measurements, visually, through the scanning electron microscope, the formation of particle agglomerates and the tendency to reduce the number of particles with smaller diameters was evident.

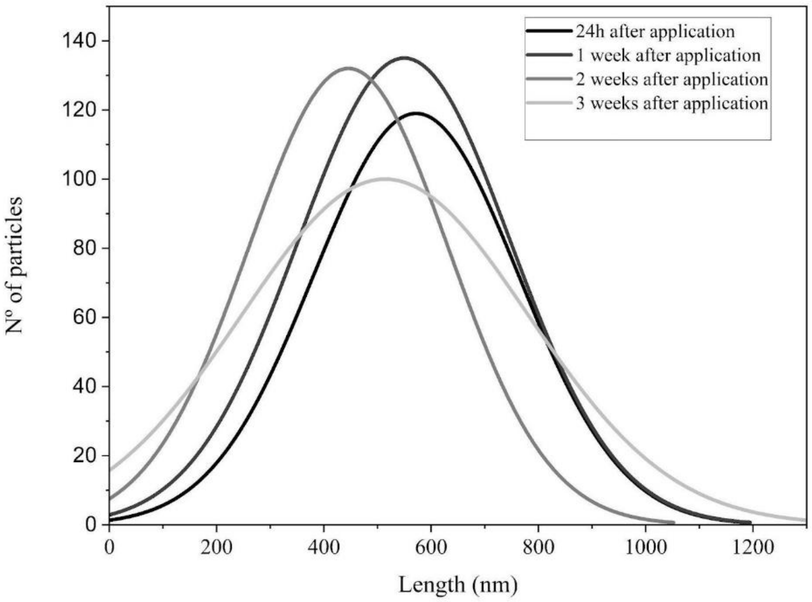

Figure 22: Histogram of the longitudinal length of the Zn nanoparticles. Longitudinal refers to a direction parallel or aligned with the length or axis of an object.

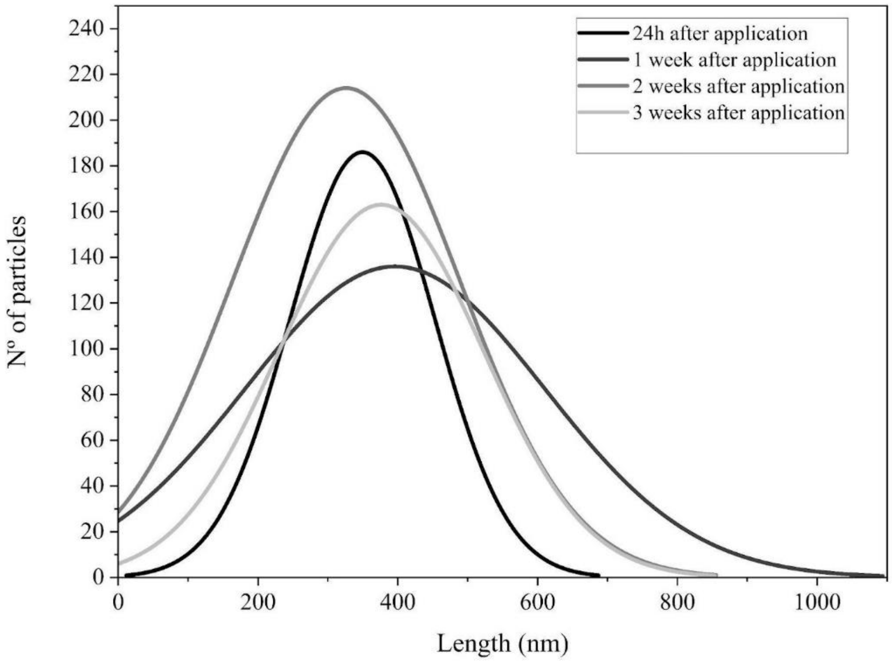

Figure 23: Histogram of the vertical length of the Zn nanoparticles. Vertical refers to a direction that is perpendicular to the ground or horizon.

Therefore, it was not certain whether the zinc clumps together and is not absorbed or whether other compounds clump together and the zinc is absorbed normally. One way of showing zinc absorption was by making chemical maps of the drops over time. As can be seen in Figures 22 and 23, the percentage of zinc on the leaf surface reduced, which proves that it did not accumulate in the cuticle. The scanning electron microscopic studies (SEM) conducted on ZnO NPs demonstrated the presence of agglomerated particles uniform distribution and nano size. The larger particle dimension observed can likely be attributed to the formation of van der Waals agglomerated formed by smaller entities, as well as interactions magnetic among the particles.

These findings align well with the observations reported by Sohail et al., providing additional support and agreement to their study. It is worth noting the characteristic decrease of oxides on the leaf surface, which is not abrupt in the short term but is considered in the longer term. Previous investigations have detected nanoparticles (NPs), by leaves application. A recent study by El-Shetehy et al. (2021) demonstrated that Zn derived from foliar application of ZnO-NPs is absorbed within the extracellular air spaces of stomata, rather than within the underlying leaf tissue. These findings provide further evidence that the absorption of Zn from ZnO-NPs occurs through a combination of ZnO particle association and dissolved Zn forms. Collectively, our study suggests that even in the form of nanoparticles, Zn can be efficiently absorbed by leaves. Being able to infer that Zn was being absorbed, most likely by the stomata, according to the study by J. Zhu (2019) NPs such as ZnO were absorbed by the stomata, forming an accumulation in the region and then moving through the apoplast.

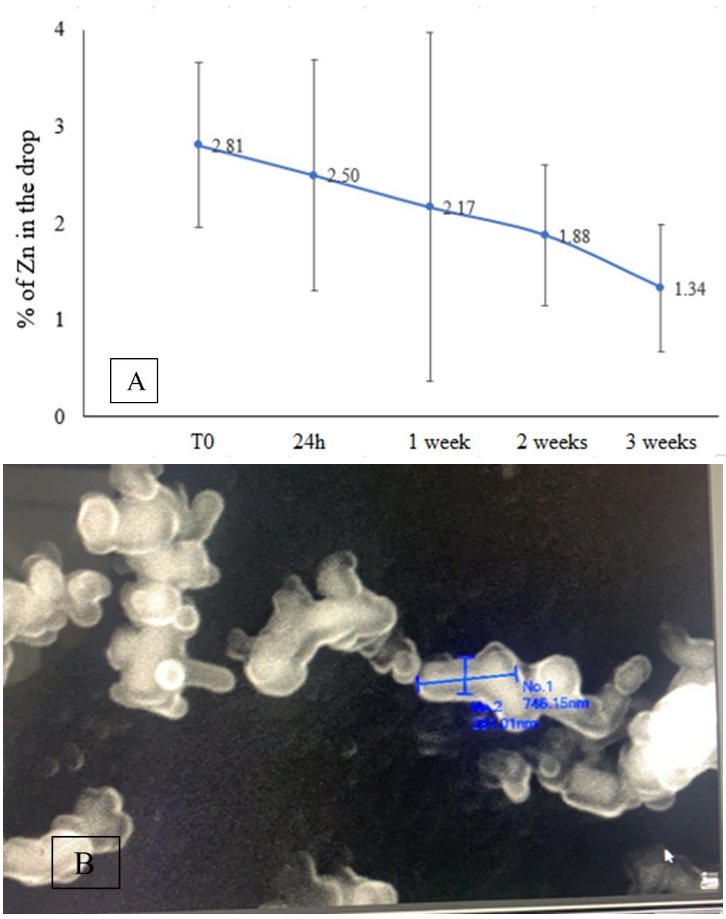

Figure 24: Percentage of Zn in the drops as a function of time (A), and image of the ZnO particle (B).

So, Zn particles formed conglomerates over time, showing that the size did not constantly decrease (Figures 22 and 23). But with the chemical map (Figure 24) we can conclude that the Zn particles were absorbed by the plant since the amount of Zn in the drop decreases with time. Using nuclear markers, the foliar absorption of sulfate and micronized zinc oxide in the soybean crop. The team observed that over 48 hours of monitoring, Zn from ZnO remained static, while Zn from $ZnSO_4$ moved, following the leaf vein through the flow via the phloem. (GOMES, 2019).

3.4 Conclusion

The results indicate that when ^{65}ZnO and $^{65}ZnSO_4$ were applied to the leaves, the soluble form, $^{65}ZnSO_4$, exhibited better translocation compared to ^{65}ZnO. This suggests that the solubility of the source material plays a crucial role in its movement within the plant. The improved translocation of $^{65}ZnSO_4$ can be attributed to its higher solubility, which enables easier absorption and transportation through the plant's vascular system. The soluble nature of $^{65}ZnSO_4$ allows for efficient uptake by the leaves and subsequent distribution to other plant tissues.

Based on the study investigating the influence of zinc (Zn) on stomata, trichomes, and vascular tissues, it can be concluded that Zn significantly affects the number of stomata and the size of the xylem, suggesting that Zn plays a role in modulating the xylem development or expansion. but does not have a significant influence on trichomes or the conductivity of phloem and vascular vessels. Also, the results from the chemical map demonstrate that when ZnO droplets were applied to the leaves, a noticeable reduction in droplet size was observed. This suggests that the leaves effectively absorbed the ZnO fertilizer, causing the droplets to shrink in size as the nutrients were taken up by the plant.

In conclusion, the study demonstrates that the translocation of soluble sources, specifically $^{65}ZnSO_4$, was superior to that of ^{65}ZnO. These findings highlight the importance of considering the solubility of foliar-applied substances for effective nutrient uptake and distribution in plants, providing valuable insights for agricultural practices and nutrient management strategies, and emphasizing the selective impact of Zn on specific anatomical structures and vascular tissues. Providing insights into the role of Zn in regulating stomatal density and xylem development and the chemical mapping study reveals that the decrease in ZnO droplet size indicates successful absorption of the foliar fertilizer by the leaves.

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ANNEX

Piracicaba: leaves collection (R4)

Itaí: application

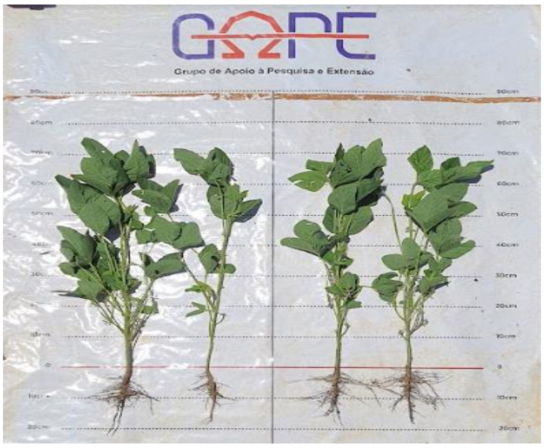
Piracicaba: stage R4 Itaí: leave fertilizer

Piracicaba: Application quality

Piracicaba (R1): T1 x T2

Piracicaba (R1): T1 x T3

Piracicaba (R1): T1 x T4



Piracicaba (R1): T1 x T5

Itaí (R1): T1 x T2

Itaí (R1): T1 x T3

Itaí (R1): T1 x T4

Itaí (R1): T1 x T5

Itaí (harvest): T1 x T2

Itaí (harvest): T1 x T3

Itaí (harvest): T1 x T4

Itaí (harvest): T1 x T5

Santa Carmem – MT (V8)

Santa Carmem – MT (V8)

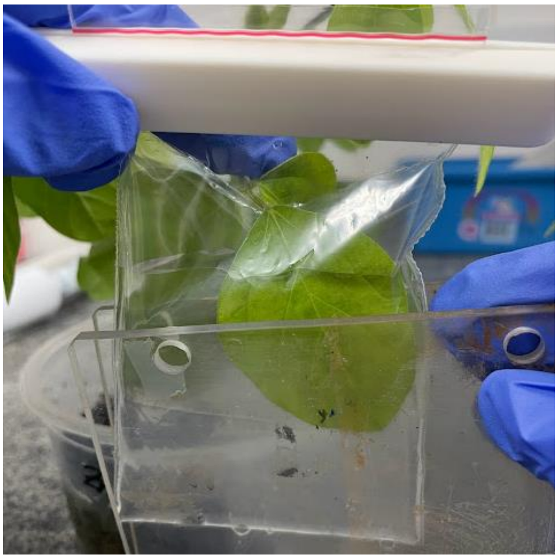
Santa Carmem – MT (V8)

Santa Carmem – MT (V8)

Greenhouse: soybean in V8 stage

Greenhouse: application of label Zn

Greenhouse: application of label Zn



Greenhouse: application of label Zn

Greenhouse: soybean in R2 stage

Left to right: control +, control -, zinc sulphate and zinc oxid.

Left to right: control +, control -, zinc sulphate and zinc oxid.

Greenhouse: soybean with label Zn

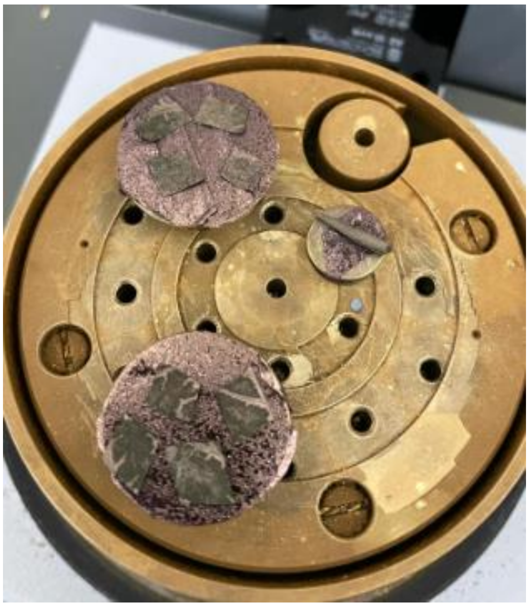
Greenhouse: soybean in R5.3 stage

LIN: preparation the lab to make the label Zn solution

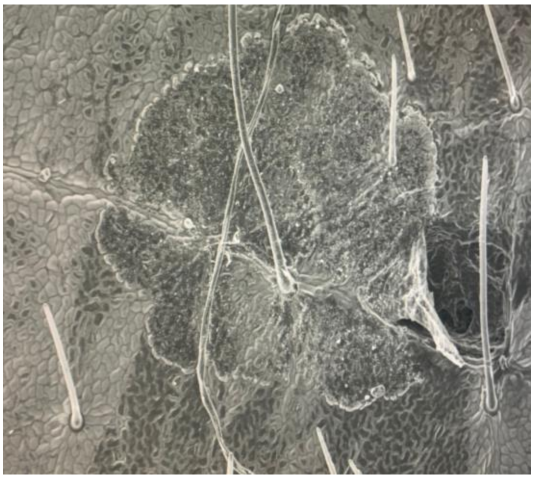
Greenhouse: sample ready to radioactivity measurement

Greenhouse experiment ZnO

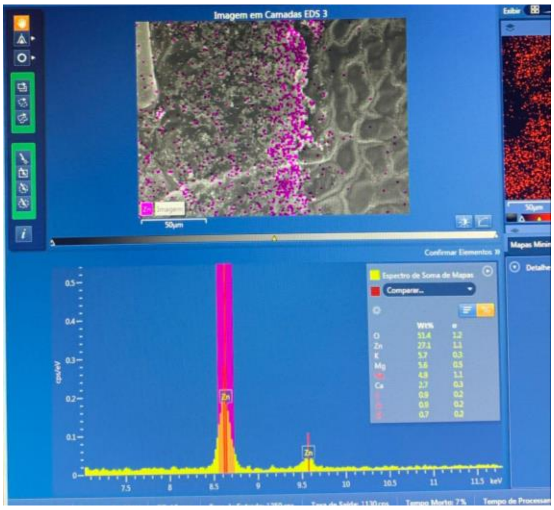
Greenhouse experiment ZnO particle monitoring



Leaves with carbon covering

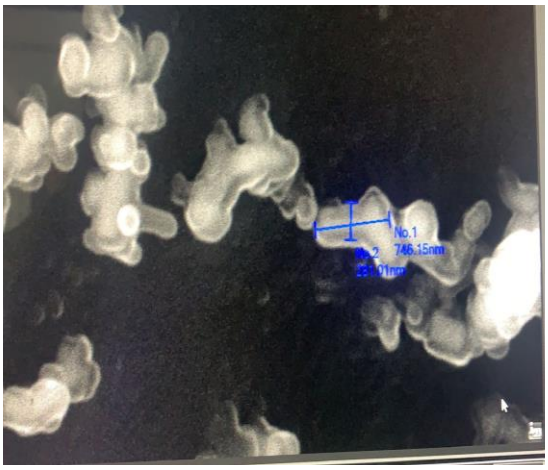


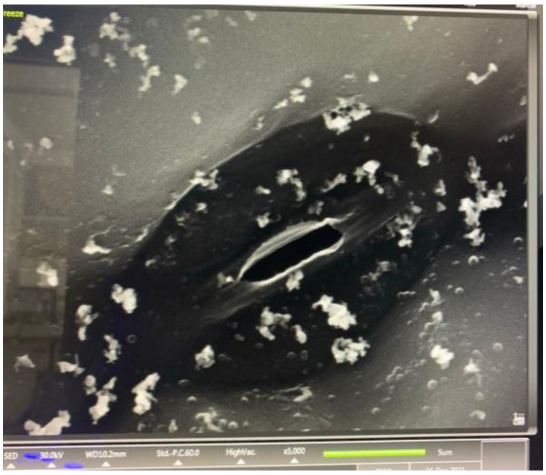
Drop by MEV



	Wt%	σ
O	51.4	1.2
Zn	27.1	1.1
K	5.7	0.3
Mg	5.6	0.5
[illegible]	4.8	1.1
Ca	2.7	0.3
[illegible]	0.9	0.2
[illegible]	0.9	0.2
[illegible]	0.7	0.2

Chemical map



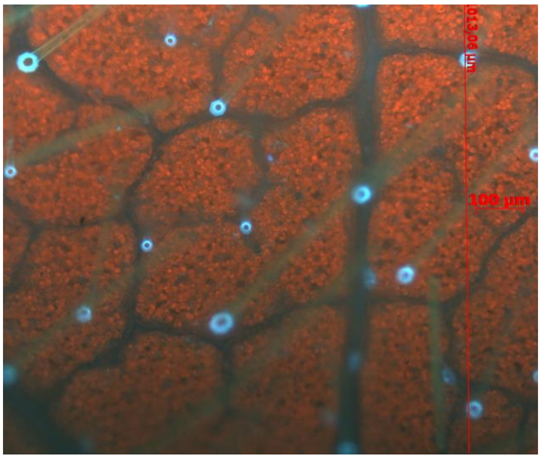


Particle size monitoring

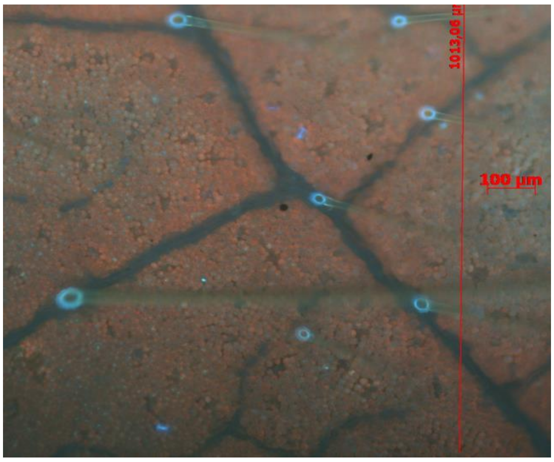
Particle size monitoring

Greenhouse experiment stem anatomy, trichomes and stomata

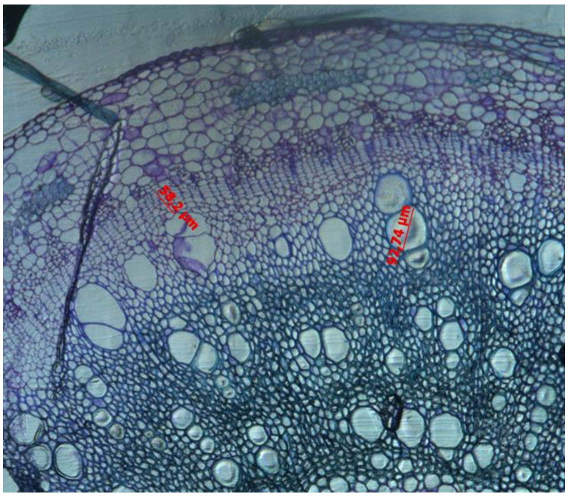
Stem in resin



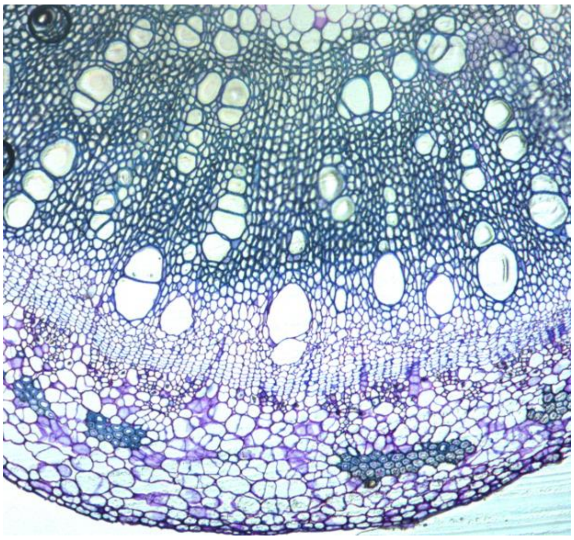
Trichomes and stomates



Trichomes and stomates



Measuring of vassel, xylem and floem



Measuring of vassel, xylem and floem