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JÉSSICA BEZERRA DE OLIVEIRA

Soybean seed treatment and foliar application with nickel sources and Ni-particle sizes: an appraisal of biological nitrogen fixation and plant growth

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Dedication

Dedicated to my parents and sisters who supported me to pursue my passions.

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Jessica Bezerra de Oliveira

“Descobri como é bom chegar quando se tem paciência. E para se chegar, onde quer que seja, aprendi que não é preciso dominar a força, mas a razão. É preciso antes de mais nada querer”

Amir Klinck

ABSTRACT

OLIVEIRA, J. B. **Soybean seed treatment and foliar application with nickel sources and Ni-particle sizes:** an appraisal of biological nitrogen fixation and plant growth. 2020. 158 p. Dissertação (Mestrado em Ciências) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

Nickel (Ni) is an essential element to N metabolism due to the fact it is a structural component of the enzymes urease and hidrogenase. Thus, this study was carried out in three different experiments with the aims of: (i) to investigate the pathway of Ni sources ($NiSO_4$; $Ni(OH)_2$ micrometric ~ 24 µm; $Ni(OH)_2$ nanometric ~ 5 nm), by µ - XRF and SEM, after dressing soybean seed with Ni in soybean plants grown on rhizotrons; (ii) to investigate the effects of Ni sources in soybean plants, under greenhouse conditions, applied via seed or leaf on BFN (by ^{15}N natural abundance method), urease, reductase nitrate, nitrogenase activity, nitrate, ammonia, ureides concentration, photosynthetic parameters and biomass in soybean plants; (iii) to examine the distribution and translocation of foliar-applied Ni in soybean leaves grown in hydroponic solutions by Synchrotron micro-X-ray fluorescence analysis and also to determine the effect of seed Ni concentration on urease activity and to establish whether an internal (presence of cotyledons) or to external (absence of cotyledons) supply of Ni can compensate for Ni nickel within the seed. The results of µ - XRF and SEM revealed that the hilum of soybean seeds coated with Ni showed areas of high spot of Ni concentrated and the seeds that received Ni based on nanoparticles had lower germination rate compared to treatments of Ni sulfate and Ni micrometric. The soybean seedlings grown in the rhizotrons showed higher quantity of Ni in roots and in the rhizosphere soil. Therefore, the Ni applied in the seed is either transported throughout the imbibition or provides a fertile microenvironment that favors the primary seedling development. Foliar application associated with nanoparticles had more positive impact on soybean growth, physiology and biological nitrogen fixation than sulfate Ni fertilizers. Regarding soybean cultivated in hydroponic solution, the results showed that leaf Ni concentrations, shoot biomass and urease activity were augmented by increasing either internal (from cotyledon seed store) or external (in solution) nickel supply. Synchrotron micro-X-ray fluorescence showed that trichomes are an important pathway for foliar Ni absorption in soybean.

Keywords: Urease activity. Foliar fertilization. Biological nitrogen fixation. X-ray fluorescence microscopy. Nickel nanoparticles. Seed treated.

RESUMO

OLIVEIRA, J. B. **Semente de soja tratada e aplicação foliar com fontes de níquel e tamanhos de partículas de Ni:** uma avaliação da fixação biológica de nitrogênio e crescimento da planta. 2020. 158 p. Dissertação (Mestrado em Ciências) – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

Níquel (Ni) é um elemento essencial para o metabolismo do nitrogênio, por ser componente estrutural da enzima urease e hidrogenase. Portanto, este trabalho foi realizado a partir do desenvolvimento de três experimentos distintos com o intuito de: (i) investigar a absorção de Ni nas sementes e o posterior transporte a longa distância nas plântulas de soja desenvolvidas em rhizotrons, por meio das técnicas analíticas de: Fluorescência de Raios X (XRF) e microscopia eletrônica de varredura (MEV), após o tratamento das sementes com as seguintes fontes de Ni: ($NiSO_4$; $Ni(OH)_2$ micrométrico ~ 24 µm; $Ni(OH)_2$ nanométrico ~ 5 nm); (ii) investigar os efeitos da aplicação das fontes de Ni nas plantas de soja (via tratamento de semente ou aplicação de Ni nas folhas), cultivadas em condições de casa-de-vegetação, na fixação biológica de nitrogênio (FBN), por meio da técnica isotópica de abundância natural de ^{15}N, pela avaliação da nodulação, pelas quantificações das atividades das enzimas urease, nitrato redutase e nitrogenase, pela determinação das concentrações de nitrato, amônia e ureideos nos tecidos vegetais, bem como pela avaliação dos parâmetros fotossintéticos (clorofila e carotenoides) e pela produção de biomassa de grãos; (iii) examinar a distribuição espacial e translocação do Ni aplicado nas folhas das plantas crescidas em solução hidropônica, por meio da técnica de espectrometria de fluorescência de Raios-X e também avaliar o efeito da contribuição do conteúdo de Ni nas sementes de soja na atividade da uréase, bem como investigar se o "*pool*" interno de Ni nos cotilédones (plântulas com a presença de cotilédone) ou baixo conteúdo de Ni (retirado dos cotilédones das plântulas) pode compensar baixa concentração de Ni na semente. Os resultados do µ - XRF e MEV revelaram que o hilo das sementes de soja tratadas com Ni apresentavam áreas com alto acúmulo de Ni e que sementes tratadas com nanopartículas de Ni apresentaram a menor taxa de germinação comparada com sementes tratadas com sulfato de Ni e $Ni(OH)_2$ micrométrico. As plântulas de soja crescidas nos rhizotrons, as quais receberam Ni obtiveram alta quantidade de Ni nas raízes e na rizosfera. Portanto, a aplicação de Ni nas sementes é ou transportado através do processo de percolação ou fornece um micro ambiente adequado, o qual favorece o desenvolvimento inicial da plântula. A aplicação foliar com nanopartículas de $Ni(OH)_2$ incrementou o desenvolvimento das plantas de soja, incrementando a eficiência da fixação biológica de nitrogênio, em relação as fontes de sulfato de Ni. Em relação a soja cultivada em solução hidropônica (experimento #3), os resultados mostraram que as concentrações de Ni na folha, a produção de biomassa da parte aérea e a atividade da urease aumentou com o suprimento de Ni (adição de Ni na solução) ou em plantas com alto conteúdo de Ni proveniente dos cotilédones. A técnica de Raios X de fluorescência (XFM) realizada no ANSTO-Sincrotron mostrou que os tricomas são uma importante via de absorção de Ni pelas folhas de soja.

Palavras- chaves: Atividade da urease. Fertilização foliar. Fixação biológica de nitrogênio. Microscopia de Raios X de fluorescência. Nanopartículas de níquel. Tratamento da semente.

SUMMARY

1 GENERAL INTRODUCTION

The last element discovered as essential nutrients for plants was nickel (Ni). The first evidence of its essentiality was verified in soybean plants (*Glycine max* [L]. Merrill) in 1983 by Eskew et al., under conditions of Ni absence, which plants showed toxicity symptoms, such as accumulation of urea in leaflet tips. The essentiality of Ni for plants has been proved four years later, when after three successive generations of growing barley plants (*Hordeum vulgare* L.) in Ni-depleted controlled conditions, plants did not produce viable grains (BROWN; WELCH; CARY, 1987).

Nickel is a vital nutrient because it performs structural component of several enzyme systems (NiFe-hydrogenase, carbon monoxide dehydrogenase, acetyl-CoA decarbonylase synthase, methyl-coenzima M reductase, superoxide dismutase, Ni dependent glyoxylase, aci-reductone dioxygenae, and methyleneurase) in bacteria and lower plants that are activated by Ni (MULROONEY; HAUSINGER, 2003; WALSH; ORME-JOHNSON, 1987). However, the activation of urease was discovered, to date, to be the only enzymatic function of Ni in higher plants (GERENDAS, 1998). Urease is responsible for hydrolysis of urea into two molecules of ammonia and one of carbon dioxide (POLACCO; MAZZAFERA; TEZOTTO, 2013; WITTE, 2011).

Nevertheless, Ni acts directly in the biological nitrogen fixation (BNF), inasmuch as it constitutes the hydrogenase enzyme, which it is responsible to recycling H_2, a product formed in the process of the nitrogenase complex to convert atmospheric N_2 into NH_3 in the root nodules (BAGYINKA, 2014; BRAZZOLOTTO et al., 2016; SHAFAAT et al., 2013). This NH_3, ammonia, is converted into ureides (allantoin and allantoic acid), which are the main forms of nitrogen exported to the shoot in plants (COLLIER; TEGEDER, 2012). In the leaves, ureides may be converted to urea, via the purines degradation pathway, being then metabolized by urease (ZRENNER et al., 2006).

Soybean is economically the greatest significant crop in the world, providing vegetable protein for millions of people and ingredients for hundreds of chemical products. The soybean is one of the richest and cheapest sources of protein and it a staple in the diets of people. The seeds contains 17% of oil, 63% of meal, and 50% of protein (FAO, 2020).

Current investigations revealed that fertilization with Ni can increase N assimilation and N metabolite levels in plants (DALIR; KHOSHGOFTARMANESH, 2015; KHOSHGOFTARMANESH; HOSSEINI; AFYUNI, 2011; TAN; IKEDA; ODA, 2000; URUÇ PARLAK, 2016). The fertilization can be done by soil either seed or leaves. Micronutrient seed treatments has potential for improving crop growth and grain nutrient enrichment (FAROOQ; WAHID; SIDDIQUE, 2012). Besides of that, foliar-application is a great alternative to avoid the risks of nutrient leaching as well as increase of nutrient assimilation (FERNÁNDEZ; SOTIROPOULOS; BROWN, 2013) and have been more effective in yield improvement and grain enrichment (JOHNSON et al., 2005). Recently, fertilization via foliar is used to treat nutrient deficiencies (DEL AMOR et al., 2009; HANNAM et al., 1984; KAYA; HIGGS, 2002; SAXENA; MALHOTRA; SINGH, 1990; SHORROCKS, 1997), and when soil conditions limit availability of soil applied nutrients (FERNÁNDEZ; SOTIROPOULOS; BROWN, 2013).

Another alternative to improve plant growth and productivity is the use of nanoparticles. Nano-technologies are already applied to production, processing, storage, packing and transportation of agricultural products (KHOT et al., 2012; NAIR et al., 2010). Nano fertilizers have proven to be convenient for crops development, once they can delivery nutrients gradually and in a more controlled manner than common fertilizers (KAH et al., 2018; SUBRAMANIAN et al., 2015).

Then, in this context, the aims of this investigation were: (i) firstly, understand the translocation and distribution of treated seed and foliar-applied Ni in soybean plants by µ-XRF on bench, synchrotron and SEM; (ii) evaluate the effects of Ni fertilization based on nanomaterial associated with foliar application in soybean plants measured by: biological nitrogen fixation determined by ^{15}N natural abundance ($\delta\ ^{15}N$ ‰), ureides, ammonia and nitrate content, urease, nitrate reductase, nitrogenase (N-ase) activity, photosynthetic parameters, and plant growth.

2 REVIEW

2.1 Soybean crop

Soybean [*Glycine max* (L.) Merrill] is an annual cycle crop, belongs to the family Fabaceae, which refers to the fruits of the flowering plants, legumes. The evidence indicates that the soybean was originated from East Asia, specifically Northern China. Since soybean has been cultivated and incorporated as food and medicine in people's daily lives for the past 5000 years. According to records, soybean was introduced in Brazil in 1882, when some tests were carried out in Bahia. However, it was only in 1949 that Brazil becomes a soybean producer (BONATO; BONATO, 1987).

Over recent decades, soybean has undergone the greatest expansion of any global crop. In the last 50 years, soybean production has grown from 27 to 269 million tons (FAO, 2019). Soybean is high in protein (around 40%) and energy, more than any other major crop. Soybeans can be eaten directly by humans, such as vegetable oil and by-products as lecithin, natural emulsifiers, and has an essential function also for animals, as livestock feed. The vegetable oil is used in food and cosmetics, soap, and biofuel.

The Brazilian Company Supply (CONAB, 2020) estimated the country's soybean yield in the 2019-2020 growing season at 124.8 million metric tons, an increase to 3.32 metric tons per hectare of area planted, up from 3.21 tons per hectare harvested in the preceding crop year, with average yield of 3.379 kg ha^{-1}. The state of Mato Grosso alone represented 28.5 percent of the Brazilian soybean export values (SHAHBANDEH, 2020).

The crop is adapted to a wide range of climate, with optimum growing conditions in mean temperatures of 20 to 30 ℃. They can grow in a wide range of soils, in near neutral soils and limed acid soils. Under great growing conditions with adequate N fixation, grain yields of 3-4 tonnes ha^{-1} can be obtained (BOARD; KAHLON, 2013).

2.2 Nitrogen assimilation: Biological Nitrogen Fixation

The nitrogen (N) is an essential element for plant growth and development. Nitrogen is required by the soybean plant in large quantities during both vegetative and reproductive stages, with the concentration value in the grain ranging from 45 to 65 g kg^{-1}. To achieve an average productivity of 3000 kg ha^{-1}, in average 240 kg ha^{-1} of N are required (ALVES; BODDEY; URQUIAGA, 2002; JUNIOR et al., 2010). Then, to obtain high seed yield of soybean, nitrogen

fixation activity are very important. Once, the availability of soil N is generally insufficient to support soybean growth. Also, the BNF offers a natural means of providing nitrogen for plants.

Legumes may feed on three different sources of nitrogen: nitrate, ammonium, and, due to symbiotic N_2 fixation (LASA et al., 2002; OH; KATO; XU, 2008; WICKERT et al., 2007). All cases ammonium is finally assimilated by glutamine synthetase (GS)/ glutamate synthase (GOGAT) system. NH_4^+ is absorbed by root cells. All inorganic N is first reduced to ammonium, because it is the only reduced N form available to plants for assimilation to amino acids (RUIZ; RIVERO; ROMERO, 2007). In the cytosol, NH_4^+ is incorporated into glutamate by glutamine synthetase (GS) forming glutamine. A transferase reaction catalyzed by glutamine oxoglutarate aminotransferase (GOGAT, glutamate synthase) produces two molecules of glutamate by transferring the amino group from glutamine to oxoglutarate (SCHUBERT, 1986). This pathway of NH_4^+ incorporation into amino acids for all three sources of nitrogen. As a cation NH_4^+ is attracted by the negative charge and enters the cell passively via a specific transport system (BERLT; FELLE; BENTRUP, 1984).

Ammonium is toxic to plants, once it can cause proton extrusion, which is associated with ammonium uptake, changes in cytosolic pH and uncoupling of photophosphorylation in plants (WANG et al., 2007). Then, ammonia is quickly converted into the organic form (amino acids) to avoid negative effects and provide nitrogenous forms suitable for source-sink transport (MASCLAUX-DAUBRESSE et al., 2006).

The nitrate reduction, catalyzed by nitrate reductase (NR) occurs in the cytosol and generates NO_2^-. This phenomenon happens due to the fact that NO_2^- is reduced to NH_4^+ in plastids, NO_2^- has to overcome two membranes of the organelle envelope (HEBER; HELDT, 1981). Nitrate assimilation occurs predominantly in the roots of the plant, being strongly dependent on the age and limitation of space for root growth (Marquez et al, 2007). Nitrate is reduced to nitrite by NR (CAO et al., 2008; KOUADIO et al., 2007; ROSALES et al., 2011). Nitrite is highly reactive, plant cells immediately transport the nitrite from the cytosol into chloroplasts in leaves and plastids in roots. In these organelles, nitrite is further reduced to NH_4^+ by NR (ROSALES et al., 2011).

The third source of N is atmospheric N_2, which can only be made available to legumes by a symbiosis with rhizobia. The biological nitrogen fixation (BNF) is a significant contributor to the global agricultural nitrogen budget, adding 21.45 million tons of fixed N each year (HERRIDGE; PEOPLES; BODDEY, 2008). Boosting the efficiency of N fixation in agricultural systems could be a powerful tool in decreasing chemical fertilizers and sustainably increasing agricultural production.

Soybean N-fixation from atmospheric N_2 are influenced by various soil conditions such as water content, pH, aeration, nutrition, and climatic conditions such as solar radiation, temperature, and rainfall (MEDEIROS et al., 2007; OHYAMA et al., 2017).

Many microorganisms such as *Azorhizobium*, *Bradyrhizobium*, *Photorhizobium*, *Rhizobium*, and *Sinorhizobium* can fix nitrogen symbiotically by partnering with a host plant. The plant provides sugar from photosynthesis that are utilized by the nitrogen-fixing microorganism for the energy it needs for nitrogen fixation. In exchange for theses carbon sources, the microbe provides fixed nitrogen to the host plant for its growth (ALVES; BODDEY; URQUIAGA, 2003). The *Bradyrhizobium* is the most commonly genus found in commercial products and associated with soybean plants (HOWARD; REES, 1996; CHUEIRE et al., 2003; TAIZ; ZEIGER, 2004).

The *Bradyrhizobium* and *Rhizobium* bacteria colonize the soybean plant's root system and cause the roots to form nodules to house the bacteria, where atmospheric nitrogen is converted in ammonia (NH_3) (JUNIOR et al., 2010; REIS et al., 2004). The bacteria then begin to fix nitrogen required by the plant. The process occurs in a two-step: first, the bacteria attach using Ca^{+2} binding protein called rhicadhesin. Second, the bacteria accumulate and anchor themselves to the root hair surface, a firmer attachment that involves lectins and/cellulose produced by the host plant and bacteria. This step is performed by the protein called NodD. The NodD is a flavonoid that introduces the transcription of nodulation genes (*nod*). The *nod* genes are involved in the production of Nod factors, nodulation signaling molecules, which activate lectins, legume receptors for nod factor, binding the rhizobium to the cell wall of root hair. From this signaling, the rhizobium releases Nod factors that introduce the winding of the cells of root hair and the degradation of the cell wall, then the bacteria enter in the external region of the plasmatic membrane of the cells and forms an infection channel, and so the nodules are formed (MEDEIROS et al., 2007; TAIZ; ZEIGER, 2004).

The process of fix atmospheric N to NH_3 is catalyzed by the nitrogenase enzyme complex, which is formed by a two component system comprising an iron-protein (Fe-protein) and a molyolybdenum-iron (MoFe) protein, each containing FeS clusters which are responsible for the electron flow during the process of substrate reduction. The limiting enzymatic stoichiometry for enzyme-catalyzed nitrogen fixation is given by equation bellow:

$$N_2 + 8\,e^- + 16ATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

For this phenomenon to happen, the condition of anaerobiosis is necessary, since oxygen (O_2) irreversibly inactivates the nitrogenase complex (MYLONA; PAWLOWSKI; BISSELING, 1995). Leghaemoglobin is a hemoprotein in N_2-fixing nodules that facilitates O_2 diffusion to respiring bacteroids in the infected cell (APPLEBY, 1984). The ATP produced from bacteroid respiration via oxidative phosphorylation is used to reduce N_2 to form NH_3. However, NH_3 is toxicity to plants, then two more steps happens (1) NH_3 is transformed into NH_4^+ in the nodules, which is assimilated by GS and GOGAT cycles and transported by xylem in the form of asparagine or (2) ureides (MALAVOLTA, 2006; TAIZ; ZEIGER, 2004; MARSCHNER, 2012).

The product of N_2 fixation is released into the cytosol by passive diffusion across the cell membranes (KLEINER, 1981; STREETER, 1989). Ureides, in tropical legumes, or amides, then, are exported to the shoot via the xylem.

The nitrogenase also produces H_2 as a by-product, and H_2 production represents a loss of energy that otherwise would be available for N_2 fixation (ALBRECHT et al., 1979; BURRIS, 1988). The hydrogenase is an enzyme that catalyzes the reversible oxidation (recycle) of H_2 inside the nodule (EVANS et al., 1987). The mostly hydrogenases are known as [NiFe]. The active center is bimetallic: nickel and iron. The nickel is coordinated by 4 cysteines, two of which bridge with iron (DE LACEY et al., 2007; FONTECILLA-CAMPS et al., 2007).

2.3 Nickel in soybean

Nickel is a transition metal very abundant in the lithosphere, and less abundant in an aqueous system. Due to the low concentrations of the element in aqueous environments, its importance in biological systems was ignored for a long time until evidence accumulated in the 1970s that nickel was essential for optimal growth of certain microorganisms (DIXON et al., 1975; THAUER et al., 2010).

Nickel was the latest element to have its nutritional essentiality recognized and confirmed for plant species (DIXON et al., 1975; SHIMADA; ANDO, 1980; SHIMADA et al., 1980; ESKEW; WELCH 1983; BROWN et al., 1987). However, the amounts of this nutrient required by plants are low compared to other micronutrients (EPSTEIN; BLOOM, 2005; CHEN, 2014). The Ni^{+2} cation is generally the metal species that is specifically transported by microorganism.

Nickel has a vital function in nitrogen metabolism from seed germination to vegetative growth (BROWN; SUNDERMAN JUNIOR, 1980). It is a structural and functional component of the metalloenzyme urease, which acts on the degradation of urea to CO_2 and NH_4^+

(BAI; REILLY; WOOD, 2006; DIXON et al., 1975). Therefore, Ni deficiency disrupts amino acid metabolism in Ni-deficient tissue, which in turn results in accumulation of glycine, valine, isoleucine, tyrosine, tryptophan, arginine, and total free amino acids, and lower concentrations of histidine and glutamic acid (BAI; REILLY; WOOD, 2006). Ni deficiency also disrupts the citric acid cycle, the second stage of respiration, where Ni-deficient tissues contained very low levels of citrate compared to Ni-well-nourished tissues. Disruption of carbon metabolism was also pointed out by Bai et al. (2006), as consequence of accumulation of lactic and oxalic acids., which results in toxicity and decreased plant growth (BAI; REILLY; WOOD, 2006; BROWN; WELCH; MADISON, 1990; MACEDO et al., 2016; ESKEW; WELCH, 1983; MACEDO et al., 2016; WITTE, 2011).

The suitable supply of Ni also increase hydrogenase activity in *Rhizobium*, augmenting nodulation and BNF in soybean (KLUCAS et al., 1983; URETA et al., 2005).The fixation of nitrogen transport is finished by ureides that catalyze the end of transport of urea molecule, which is metabolized by the urease enzyme (FABIANO et al., 2015).

The first studies on Ni deficiency were reported in Pecan [(*Carya illinoinensis* (Wangenh.) K. Koch] by Marz (1918). The visual symptoms were initially exhibited by yard trees within certain Florida, southern Mississippi, and southeastern Georgie (DEMAREEE, 1929). The symptomology was called as “Mouse-ear”, which causes range from mild morphological distortion of leaflets to gross deformity of shoot, foliar, and reproductive organs (WOOD; REILLY; NYCZEPIR, 2004). Differently, soybean does not show Ni deficiency in the same mode as pecan, demonstrating that it requires a noticeably lower amount of this micronutrient to support adequate development. Hence, there is a lack of information about Ni deficiency in soybean (LAVRES; FRANCO; DE SOUSA CÂMARA, 2016), however, recent investigations demonstrate that soybean genotypes show positive, but differential, responsiveness to Ni-supply (FREITAS et al., 2018; 2019). In addition, yield increases in field-grown soybean due to Ni fertilization were first reported relatively recently under greenhouse conditions (MACEDO et al., 2016; FREITAS et al., 2019; KUTMAN; KUTMAN; CAKMAK, 2013a; LAVRES; FRANCO; DE SOUSA CÂMARA, 2016; RODAK et al., 2015), and even more recently under field conditions (FREITAS et al., 2019). A previous study showed that Ni deficiency occurs in field-grown soybean plants with no visible leaf symptoms, i.e., it is a hidden deficiency, which is difficult to detect (FREITAS et al., 2018). However, the effect of Ni supply in the crop, as regard to leaf uptake, and to seed treatment is still unclear (ALOVISI; ALESSANDRA; EBERHARD, 2011).

2.4 Nanoparticles in plants

Current advances in nanotechnology has affected industries including manufacturing, biomedical applications, electronics/telecommunications, agriculture and renewable energy, among others (MA et al., 2015). Nanoparticles (NPs) are roughly defined as particles having at least one dimension between 1 and 100 nm in diameter (AUFFAN et al., 2009). The word "Nano" was extracted from the Greek term Nanos that means "dwarf" and it can be explained as 10^{-9} of any value or unit.

The synthesis of NPs mainly occur in two processes: "Top down" and "Bottom up" approaches. "Top down" are used to break down materials into substances at nano-scale. Whereas, the process "Bottom top" is contrarily, when atoms self-assemble to new nuclei and subsequently grow into particles with nano-scales and include physical and chemical methods to synthesize nanomaterial (KULKARNI; MUDDAPUR, 2014). These methods are mostly based on complicated procedures. In most cases severe conditions such as high temperatures, and toxic starting material need to be used, which leads to the high cost and increases minor poisonous contamination on the final products. Many attempts have been devoted to using biological catalysts (such as plants, bacteria, fungi) as an environmentally friendly approach to synthesizing of nanoparticles (SINGH et al., 2017).

Several techniques are employed for characterization of the nanomaterial structures: spectroscopy (X-ray, Zeta potential e etc.) and microscopy (Transmission electron microscopy, scanning electron microscopy, etc.) strategies are the most commonly used techniques. They are used for the determination of nanoparticle size distribution, shape, mass, chemical composition and among others (IOANNOU et al., 2020).

Therefore, these unique physicochemical properties and original features, NPs have been extensively used in many aspects of daily life and energy production (NEL et al., 2006). They are characterized of higher reactivity, solubility and biochemical activity depending on their high surface energy and the high surface-to-volume ratio (DUBCHAK et al., 2010).

The application of fertilizers to supplement natural soil fertility is a routine practice in modern agriculture, although temperate and tropical soils commonly remain deficient in micronutrients (BARKER; PILBEAM, 2007; KAYA et al., 2009). Applications of this new technology are found in agriculture and nano-technologies are already applied to production, processing, storage, packing and transportation of agricultural products (KHOT et al., 2012; NAIR et al., 2010; KHOT et al, 2012; NAIR et al., 2010). Plants serve as a potential pathway for the transportation of NPs (RICO et al., 2011). To date, studies in this area has been focused on the interaction between plants and NPs, and the effects of NPs on environment,

the food chain and human health; evaluating the pros and cons of NPs requires interdisciplinary knowledge (TOLAYMAT et al., 2010).

The application of NPs in agricultural industries in order to increase productivity of lands and crops, especially under suboptimal situations, started at the beginning of the 21th century (DUHAN et al., 2017; HE et al., 2018). However, nanotechnology remains a relatively under-explored area in agricultural science (HE et al., 2018; KHOT et al., 2012; WU et al., 2017). Numerous of nanomaterials with great capacities to revolutionize the agriculture industry have been introduced, characterized by a number of advantages and disadvantages (CHAUDHRY et al., 2018; HE et al., 2018; KHOT et al., 2012).

Nanoparticles have a varied effects on plants by helping them to improve growth, productivity and development (SINGH et al., 2016; SHWETA et al., 2016; TRIPATHI et al., 2017). Additionally, NPs has a noteworthy role in the protection of plants against various abiotic stress factors. They have shown a powerful tool to reduce the activity of reactive oxygen species (ROS) (RICO; PERALTA-VIDE; GARDEA-TORRESDEY, 2015), such as protection to photosynthetic system and enhancing better photosynthesis by suppressing oxidative and osmotic stress (GOHARI et al., 2020; QI; LIU; LI, 2013).

Nanomaterials have been tested masses in recent years to investigate their potential into growth promotion and stress amelioration in plants such titanium oxide (TiO_2), cerium dioxide (CeO_2), zinc oxide (ZnO), gold, silver and several more. However, a lot of materials after applied at higher concentrations may lead to toxicity symptoms particular (BEGUM; FUGETSU, 2012; GOHARI et al., 2020). The application of 100 mg L^{-1} of carbon nanotubes showed toxicity and aggregation in roots of *Ocimum basiculum L.* (GOHARI et al., 2020). Also some studies of exposure of NPs exhibited prompts oxidative stress and causes decrease in germination rate, root and shoot length and crop yields (BARHOUMI et al., 2015; DA COSTA; SHARMA, 2016).

This methodology is very promising the agricultural field, as protection against pathogens and diseases, enhanced uptake of nutrients, minimized nutrient loss, increased efficiency of pesticides, fungicides, herbicides, leading to greater plant growth and controlled release, site-specific delivery of fertilizers (DA CRUZ et al., 2019; HE et al., 2018; KASHYAP; XIANG; HEIDEN, 2015; SOLANKI et al., 2015; WALEED FOUAD ABOBATTA, 2018).

Nanoparticle can improve the practices and development of the plants enhanced better crop protection, nutrition and management due to size, high surface, precise dosage and slow release, which decrease dose and other special features (DUHAN et al., 2017), hence,

improving food quality and safety and reduction of agricultural inputs (PRASAD; BHATTACHARYYA; NGUYEN, 2017).

Nano-fertilizer foliar sprays have proven to be convenient for field use because they can feed plants gradually and in more controlled manner than salt fertilizers (KAH et al., 2018; SUBRAMANIAN et al., 2015) thus reducing toxicity symptoms that may occur after soil application of the same microelements.

Due to the fact that nano-fertilizers can delivery micronutrients in plants slowly and more tailored, they exhibit potential to avoid the induction of phytotoxicity in plants. In addition, it can decrease potential soil pollution and other environmental risks that may occur when using chemical fertilizers directly applied to the soil (SOLANKI et al., 2015). Another benefit described of using nano-fertilizers is that application can be made in smaller amounts than common fertilizers (DAVARPANAH et al., 2016). Rui et al. (2016) studying application of NPs of Fe_2O_3 in peanut (*Arachis hypogea* L.) discovered that this source can replace traditional Fe fertilizers in the cultivation in sandy soil. Another investigation in apple (*Malus pumila* Miller) presented that plant growth characteristics, such as plant weight, diameter, leaf number and leaf area) increased with Fe and Zn nano-fertilizer treatment (MOHASEDAT et al., 2018). Results benefits of ZnO NPs were informed on seed germination, seedling vigor, leaf chlorophyll content, stem and root growth in peanut (PRASAD et al., 2012) and pomegranate (DAVARPANAH et al., 2016). All this works showed positive responses to the use of nano-fertilizer (NPs) in the crop growth, however, nano-fertilizers may also induce deleterious effects, such as oxidative stress and toxicity symptoms in plants (NHAN et al., 2015) and other organisms in the ecosystem (BAEK; AN, 2011; HAJIPOUR et al., 2012; HEINLAAN et al., 2008). Nonetheless, recent study concluded that nanotoxicity depends both the nanoparticle composition and the plant species exposed (NHAN et al., 2015). The application of 200 mg/L of ZnO NPs (zinc, 35 nm) and ZnO (zinc oxide, 20 nm) inhibit germination in ryegrass and corn, respectively. The root growth of radish, rape canola, ryegrass, lettuce, corn, and cucumber species was inhibited upon exposure to 2000 mg/L nanosized Zn and ZnO (LIN; XING, 2008).

2.5 Ni application via seed and foliar

Nickel application are reported to enhance the resistance to rust diseases in several crops (GRAHAM; WELCH; WALKER, 1985; BARCELOS et al., 2018). A lot of groups of researches studied the positive effects of Ni nutrition on crops in relation to different sources of Ni. Plants, such as rice, tomato, canola and wheat when they were supplied with Ni in the

nutrient solution in hydroponics experiments, have been reported to increase development in the growing plant (KROGMEIER et al., 1991; NICOULAUD; BLOOM, 1998). The seed treatment with micronutrients, Mo and Co has been done with the aim of improving BNF (LAVRES; FRANCO; DE SOUSA CÂMARA, 2016), then minimum rates of Mo and Co have already been defined by researches and widely applied by farmers. Seeds can be treated with micronutrients either by soaking in nutrient solution of a specific concentration for a specific duration (seed priming) or by coating with micronutrients (FAROOQ; WAHID; SIDDIQUE, 2012).

For plant metabolism, Ni is required in amounts of less than 0.01 mg kg^{-1}, dry weight. Despite this information, fertilization with Ni in soybean growth is still not officially recommended (LAVRES; FRANCO; DE SOUSA CÂMARA, 2016). However, recent works recommend a rate of 45 mg kg^{-1} of Ni in the treatment of the seed, which stimulated the BNF process, due to the higher activity of the hydrogenase enzyme, which reflected directly in higher accumulation of total N in the aerial parts and grains and the increase of urease activity (LAVRES; FRANCO; DE SOUSA CÂMARA, 2016). Several reports indicated the potential of seeds treated in improving wheat yields (MARCAR; GRAHAM, 1986; WILHELM; GRAHAM; ROVIRA, 1988) and forage legumes (SHERRELL, 1984). However, one study indicates seed damage and germination inhibition by priming at higher nutrient concentrations.

The application of nutrient solutions to plant's foliage as an alternative means to fertilize crops was noted in the early 19^{th} century (GRIS, 1843). This method can support the nutrient supply; however, the factors influencing foliar applications' effectiveness are not well understood (FERNANDEZ; EICHERT, 2009).

Foliar application of Ni can prevent toxic symptoms caused by urea accumulation in leaf tissue, and glyphosate drift symptoms (ESKEW et al., 1984; KUTMAN et al., 2013; 2014). Despite the importance of foliar fertilization, including for Ni, there remains much uncertainty as to the underlying processes by which foliar-applied nutrients move across the leaf surface into the underlying plant tissue (FERNÁNDEZ et al., 2017; LI et al., 2018).

The possible physiological interactions between soybean and types of Ni application: seed and foliar has not been extensively explored and, to date, only a few studies have been published on foliar fertilization (WANG; NGUYEN, 2018).

2.6 New approaches to determined distribution and translocation of nutrients over the plant tissues

The distribution and translocation of elements, essential or nonessential, inside plant organs change over time in response to physiological stimuli, developmental stage, and the external environment (KOPITTKE et al., 2018). The distribution of elements within plant tissues is informative for studies ranging from functional characterization, in molecular biology, improving human nutrition, plant health and climate adaptability, to the movement and deleterious effects of contaminants throughout the food chain.

The techniques suitable for investigating the content and distribution of elements within plants include x-ray fluorescence microscopy (XFM) from benchtop or based on synchrotron, scanning electron microscopy (SEM) coupled with energy-dispersive x-ray spectroscopy and among others. These several methods all have benefits and disadvantages, opposing not only in the range of sample preparation required but also in sensitivity (detect limit), resolution, detectable elements, and probed volume (LOMBI; SCHECKEL; KEMPSON, 2011).

Synchrotrons were first used in plant analysis in the 1990s (GILFRICH et al., 1991). These previous studies were essentially a proof of concept rather than an attempt to address specific questions. The utility of XFM in plant sciences became apparent from these early studies in hyperaccumulator plants (FREEMAN et al., 2006; MCNEAR et al., 2005; PICKERING et al., 2000; 2006; SCHECKEL et al., 2007; TAPPERO et al., 2007).

X-ray fluorescence microscopy offers varieties of single abilities, including allowing for in vivo analyses at room temperature and pressure, providing good detection limits (approximately 1-100 mg kg^{-1}) and an excellent resolution (down to 50nm), and having no theoretical restrictions on sample (KOPITTKE et al., 2018). Synchrotron-based XFM analysis are typically orders of magnitude faster than benchtop systems, being a powerful technique that allows sub-micron scale mapping of endogenous and exogenous elements in plants specimens (DE JONGE et al., 2010; DE JONGE; VOGT, 2010; HACKETT et al., 2019; HARE et al., 2015; LOMBI; SCHECKEL; KEMPSON, 2011). XFM are able to provide information on elemental distribution, not only in situ but also in vivo (BLAMEY et al., 2018; SCHECKEL et al., 2004).

X-ray fluorescence (XRF) is a method spectroanalytical, whose may be applied with minimal or no samples preparation (GREDILLA et al., 2016). However, even that may assist physiological and nutritional studies, there are some concerns about the XRF *in vivo* approach regarding the damage which X-ray radiation may causes on living tissues (KOPITTKE et al., 2018; LOMBI; SCHECKEL; KEMPSON, 2011).

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3 Do nickel hydroxide nano- microparticles or sulfate improve seed (Glycine max) germination? A multifacetated overview

Abstract

The pathway whereby the ions moves after application of different sources of nickel (Ni) in soybean seeds [*Glycine max* (L.) merril] from seed tegument to another soybean seedlings remains unclear. The use of nanoparticles (NPs) in agriculture remains vague in relation to their potential in the main crops in the world, such as soybeans. Therefore, this study aimed at investigating the effects of Ni sources and particle size after soybean seed treatment in order to observe the Ni-translocation from seeds to seedlings tissues during plants development. The soybean seeds were treated with three Ni sources: Ni Sulfate ($NiSO_4$), Ni hydroxide nanoparticles (5 nm), and Ni hydroxide micrometric ($Ni(OH)_2$) (24 µm) at rate of 360 mg kg^{-1} of seeds. Firstly, the seeds treated were placed in a petri dish and examined after the emergence of the radicle. Then, the seeds treated also were placed in rhizotrons with soil to germinate per 21 days until the roots reach the maximum size inside the rhizotrons. These experiments were developed to characterize the pathway of Ni absorption on the cotyledon tissue and its long-distance translocation along seedlings tissues. Using Microprobe X-ray fluorescence spectroscopy (µ - XRF) and Scanning Electron Microscope with energy-dispersive X-ray (SEM-EDX), the distribution and translocation of Ni were studied in soybean seedlings. The concentration of Ni was also examined using an inductively coupled plasma-optical emission spectrometer (ICP- OES). To characterize the nano and micro particles of Ni the transmission electron microscopy (TEM) and X-ray diffractometry (XRD) were performed. The results indicated that seeds treated with Ni-nanoparticles presented better distribution around the seeds and roots than those treated with Ni sulfate and Ni hydroxide micrometric. While, the treatment with Ni hydroxide micrometric exhibited the highest rate germination, however the worst distribution around the seeds, with hot spots areas in specific locations compared to the treatments with the seeds treated with nanoparticles or sulfate. Interestingly, regardless of all treatments the seeds coated presented high spots of Ni concentrated in the hilum and did not enter the cotyledon. The soybean seedlings developed in the rhizotrons showed higher average of Ni in roots and in the soil that received Ni. Therefore, the Ni applied in the seed is either transported throughout the imbibition or provides a fertile microenvironment that favors the primary seedling development. The three Ni sources had an impact in soybean early –seedling development: germination rate, length of roots and distribution of Ni across the soybean tissues.

Keywords: seed**,** hilum, nanoparticles, X – ray fluorescence, *Glycine max*, soybean, germination, Ni sources.

3.1 Introduction

Nickel (Ni) was considered the latest essential element for higher plants (BROWN et al., 1987), due to its key role in urease, an important enzyme involved directly into the nitrogen metabolism (DIXON et al., 1975; ESKEW et al., 1983; HOGAN et al., 1983; POLACCO et al., 2013; WITTE, 2011). Ni also is an essential catalytic cofactor of seven other enzymes found in microorganisms, as [Ni-Fe] – hydrogenase (EVANS et al., 1987; LI; ZAMBLE, 2009). The importance of this enzyme is also found in some symbiose bacteria that are able to capture atmospheric N_2 and convert into ammonia for plants by nitrogenase activity (RUIZ-ARGÜESO et al., 2001). Soybeans are dependent on N_2 fixation, and as a consequence the inadequate Ni supply can negatively influence the growth and development of the plant. As known, soybean (*Glycine max* [L.] Merril) has been cultivated for thousands of years in Asia, and over the last century, cultivation has expanded dramatically (BRUINSMA, 2009). Soybeans is the most significant crop in Brazilian agriculture, its production was measured around 123.000 million metric tons in crop year 2019-2020, i.e, the productivity was estimated more than 3333 kg ha^{-1}, over the USA. In this year, the area planted with soybean in Brazil added up to nearly 37 million hectares (SHAHBANDEH, 2020).

In the absence of this element, bad outcomes are expected, such as quality seed decrease and reduced productivity (KAPPES et al., 2008). According to Freitas et al. (2018), Ni deficiency occurs in field-grown soybean plants with no visual leaf symptoms, i.e., it is a hidden deficiency, which is difficult to detect. Nickel is important in the process of Biological Nitrogen Fixation (BNF), which is involved in the expression and biosynthesis of hydrogenase (KLUCAS et al., 1983). This is changed by the increment of small quantities of Ni into liquid fertilizers (BROWN et al., 1987; ESKEW et al., 1983). Another strategy is to deliver the right amount of Ni to plants at the precise spot using the seed treatment technique (LAVRES et al., 2016). Consequently, Ni availability for plants can improve seed germination and seedlings vigor.

Nickel plays a vital role widespread of physiological processes, from seed germination to vegetative growth. Ni distribution and translocation on the plants is mainly carried out through the root system via passive diffusion and active transport (SEREGIN; KOZHEVNIKOVA, 2006). The uptake of Ni into in the plant varies between active and passive transport which differs with the species, form of Ni and concentration in the soil or nutrient solution (DAN et al., 2002; VOGEL-MIKUŠ et al., 2005). Besides that it depends on the Ni concentration, plant metabolism, the pH values of soil or solution, the presence of other metals

and organic matter composition (CHEN et al., 2009). Moreover being absorbed by roots, Ni can also enter into the plants via leaves (SAJWAN et al., 1996).

To improve the understanding of physiological processes, which control plant growing, it is necessary to comprise these changes statically and dynamically at the plant tissue scale. The distribution of elements within tissue plant is very informative for studies that undergo from a functional characterization in molecular biology to those studies related to improving plants health and shape it according to environmental stimuli (KOPITTKE et al., 2018). Understanding the process of how the nutrient applied via seed treatment is absorbed is the first step to determine the contribution of better nutritional management.

The use of X-ray fluorescence (XRF) instruments provides an effective, non-destructive method for the systemic quantitative assessment of elemental compositions in plants (VAN DER ENT et al., 2019; VIDIRO GEI et al., 2018). Then, this technique non-destructive may allow determine the concentration, distribution, and chemical environment of elements, in real-time assays in hydrated tissues, at room temperature and pressure. As XRF use minimum or none sample preparation, this technique allow analysis in situ (KOPITTKE et al., 2018). Understanding the process of how the nutrient applied via seed treatment is absorbed is the first step to determine the contribution of better nutritional management.

Nanotechnology has filling agriculture-related gaps with the good improvement compared to conventional systems (JOSEPH; MORRISON, 2006), not only to accelerate plant growth and improve yields, but also for disease control (WANG et al., 2016). This is related to the smaller particle size and larger contact surface, and hence to reach the desired target faster and more efficiently, without major losses (NAIR et al., 2010). Therefore, these happenings has inspired the scientific community to conduct investigations to understand the impacts of nanomaterials on agriculture, including possible negative impacts. Nanoparticles (NPs) received considerable attention in the fields of science, showing the advance of consumer products based on nano-sized heavy metal. Nanotechnology have been employed in curing diseases, in biomedical instrumentations, nanosensors, environmental protection and agricultural technology (KHAN et al., 2019; NEL et al., 2006).

As known, there still a lack of information on the potential use of NPs as fertilizer of plants. In this sense, more studies are still needed in order to understand the uptake of Ni applied in seed treatment. Furthermore, the use of μ- XRF and SEM-EDX techniques may help a better understanding on how Ni is absorbed and translocate from the seed treatments to plant germination, as well as to understand some aspects of possible effects of NPs. Doubtless the use of NPs in agriculture is promising. Therefore, this study aimed at investigating and to gain

insight into the Ni distribution in seeds and the pathway of Ni from seeds to roots and soil solution. For this, seeds of soybean were treated with different sources of Ni at the early stage, (V1- one set of unfolded trifoliolate leaves) and also evaluating the effect of the use of Ni-NPs.

3.2 Material and Methods

3.2.1 Seed treatment

The species grown was [*Glycine max* (L) Merrill], cultivar 6266 RSF IPRO (85% germination rate). This genotype presents indeterminate growth habit, early cycle and maturity group number 6.6. The seeds were treated with three Ni sources, Nickel sulfate ($NiSO_4$) (*Synth*), Ni hydroxide [$Ni(OH)_2$)] micrometric (24 µm) prepare according to Ramesh and Kamath (2006) and Ni hydroxide [$Ni(OH)_2$)] nanometric (5nm). The seeds were soaked into the Ni based solution and dispersions, 360 mg kg^{-1} (limit detection of equipment) - which corresponds to 20 g per hectare - and 100 g of the seeds were shaken inside a plastic bag for 5 minutes to promote uniform covering of the seeds surface and then put to dry in a fume hood for 24 h at room temperature (Figure Suppl. 1 - Appendix).

3.2.2 Characterization of materials

The nickel hydroxide NPs was prepared according to the method described by Danczuk et al. (2014), by the reaction of nickel acetate with sodium hydroxide in 1:2 M ratio, instead of KOH and nickel hydroxide micrometric was prepared according to the method described by Ramesh and Kamath (2006), by precipitation from a suitable solution containing dissolved Ni^{+2} ions is described (Figure Suppl.2 - Appendix). Nano and micrometric particles were characterized using X-ray diffractometry (XRD) (Figure Suppl. 3 - Appendix) patterns and Anlysette 22 MicroTec Plus, Frtsch. (Figure Suppl. 4 - Appendix).

3.2.3 Nickel distribution on coated seeds by X- ray fluorescence spectrocopy micro analysis (µ- XRF)

After 72 h drying, the soybean seeds were longitudinally sliced at the hilum region (Figure Suppl. 5 - Appendix). Then, 12 seeds were linescanned by microprobe X-ray fluorescence spectroscopy. Sixteen points were measured for 20 s each. All X-ray fluorescence results reported herein were carried out using an Orbis PC, EDAX, USA.

The Rh anode operated at 40 kV and 700 µA, a 250 µm thick Al primary filter was employed to improve the signal-to-noise ratio. The 30 um X-ray beam, at Mo Ka, was focused by polycapillary optics.

3.2.4 Cultivation assay

The soil used to grow the plants was of sandy texture and had low Ni concentration. The chemical characteristics were pH (CaCl2) 4.2; organic matter : 5 g dm^{-3}, P (resin) 2 mg dm^{-3}, K 0.3 mmolc dm^{-3}, Ca 1 mmolc dm^{-3}, Mg 1 mmolc dm^{-3}, H + Al 25 mmolc dm^{-3}, Al 3 mmolc dm^{-3}, CTC 27 mmolc dm^{-3}, S 6 mg dm^{-3}.

The seeds were sown at a depth of 2.0 cm in acrylic sample holders (rhizotrons), which an external and removable lid was covered with polypropylene film (7.6 thickness µm), a sponge was placed on the bottom to prevent soil loss. Two soybean seeds were sowed in each rhizotrons. After the emergence, only the healthier seedlings were left in the rhizotrons. Plants were germinated in growth chamber at 27± 3°C, 12 h photoperiod illuminated by LED lamps (Golden, Brazil) at a photon lux 250 µmol photons s^{-1} m^{-2} and were irrigated with deionized water daily. The experiment was carried out using three replicates per treatment. Summing up twelve samples (n = 12). Then, past 21 days (completely developed unifoliolate leaves), the outer lids of the rizotrons were removed, and the roots and their immediate neighboring soil were investigated by µ-XRF. Each spot was investigated during 200 s by a 30-µm X-ray beam focused by a polycapillary optical element. The XRF spectra were fitted and the Ni Kα net counts were determined. Only the Ni Kα signals above the instrumental limit of detection (LOD) were considered.

These analyses aimed at observing the translocation of Ni from seed-soil-root following the treatment of soybean seeds. For this, the analysis was performed at new and old roots, as well in the neighboring rhizosphere soil (Figure Suppl.6-Appendix). Six soil and six root points were probed per seedling.

3.2.5 Germination assay and root tip analysis

At the same time, the treated seeds were then transferred to 15 cm Petri dishes covered with filter paper. The paper was moistened with 10 mL of deionized water. Three replicates were used each treatment. The petri dishes were sealed with plastic film (Parafilm) in the edges, to avoid loss of moisture. The seeds were incubated for 2 days, until the moment the radicle emergence, in a dark ventilated germination chamber (TE, Tecnal, Brazil) at 27° C.

Then, the root tips were analyzed by µ- XRF. For catch root tip 12 points were recorded using 200 s of dwell time (Figure Suppl.7-Appendix).

3.2.6 X-ray fluorescence spectral processing

To ensure Ni signal reliability, Ni threshold was calculated as shown in Equation 1:

$$\text{Threshold (cps)} = 9 \text{ x } \sqrt{(\frac{\text{BG(average)}}{\text{t(s)}})} \quad (1), \text{ where}$$

BG(average) is the background average under the Ni Kα XRF peak of sixteen points of line scan and t (s) is the dwell time per point.

3.2.7 Scanning electron microscopy (SEM) analysis of the seed and root surface

We performed these analyses in two steps: (1) Soybean seed treated with different source of Ni were longitudinally sliced at the hilum region, similar processes described above to perform µ-XRF. (2) We cut of root tips from the emerged seeds. The samples were immersed in Karnovsky (M.J., 1985) fixative solution for 48 h. Afterward, the samples were sequentially dehydrated in ethanolic series (10, 30, 50, 70, 90 and 100%, (3x) for 30 min each step. Lastly, the samples were dried at their critical point (LEICA CPD 300), glued on aluminum stubs and carbon-coated (Bal-tec model SCD 050). The samples were analyzed under SEM-EDX (Jeol JSM IT 300, Japan) at 15kV and digitally recorded at a working distance of 15 mm and 3D images was obtained by digital microscopic (Hirox digital microscope, kh – 8700).

3.2.8 Transmission electron microscopy (TEM)

The nanometric nickel hydroxide dispersion was diluted in a 2:1 ratio. The $Ni(OH)_2$ aqueous dispersion was prepared in deionized water at 1000 µ Ni L^{-1}. Afterward, one droplet this solution was placed one a small screen, which was covered with Formvas film and it had 30 mesh , where was kept it for 5 minutes. Then, the images were recorded by transmission electron microscopy (Tecnai Spiri; FEI Company, Hillsboro, OR, USA) operating at 60kV in order to determine particle size and shape.

3.2.9 Nutrient analyses

The concentration of Ni on the seed was determined using 0.25 g of dried seeds material. Samples were digested in acid solution (65% HNO_3 and 30% H_2O_2) in a digestion blocks until it reaches 130°C. Concentrations of Ni were determined using a inductively coupled plasma-optical emission spectrometer (ICP-OES) (iCAP 7000 Plus Series, ThermoFisher Scientific).

3.2.10 Statistical analysis of Germination

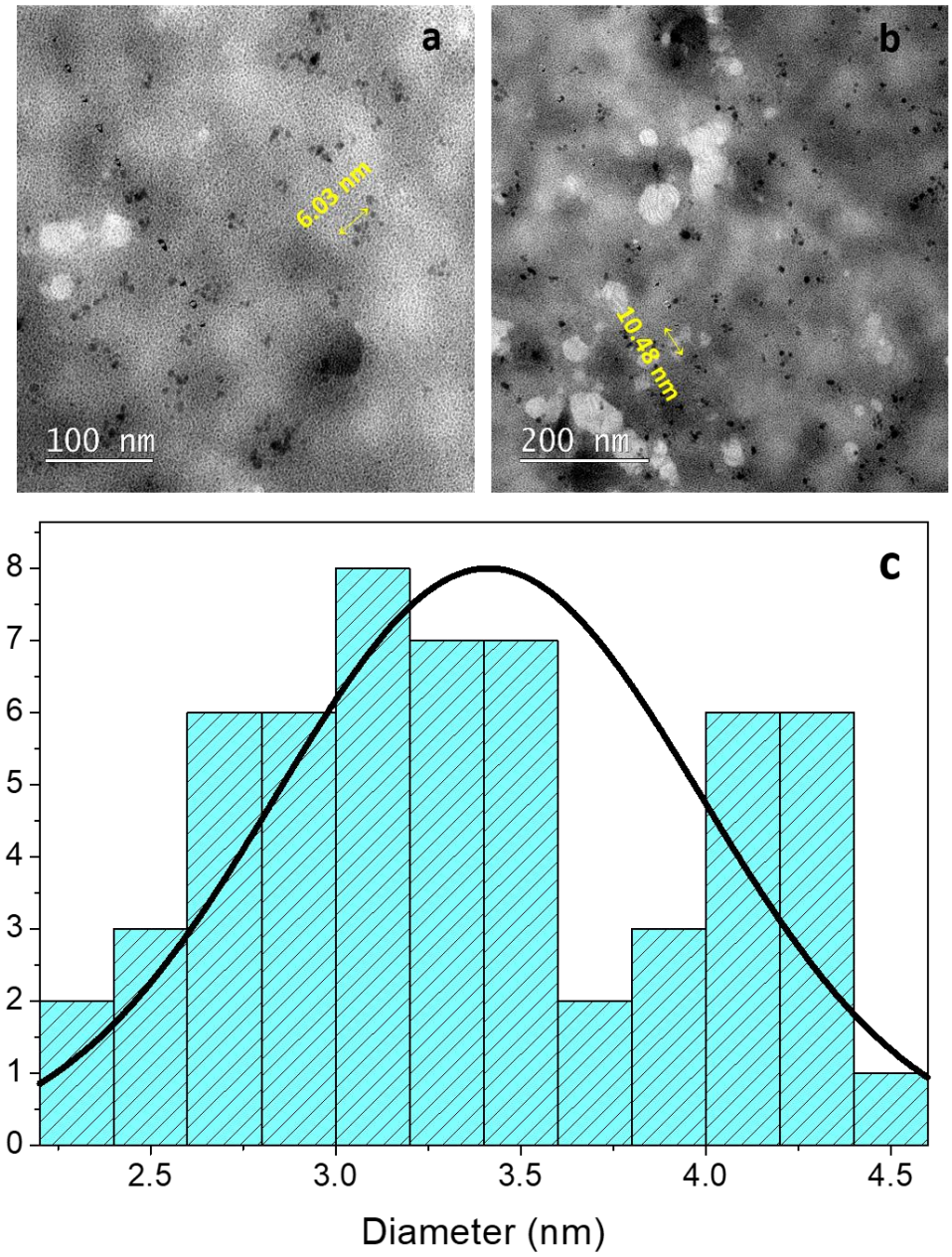
The germination assays were performed with the R Development Core team software R (version 4.0.2) (2015). The data were analyzed by calculating the descriptive parameters mean, standard deviation, and coefficient of variation. The data were also submitted to analysis of variance by the F-test software R (versão 4.0.2). When the rate of germination was significant, we applied Tukey test. In all analyses, the level of significance was considered to be 5%.

3.3 Results

3.3.1 Characterization Ni sources ($Ni(OH)_2$ micro and NPs)

Nano and micrometric particles were characterized using X-ray diffractometry (XRD) patterns and Anlysette 22 MicroTec Plus, Frtsch, respectively. The crystallite size determined by the Scherrer equation using XRD patterns and transmission electron microscopy was employed to image the shape of the nanoparticles (Figure 1a-b). The nano particle size analysed by TEM varied from 10 nm to 6 nm and had a spherical shape. However, using the software ImageJ to calculate the size we obtain average around 3.41nm (Figure 1c), varying from 2.25nm to 4.51nm. The size of nickel hydroxide micrometric presented average of 24 µm (Figure Suppl 4 - Appendix).

Figure 1 - (a-b) Transmission electron microscopy and (c) particle size distribution histogram of spherical Ni(OH)2 nanoparticles determined from ImageJ®



3.3.2 Effect of Ni sources on seed germination

Firstly, we determined the effect of Ni sources on the morphological development of soybean seedlings. The germination rate of seeds treated with micrometric $Ni(OH)_2$ was the highest when compared to the other Ni-sources (Figure 2). The treatment with Ni-nanoparticles had a substantial difference when the germination rate was compared to the other treatments, whereas in two days the other sources have already germinated, the emergence of radicle in the NPs treatment took 5 days more to measure the germination rate. Then, after 7 days, the

germination rate of the seeds treated with micro $Ni(OH)_2$ was 87% in contrast to 74% of germination rate of the control seeds, and finally, 63% ratio for the nano $Ni(OH)_2$ treatment. There was significant difference ($P<0.05$) in the rate germination between Ni hydroxide and Ni micrometric particles treatment, by which the germination rate of the seeds exposed to nanometric $Ni(OH)_2$ was slightly less than seeds coated with micrometric $Ni(OH)_2$ (Figure 3a). The length of the radicle calculated by ImageJ® corroborates with the data of the rate germination, on which the root length of the seeds coated with hydroxide micrometric were highest compared to those coated with control, sulfate and NPs, with an average of 2.37 control; sulfate 2.89; micro 3.45 and NPs 0.48 cm (Figure 3b).

Figure 2 - Soybean *(Glycine max.*[L] Merril.) seedlings after 2 days incubation in a growth chamber: (a) Control; (b) NiSO4; (c) Ni(OH)2 nanometric and (d) Ni(OH)2 macrometric. All seeds were treated with Ni rate 360 mg $kg^{-1.}$ The treatment with nanoparticles prevented proper root development and the hydroxide micrometric presented one of the biggest root elongation

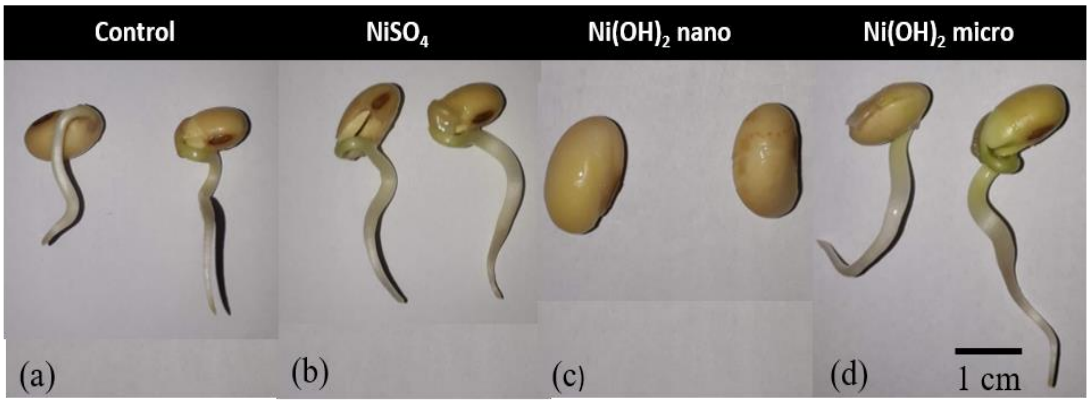
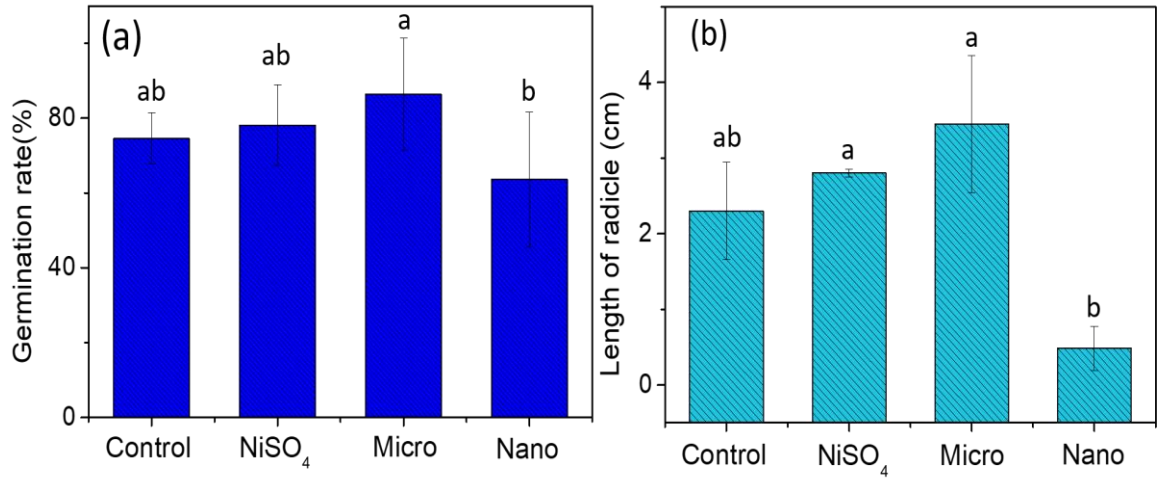


Figure 3- (a) Germination rate (%) (b) and length of the primary root 7 days after Ni sources treatment in the germination assay of soybean (*Glycine max*). Different letters indicate significant differences according to the least significant difference Tukey test



3.3.3 Distribution of Ni on seed coat and primary root after the treated seeds

Afterward dry digestion, the soybean seeds treated with Ni sources were analyzed by ICP – OES, in order to quantify the Ni-concentration in the seeds according to the Ni- treatments (Figure 4). Although applying the same dose of Ni in the all seeds/treatments (360 mg kg^{-1}), application of $NiSO_4$ treatment showed better adherence. The seeds treated with $NiSO_4$ had the highest concentration, 12.3mg kg^{-1} compared to the other treatments. While, the treatment with nanoparticles and Ni micrometric presented the lowest concentration 6.13 and 6.97 mg kg^{-1}, respectively. However, it is important to note that both treatment (micrometric and nanometric $Ni(OH)_2$) presented uniformity in the Ni- absorption into the seed-interface (Figure 5).

Scanning Electron Microscopy coupled with Energy Dispersive X-ray demonstrated the spatial distribution of the different Ni sources on treated seeds coats (Figure 5) and primary root (Figure 6). Our observation revealed that the micrometric $Ni(OH)_2$ source forms clusters of the particles on the seed coat surface, while the other sources treatments (NPs and sulfate) were uniformity and well distributed on the seed coat. The seeds coated with Ni sulfate exhibited a layer of crystals residue that partially covered the tegument (Figure 5g-l). On the other hand, 48 hours after the emergence of the root only captures the surface of the tissues, i.e., there is no X-ray penetration to detect the presence or absence of the element within the tissue (Figure 6). Thus, in the $NiSO_4$ treatment was not possible to detect Ni counts for this technique, as well the

control group (Figure 6a-d). On the other hand, we observed Ni counts at the radicle tips whose seeds were treated with micro and NPs $Ni(OH)_2$. Interestingly, the seeds coated with NPs revealed more Ni counts at the tip of the radicle and a distribution more uniform around the radicle tip compared to the seed coated with micrometric $Ni(OH)_2$. It is also possible to observe accumulation of Ni by the radicle tissue in the micrometric $Ni(OH)_2$ treatment and to note the difference between both treatments (Figure 6e-h).

Figure 4 - Nickel concentrations on the soybean (*Glycine max*) seeds treated with Sulfate – $NiSO_4$; Hydroxide nickel micrometric – $Ni(OH)_2$; Hydroxide nickel nanometric – $Ni(OH)_2$ in rate 360 mg kg^{-1} and control – without none nickel source, determined using ICP-OES

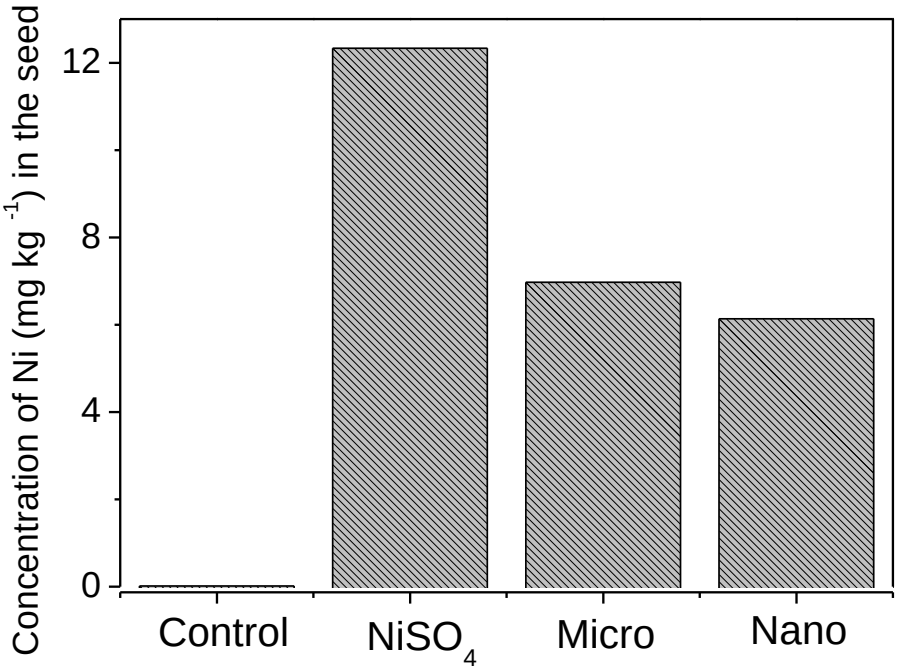


Figure 5 - Light and electron micrographs of soybean (*Glycine max*) seed treated with different sources of Ni 360 mg kg^{-1} (a,d,g,j,m,p,t w). EDX Scanning electron micrographs to observe Ni distribution in the seed coat. Hi – Hilum; Sd – Seed coat; Mi – Micropyle; Rf – rafe. Arrows indicating deposition of nickel source in seed coat on the seed. The blue color indicates Ni distributions around the seed coat. Arrows in letter h indicate eruptions, characteristic of sulfate source

*Figure next page

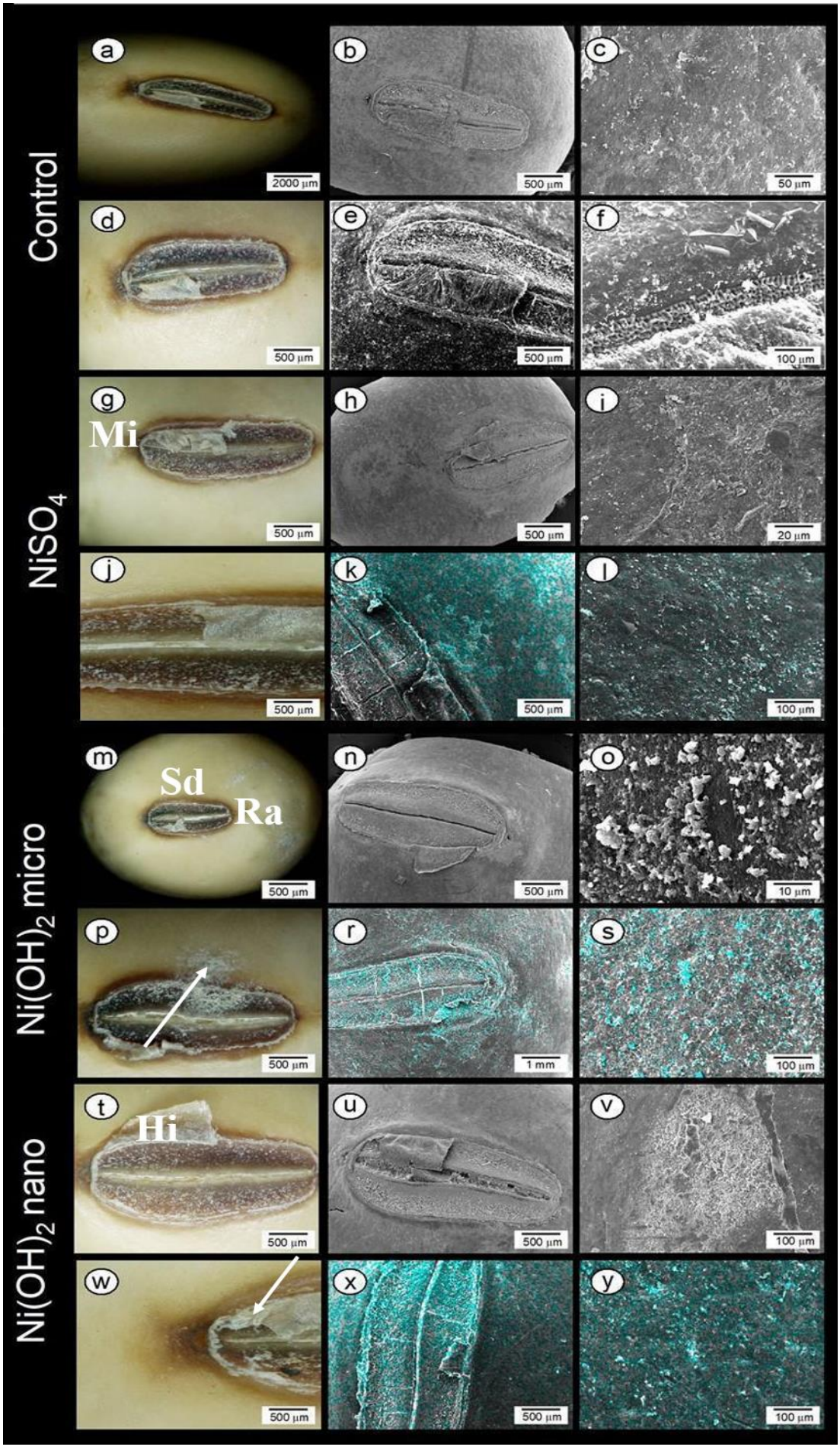
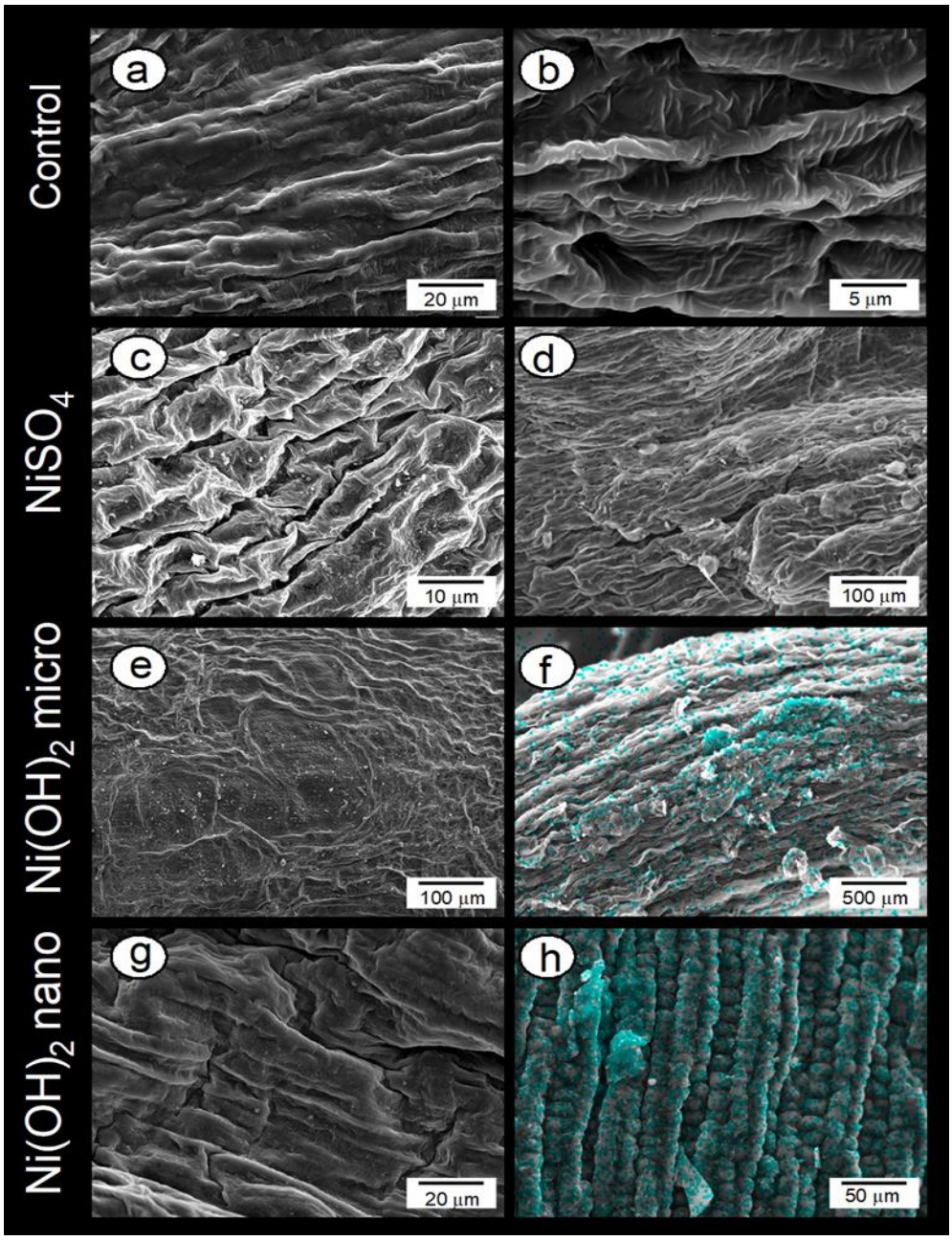

Control
NiSO4
Ni(OH)2 micro
Ni(OH)2 nano
a
b
c
d
e
f
g
Mi
h
i
j
k
l
m
Sd
Ra
n
o
p
r
s
t
Hi
u
v
w
x
y
2000 µm
500 µm
50 µm
100 µm
20 µm
10 µm
1 mm

Figure 6 - EDX Scanning electron micrographs of roots tip after 48 hours germination of soybean (*Glycine max*) seed with Ni-source. The blue color indicates the distribution of Ni by the tip root. The control and sulfate were not detected in the element by technique



3.3.4 Distribution and translocation of Ni on seed-radicle by µ - XRF

After dried, seeds coated with Ni sources (micrometric, nanometric $Ni(OH)_2$ and $NiSO_4$) were analyzed by µ - XRF. The seeds treated with $NiSO_4$ presented higher distribution (based on the count net Ni Kα) than the ones treated with micrometric $Ni(OH)_2$ source, ca. of

6% highest that Ni micrometric treatment. While the Ni-NPs showed better distribution, ca. of 83% higher than the $NiSO_4$ treatment. Most of the Ni remained trapped in the seed coat (Figure 7) directly enriching the cotyledon. Interestingly, regardless of the treatment, the seeds coated with Ni presented points of accumulation of Ni specifically in the hilum region, and then, it sharply decreased at the seed coat-cotyledon interface. The Ni-NPs and $NiSO_4$ treatment presented the highest Ni counts in the specific area of hilum an average of 1177.83 and 1044.86 count net Ni Kα, respectively (Figure 7b-d). Whereas the furthest point from the hilum was not even possible to detect any Ni, only signal-to-noise-ratio. On the order hand seeds from the micrometric $Ni(OH)_2$ treatment (Figure 7c) had the lowest concentration, Ni counts in the hilum region had an average of 699.16 count net Ni Kα. The control treatment did not detected the element (Figure 7a).

X-ray fluorescence microanalysis (µ-XRF) also showed the presence of Ni which it was also verified at the radicle tip after emergence (Figure. 8). This technique can detect the element beyond the surfaces, *i.e.*, inside the tissues. Remarkably, a uniform distribution of $NiSO_4$ treatment (Figure 8b) was observed in this technique differently of the SEM- EDX technique used previously. Regardless of the treatments, there was a pattern, indicating the Ni counts was higher at the tip of the radicle and move towards to the other soybean tissues. The NPs $Ni(OH)_2$ treatment showed 63.24% more counts of Ni at the radicle tip compared to the furthest tissue. Micrometric $Ni(OH)_2$ and NiSO4 treatments presented correspondingly results of Ni counts, 26% and 17%, respectively.

Figure 7 - Distribution in the seeds by non-invasive and in vivo fluorescence (μ-XRF.) The black curve show the number of Ni Kα count rate (cps) for seed (a) control; (b) $NiSO_4$ (c) $Ni(OH)_2$ micro; (d) $Ni(OH)_2$ nano. The red curve refers to the thereshold. From the image it is possible to infer that the treatment adheres more to hilum on the seed coat

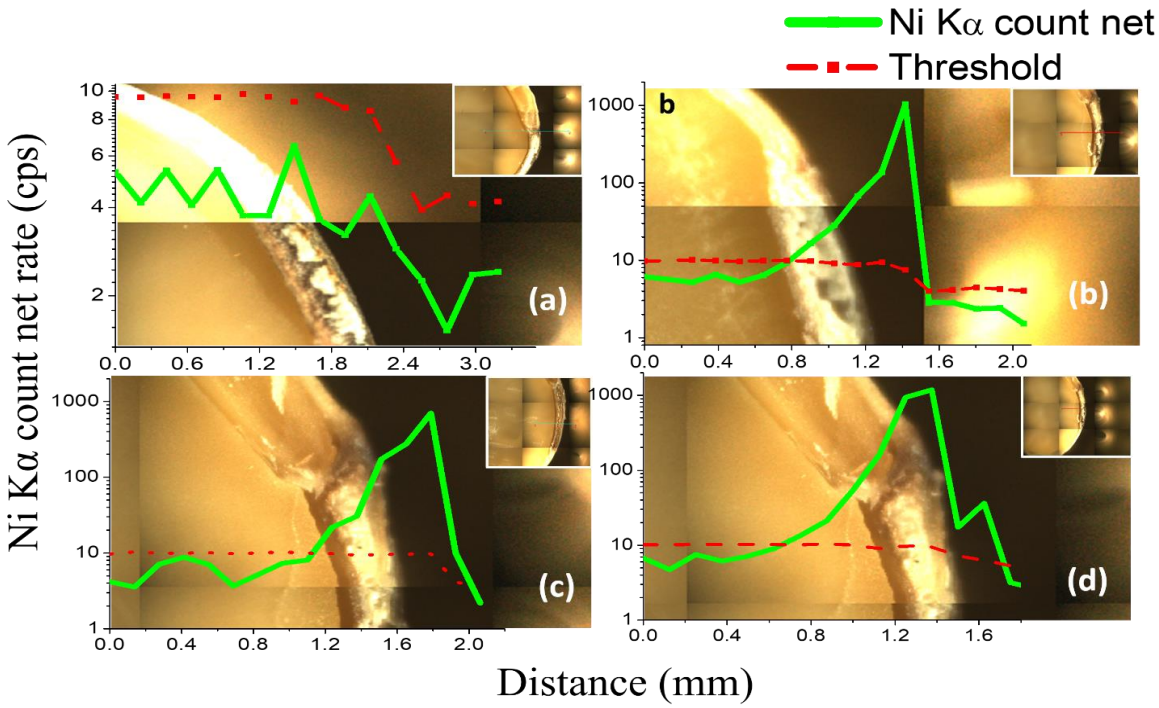
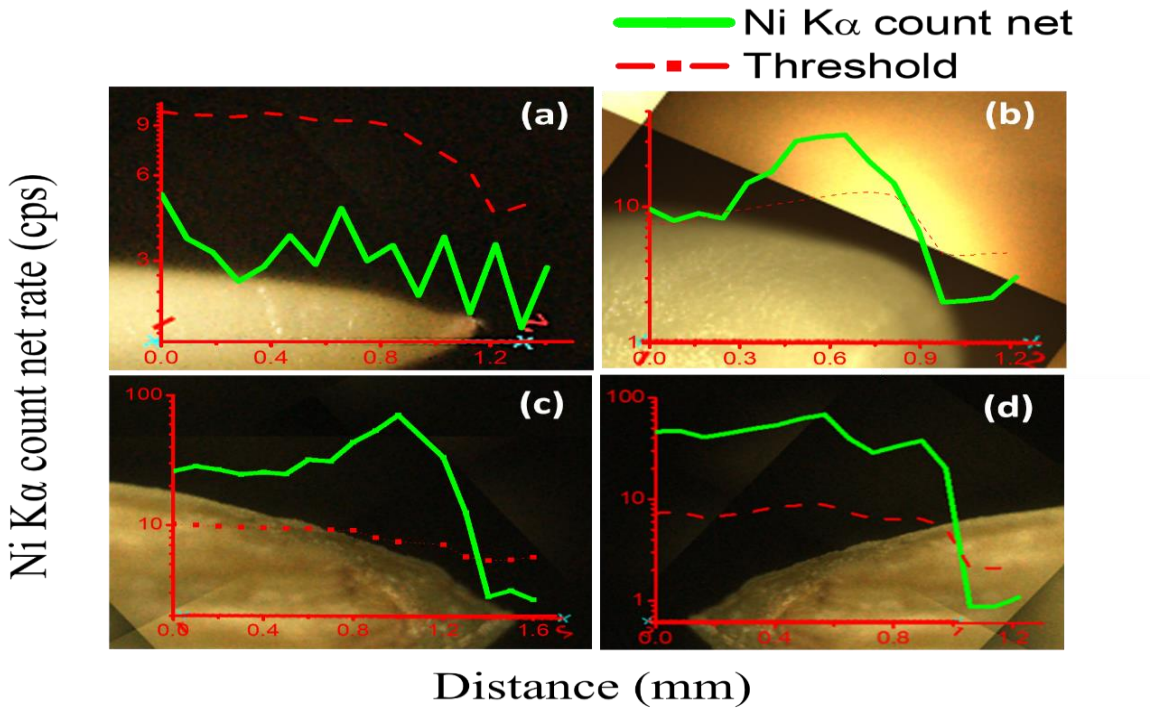


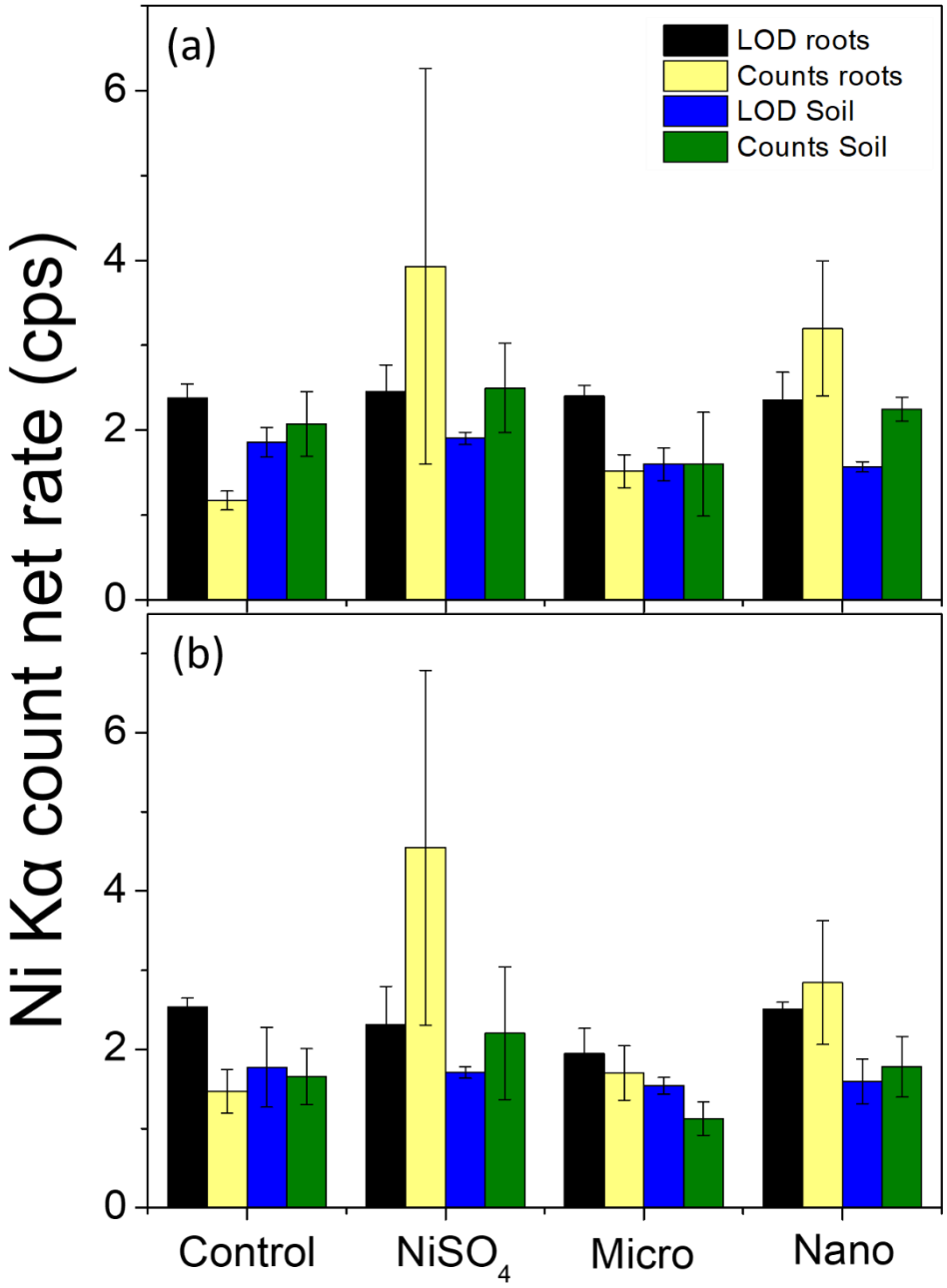
Figure 8 - Distribution in the roots by non-invasive and in vivo fluorescence (µ-XRF.) The red curve show the number of Ni Kα count net rate for root tips (a) control; (b) NiSO4 (c) $Ni(OH)_2$ micro; (d) $Ni(OH)_2$ nano. The red curve refers to the thereshold. From the image, it is possible to infer that the treatment adheres more to the seed coat



3.3.5 Tracing Ni transference from seed coated to soil and roots

Figure Suppl 6 (Appendix). shows the experimental setup built to evaluate the presence of Ni in the rhizosphere of soybean plants. Plant roots and soil were analysed in situ by µ -XRF. This experiment was carried out in different types of root parts (old and new roots) to observe the translocation of Ni through the root. Old and new root no statistical difference in the distribution of Ni over the roots (Figure 9). However, the $NiSO_4$ treatment showed highest Ni counts at the roots, an average of 3.92 and 4.54 at old and new roots, respectively. While the NPs $Ni(OH)_2$ treatment presented Ni counts an average of 3.19 and 2.84 at old and new roots, respectively. The control and micrometric $Ni(OH)_2$ treatment did not detected Ni even in the old and new roots. There was a correlation between Ni sources found in the root and the neighboring soils. The $NiSO_4$ treatment was observed that Ni counts in the new root, also was observed Ni counts neighboring soils, an average of 2.49 Ni counts in the neighboring rhizosphere soils. The same occur with NPs $Ni(OH)_2$ treatment, which showed 1.78 Ni counts in the neighboring rhizosphere soil close to the old roots. This pattern also follows with old roots analysed.

Figure 9 - Counts of Ni Kα in the old and new roots and neighboring soils by non-invasive and in vivo fluorescence (μ-XRF) microanalysis. (a) Ni Kα counts net cps in old roots and neighboring soils with threshold of detection (cps); (b) Ni Kα counts net cps in new roots and neighboring soils with threshold of detection (cps)



3.4 Discussion

Our study was carried out to evaluate the features and effect of Ni sources, a question that is not comprehensively addressed for the use of this micronutrient. In the present study,

we investigated the particle sizes, rate germination, uptake and translocation of Ni by roots and seeds - on soybean, which different techniques were employed.

The better germination rate and the greatest radicle elongation was found at the micrometric $Ni(OH)_2$ treatment. Herein, the primary roots development were expressively higher when compared to the other treatments (Figure 2). Owing to the fact that this is an insoluble source, *i.e.*, low solubility in water, and the size of the particle, an average of 24µm (Figure Suppl. 4 -Appendix), there was an uneven distribution of the Ni in the seed coat. As a result, fascinatingly the germination rate of seeds increased. This may be justified by the reason that the micrometric source not causing any interference in the seed. Once this source associated with the other results (SEM EDX, µ- XRF at the seeds and radicles) shows that there were little or none absorption of micrometric $Ni(OH)_2$ by the tissues of soybean plant. Hence, this source probably are not able to change any morpho-physiological, therefore, are not capable to cause deleterious effects on the development of soybean plants.

On the other hand, the NPs $Ni(OH)_2$ treatment decreased the germination rate of soybean seeds, presented decrease of 23% than the seeds treated with micrometric $Ni(OH)_2$, which showed germination rate by 86%. This result totally unlike from the other described previous can be explained because of the features of the source. The NPs $Ni(OH)_2$ size varied from 10 nm to 6 nm and had a spherical shape (Figure 1) and similar results were presented Gonçalves et al. (2018) which detected an average diameter of 5nm (4.7 ± 1.8nm) by TEM of the nickel hydroxide nanoparticles. Therefore, this source has a minimum of contact surface, which facilitates the absorption of Ni by the soybean tissues and associated with the high dosage of Ni (360 mg kg^{-1}) this treatment had a negative impact on germination.

Several studies also related that the toxicity of NPs were linked to their properties: hydronamic sizes, concentration, surface chemistry shape and chemical characteristics of the exposure (GRIFFITT et al., 2008; JU-NAM; LEAD, 2008; XIA et al., 2008; JIANG et al., 2009). Similar results also were presented for *Lemma Gibba* L. suggesting the nickel oxide nanoparticles is known to be practically insoluble in water limiting considerably the release of free ionic Ni. On the order hand, the physicochemical conditions of the aqueous solution may alter the properties of nickel oxide nanoparticles, modifying their bioavailability by changing their surface contact, charge and solubility (OUKARROUM et al., 2015).

Nanoparticles mostly are known for your damage effect. Soares et al. (2016) shows that nickel oxide nanoparticles (NiO-NPs) was toxic in barley, inducing a more negative response in their metabolism. Similar results were found in roots of tomato seedlings, which reported that NiO-NPs treatment reduced root elongation, increased the levels of ROS, lipid

peroxidation, glutathione and superoxide dismutase activities and induced cell death as indicated by the increased number of apoptotic and necrotic cells (FAISAL et al., 2013). On the other hand, Saleh et al. (2019) pointed out an interaction of NiO-NPs with CO2 in wheat induced recovery of the damage effect of Ni on photosynthesis and inhibition of photorespiration and therefore decreasing the production of H_2O_2 and hence, reduced cellular damage. Another study demonstrated that a combined application of SiO_2 and NiO nanomaterials resulted in a positive response of the antioxidant enzymatic system in both leaves and roots of barley plants exposed to high levels of nano-NiO (SOARES et al., 2018). Considering this results, there are a considerable lack of information about plants responses to nanoparticles. Then, to the best of our knowledge, this is the first study to examine the seeds treated with micrometric and NPs Ni(OH)2¸ therefore, further studies are necessary to understand exactly the behavior of these sources in plants.

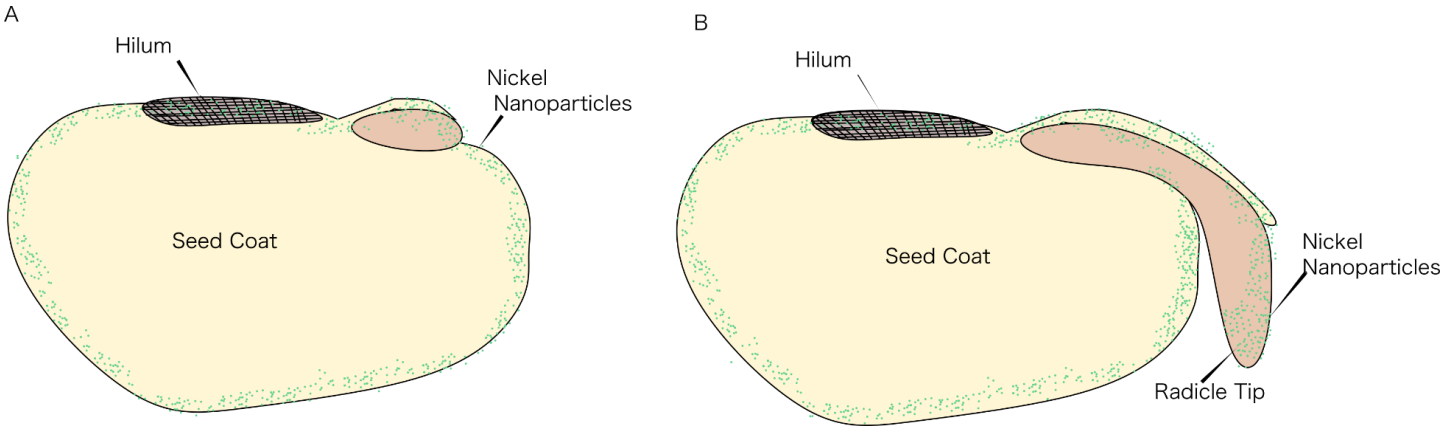
In this sense, the µ- XRF linescans showed that seeds coats inhibit the Ni movement into the cotyledon (MONTANHA et al., 2020). The mapping of seed treated with $NiSO_4$ can be observed this phenomenon (Figure Suppl. 8- Appendix), whither the Ni deposition is clearly noted on specific area: hilum. The Ni counts in the soybean seeds (Figure 8) were varied widely between the hilum and furthest tissues. It was also observed that in the hilum the NPs $Ni(OH)_2$ and $NiSO_4$ treatment presented the highest Ni counts. Savassa et al. (2018) also observed in zinc treated Phaseolus vulgaris seeds, including nanoparticles of different sizes that Zn treatment is not homogenous in the seed and showed hotspots of Zn accumulation in the hilum region. Another study also had similar results with ZnSO4; ZnO 5 µm and ZnO 40 nm in soybean plants (MONTANHA et al., 2020). The importance of seed coat is it act as a barrier for seed protection, due to it is lignified tissue, whereas the hilum a part of the seed responsible for the imbibition processes (RADCHUK; BORISJUK, 2014; SMÝKAL et al., 2014).

Likewise, the data obtained by SEM-EDX in the seeds shows that the micrometric $Ni(OH)_2$ treatment does not present good distribution throughout the seed, whilst the NPs $Ni(OH)_2$ and $NiSO_4$ treatments presented better distribution. Whereas, SEM-EDX performed at the radicle tips NiSO4 treatment was not possible to detect or detect very few Ni counts (Figure 8). However by µ- XRF linescans was possible to detect Ni in the radicle tips. A possible explanation for this phenomenon is that the $NiSO_4$ source is a water-soluble source and then probably the Ni has already absorbed by the radicle tissue. Another possible reason is that the $NiSO_4$ source may have been lost through the irrigation, undergoing a process of leaching from the seed to water (ROY et al., 2006; OTIENO et al., 2009). Therefore, in the surface of the radicle tip was not more possible to detect the element. The micrometric and NPs

$Ni(OH)_2$ treatment even by SEM-EDX and µ- XRF technique in the radicle tips in addition to Ni detection were also possible to observe the distribution of these sources in this soybean tissue. This event can be justified by the fact that the sources are insoluble in water, hence, the loss of Ni by leaching occurs in less quantity. Another reasons is likely due to the low solubility of hydroxides, most of Ni remained on the tissue surface. Besides that, these sources tend to form an agglomeration, which is more able and easy to be detect by theses technique. Scanning electron microscopy of the tips roots micrometric treatment (Figure Suppl. 9 - Appendix) observed the stomata at the caulicle and the images shows the micrometric $Ni(OH)_2$ crowded very close to the stomata.

In general, the treated seed particles adhere to the seed coat, especially the hilum, then when root emergencies occur, the particles remain aggregated in the root tissue, as shown in the scheme (Figure 10).

Figure 10 - Scheme showing the adherence of nanoparticles in the seed coat and radicle tip. (A) It is possible to see that the nanoparticles are along the seed coat and (B) when the root emergence, as the root grows the particle remains aggregated in the root tissue



Rhizotrons are techniques to conserve the tissue intact, with minimal damage, repeated observations and measurements of root systems (KLEPPER; KASPAR, 1994). The analysis of old and new roots in the rhizotrons had no statistical difference in the Ni- distribution (spatial distribution) along the roots. The µ- XRF intensities at the different parts of roots and neighboring soil showed that the seed coating enriches the rhizosphere with Ni. The $NiSO_4$ and NPs $Ni(OH)_2$ treatments showed this trend among the analysed parts: old and new roots. This

data shows a possible translocation of Ni after the seed coated and soaked in the soil. Therefore, it can occur a percolation of Ni from the root or seed treated with Ni by irrigation into the soil.

Interestingly, for the micrometric $Ni(OH)_2$ treatment it was not find Ni counts even in old and news roots, include the neighboring soil analysed. The possible explanation to NPs $Ni(OH)_2$ having been found it in the roots and neighboring soil is the presence of smaller particle size and, hence, larger contact surface and to reach the desired target faster and more efficiently (NAIR et al., 2010). The nanoparticles size increases the surface- volume ratio, so an important fraction of atoms stay on the surface (ZANCHET et al., 1999). The overall uptake of Ni by plants depends on the concentration of Ni^{+2}, plant metabolism, pH value of soil or solution, the presence of other metals and organic matter composition (CHEN et al., 2009). In other words, observing all of these results shown using smaller particle size sources may increase the effect of nutrient absorption efficiency. The mostly Ni absorbed in plants is retained in the roots (CATALDO et al., 1978). This may be due to the sequestration in the cation exchange sites of the walls of xylem parenchyma cells and immobilization in the vacuoles of the roots (SEREGIN; KOZHEVNIKOVA, 2006). Then the uptake of Ni, regardless of the type of source used, is predominates via roots (YUSUF et al., 2011).

The rate used of this work (360 mg kg^{-1}) is considered toxic for plant development (LAVRES et al., 2016). Therefore, we did not go forward in the soybean cycle, this dose was chosen due to the techniques used that require limit of detection (threshold) to quantify this element.

3.5 Conclusion

To summarize, this study showed that the three Ni sources at 360 mg kg^{-1} affected soybean seeds germination. The seeds coated with NPs $Ni(OH)_2$ showed lowest germination, although of having good spatial distribution along of the seed, radicle tip and roots.

Regardless of the treatment, Ni remained on the seed coat, specifically in the hilum, therefore, Ni does not move from the seeds coating towards the cotyledons. Remained unclear how this element adhered on the seed coat is further absorbed by the plant. Possibly this element is delivered in the soil rhizosphere.

The seed treatment with nanoparticles still left many gaps to be understood. However, it was clear that the particle size influences the highest efficiency in the uptake of the element by the plant. That is, the source having smaller particle will reach the desired target. Nevertheless, it is not yet known whether this will be positive for all plant development.

In this context, $NiSO_4$ treatment represents a better alternative, as it has more studies and evidence that this source has positive effects on soybean plants development and growth. Further studies are necessary to investigate the impact of these sources on the life cycle of soybean plants.

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4 Soybean seed treatment and foliar fertilization based on macro, micro and nanoparticles of nickel to improve biological nitrogen fixation and plant growth

Abstract

Biological nitrogen fixation (BNF) is the main source to obtain nitrogen (N) by soybean crop [*Glycine max* (L.) Merrill]. To achieve the highest BNF, nickel (Ni) fertilization has been widely practiced to improve crops. Since, Ni is a micronutrient required for plants metabolism due to its role as a structural component of urease and hydrogenase, which, in turn, succeeds N metabolism in soybean plants. In addition, foliar Ni fertilization linked with application of nano materials might improve the efficiency of BNF, boosting the biomass, and the N content. In the current study, soybean plants were conducted with the aim to understand the effects of Ni fertilization by seed or foliar spray in V4 phenological stage using different Ni sources: nickel sulfate ($Ni(SO)_4$); hydroxide nickel micrometric ($Ni(OH)_2$ ~ 24 µm), and hydroxide nickel nanometric (Ni-NPs- $Ni(OH)_2$ ~ 5 nm). Soybean plants (cultivar IPRO flecha) were grown in greenhouse conditions in a soil sandy and treated with 45 mg per kg^{-1} of seed of Ni (application via seed) and 20 g ha^{-1} of Ni via leaves as either nickel sulfate, hydroxide nickel micrometric or hydroxide nickel nanometric and compared to untreated control plants up to the R7 developmental stage. This study evaluated the BNF, directly by the ^{15}N natural abundance method ($\delta^{15}N‰$), by measuring urease, nitrate reductase activity and nitrogenase (N-ase) activity, ammonia, nitrate and ureides content at Vn (V stages continue with the unfolding of trifoliate leaves), R2, R5 (flowering stage) and R7 phenological stage (grain maturity). Foliar Ni fertilization, overall, regardless of the Ni sources, improved by 32% of biomass of soybean, 27% of urease activity, 9% of BNF compared to plants that received Ni only via seed. The proportion of N derived from N_2 fixation varied from 75 to 100% in leaves using the natural ^{15}N abundance method and non-nodulating *Oriza sativa* as reference. In the grains the BNF range from 69 to 82%, where plant supplied via seed by Ni-NPs showed the highest results (ca. of 92% of BNF). Plants treated based on Ni-NPs in whole plant showed an increase by 22% in biomass compared to Ni micrometric and Ni sulfate, regardless of the Ni form application. However, plants treated with Ni-NPs revealed a decrease in nitrogenase activity. In general, foliar application associated with nanoparticles had more positive impact on soybean growth, physiology and biological nitrogen fixation than conventional Ni fertilizers. These results indicate that the foliar Ni-NPs application could be considered for soybean systems to improve soybean quality.

Key word: foliar fertilization, nickel nanoparticles, biological nitrogen fixation.

4.1 Introduction

Soybean crops afford one of the world's most important sources of protein and oil. Soybean yield improvements have occurred from biomass grains and increased partitioning to the seed, which all require large amounts of N (BALBOA et al., 2018) supplied by biological nitrogen fixation (BNF) and/or the soil. The contribution of N from BNF ranges from 0 to 98% depending on many factors, the most important being rhizobial activity (SALVAGIOTTI et al., 2008). The BNF occurs due to interaction between soybean plants with bacteria belonging the *Bradyhizobium* genus, which are highly efficient in promoting BNF. Through infection of the root sytem, they form nodules within the bradyrhizobia reduce N_2 to NH_3 (CADISCH et al., 2000; GONZÁLEZ-GUERRERO et al., 2014). Biological nitrogen fixation is one of the most effective replacements to reduce the huge use of nitrogen (N) fertilizers for improving plant nutrition as well as to decrease environmental impacts (JENSEN; HAUGGAARD-NIELSEN, 2003; CAMPO et al., 2009).

Nitrogen (N) is the nutrient required in greatest amount by soybean plants (ALVES et al., 2003). Therefore, optimized BNF is fundamental to supply the N demand. Yet, the low availability of micronutrients like cupper, iron, manganese, and nickel, principally in tropical Oxisols that have received excessive lime doses, can result in nutritional disorders, hence, reducing the nitrogen fixation capacity (FAGERIA; STONE, 2008).

Based on this information, it is well recognized that Ni is an essential element for plants, it is utilized as a cofactor for several metalloenzymes such as urease and glyoxalase I (DIXON et al., 1975; BROWN et al., 1987; POLACCO et al., 2013; MARSCHNER et al., 2012). Nickel also has been reported to be involved in the expression and biosynthesis of hydrogenase, fundamental enzyme for the BNF process, which eliminate free radicals (H^+) produced by this process of conversion of N_2 atmospheric to ammonia (KLUCAS et al., 1983; GONZÁLEZ-GUERRERO et al., 2014).

Then for supply Ni and another micronutrients can be applied by soil, seed, and foliar sprayed. Treatment of seed with micronutrients is a good alternative to enhancing BNF, synchronized germination (FAROOQ et al., 2009) owing to less imbibition time (BROCKLEHURST; DEARMAN, 1983; TAYLOR et al., 1998) and build-up of germination-enhancing metabolites (BASRA et al., 2005; FAROOQ et al., 2006).

Although the required quantity of micronutrients can be supplied by any of these methods, foliar sprays has been more effective in yield improvement and grain enrichment (JOHNSON et al., 2005). Currently, foliar fertilization is ordinarily used to treat nutrient deficiencies (HANNAM et al., 1984; SAXENA et al., 1990; SHORROCKS, 1997; KAYA; HIGGS, 2002; DEL AMOR et al., 2009) or in conjunction with soil application to reduce annual use without compromising produce of the crops, as soybean, rice and maize (AKHTAR et al., 2014) and avoid toxicity symptoms (KUEPPER, 2003; FAGERIA et al., 2009; LICHTFOUSE, 2010). Foliar sprays is highly efficient, and it can decrease the lag time between application and uptake by the plant. However, the quality of foliar fertilizer depends on species and the chemical properties (BOWMAN; PAUL, 1992; STIEGLER et al., 2010). To date, there have been a few studies showing the ability of nanofertilizers in crops to enhanced good productivity.

Due to unique properties of nanoparticles, such as particles smaller than 100nm, at least in one dimension (SOLANKI et al., 2015) the use of nano fertilizers has been further advanced. The use of nano-fertilizer, as foliar sprays or by another method, have demonstrated to be suitable for crops use because they can delivery nutrient required by plant gradually and in more controlled manner than common fertilizers (SUBRAMANIAN et al., 2015; KAH et al., 2018).

The advantage of using nanoparticles in agriculture is because they exhibit potential to avoid the induction of phytotoxicity in plant via slower and more tailored delivery of essential elements while show potential to reduce soil pollution and other environmental risks that may occur when using chemical fertilizers directly applied to the soil (SOLANKI et al., 2015). Another benefits of using nano-fertilizers is that application can be done in smaller amounts that common fertilizers (DAVARPANAH et al., 2016).

The possible physiological interactions between soybean and application of Ni based on nanoparticles has not been extensively explored and, to date, only a few studies have been published on foliar fertilization (WANG; NGUYEN, 2018). Then, to understand the interactions between different Ni sources ($Ni(SO)_{4;}$ $Ni(OH)_2$ micromentric ~ 24 µm, and $Ni(OH)_2$ nanometric ~ 5 nm) and forms of Ni application in soybean plants by seed or foliar spray, the aims of this investigation were (i) to compare plant growth responses in soybean plant with different sources Ni and types of application; (ii) to analyze the activity of urease, nitrate reductase and nitrogenase activities; (iii) nitrogen accumulation by methods indirect, such ammonia, nitrate and ureides content, and (iv) evaluation of BNF directly by ^{15}N natural abundance method $\delta N^{15}‰$.

4.2 Materials and Methods

The experiment was carried out in a greenhouse in the research area of the department of Soil Science of "Luiz de Queiroz" College of Agriculture (ESALQ), from University of Sao Paulo (USP). The soil employed for the experiment, classified as Red-Yellow Latosol (Oxisol; EMBRAPA, 2006), was collected from the surface layer (0-20 cm) from an area in the municipality of Itatinga, Sao Paulo State (22º 43' 31" S e 47º 38' 57" W). The texture was sandy, with low Ni concentration (0.3 mg dm^{-3}). The chemical characteristics are: pH ($CaCl_2$) 4.2; organic matter: 5 g dm^{-3}, P (resin) 2 mg dm^{-3}, K 0.3 $mmol_c$ dm^{-3}, Ca 1 $mmol_c$ dm^{-3}, Mg 1 $mmol_c$ dm^{-3}, H + Al 25 $mmol_c$ dm^{-3}, Al 3 $mmol_c$ dm^{-3}, CTC 27 $mmol_c$ dm^{-3}, S 6 mg dm^{-3}. Based on the soil chemical analysis, soil correction was carried out individually (each pot) in order to fulfill soybeans nutritional requeriments (RAIJ et al., 2001), liming with calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$) in the proportion 3:1 mol.

4.2.1 Experimental design

The experimental design was fully randomized, with seven treatments in a factorial scheme 2x3, with the first factor is the types of Ni application: (1) via seed; and (2) foliar fertilization (first factor); and three Ni sources ($NiSO_4$; $Ni(OH)_2$ micro $Ni(OH)_2$ nano) (second factor), containing 11 eleven repetitions, being three repetitions kept until the first reproductive phenological stage of inflorescence emergence: R2 (86 d); three repetitions kept until the beginning seed, when seeds are around 3mm - R5 (98 d), the plants were harvested to evaluate the nodular development of the root system and enzyme activity urease, nitrogen, nitrate reductase, and ureides. The rest of the plants (five repetitions) were maintained until the phenological reproductive stage of fruit and seed ripening- R7 (122 d). Each pot with two plants was an experimental unit. In general, the treatments are based on in this sequence: 1- absolute control – only seed treated with Co, Mo, and inoculation with *B. japonicum*; 2- Ni-$NiSO_4$ (treated seed); 3- Ni-$NiSO_4$ (treated seed + foliar); 4- Ni-$Ni(OH)_2$ micro (treated seed);5- Ni-$Ni(OH)_2$ micro (treated seed + foliar); 6-Ni-$Ni(OH)_2$ nano (treated seed); 7- Ni-$Ni(OH)_2$ nano (treated seed + foliar).

4.2.2 Cultivation assay

The species grown was *Glycine max* (L.) Merril, cultivar 6266 RSF IPRO Flecha, which have a high potential for productivity. This genotype presents indeterminate growth habit, early cycle, and maturity group number 6.6. The experiment started on February 6, 2019 and the

plants were harvested in three different stages R2 (full flowering), R5 (beginning seed) and at the end of the reproductive cycle, corresponding to reproductive phenological stage of fruit and seed ripening. – R7 (FEHR et al., 1971).

The plants were grown in plastic pots with capacity of 3 dm^3 of soil, with two plants per pot. The soil utilized had not previously cultivated with legumes. Because of that we decided to assure initial supply of all treatments with a small dose of ammonium nitrate (NH_4NO_3), in the dosage of 166.75 mg per pot of N, equivalent to a rate of 30 kg ha^{-1}, to supply the needs for initial development of the plants (HATFIELD et al., 1974; PIEROZAN et al., 2015; LAVRES et al., 2016). The dose of K, in the form of KCl was 150 mg dm^{-3}, applied at the time of sowing, and later as top dressing during phenological stages V1 (first leaflet appearance; FEHR et al., 1971). The P rate, in the form of CaH_2PO_4, was 300 mg dm^{-3}. The micronutrients (g pot^{-1}: 0.023 of B – as boric acid; 0.038 of Fe – as ferrous sulfate; 0.026 of Cu; 0.056 of Mn and 0.087 of Zn, as zinc sulfate) were applied the day before planting. The seeds (1 kg of cultivar 6266 RSF IPRO Flecha) were treated with a commercial aqueous product containing molybdenum and cobalt, in the rate of 0.17µliter, equivalent to a dose of 90 mL ha^{-1}. Afterward, it was performed application of Ni for each source at rate of 45 mg kg^{-1} of seeds – it corresponds to 2.5 g per hectare. We used three sources of Ni, sulfate of Ni ($NiSO_4$); hydroxide of Ni micrometric ($Ni(OH)_2$) – 24 µm of diameter; hydroxide of Ni nanometric – 5nm of diameter and absolute control. The solution had been previously fortified with 2 µL of the commercial liquid product "Semia 5019" and "Semia 5079", which contain strains of *Bradyrhizobium elkanii* e *Bradyrhizobium japonicum*, respectively. The seeds were treated early in the morning and then were sown at a depth of 2.0 cm in the pots. Firstly, sowing 10 seeds per pot, and in the stage V1 it was removed, leaving only two plants per pot. The remaining plants not used were discarded in a suitable place. No fungicide or insecticide was added at the time of seed treatment. The soil moisture was corrected during the experiment to keep it at 70% of maximum water retention capacity, by means tensiometers. Rice (*Oriza sativa*) was sowing in the same soil and same conditions in the greenhouse that was not atmospheric nitrogen fixers – to serve as reference to indicate enrichment of ^{15}N available in the soil (PEOPLES et al., 1989; BODDEY et al., 2001; BRITO et al., 2009; URQUIAGA et al., 2012; LAVRES et al., 2016).

The treatments were foliar applied when the plants reached the V4 phenological stage (with two trifoliate leaves completely expanded). The treatments consisted of foliar application of aqueous solutions/dispersion of the following Ni sources: Ni sulfate - $NiSO_4$; hydroxide of Ni micrometric - $Ni(OH)_2$; hydroxide of Ni nanometric - $Ni(OH)_2$. The dose used was based on the study by Barcelos et al. (2017), which recommends 20 g ha^{-1} of Ni. We carry one solution

to each plant to avoid loss of the nutrient at the bottom the bottle, once hydroxide is an insoluble source. Tthe solution was carefully applied to not spill any quantity on the soil, then each plant was sprayed separately, and an aluminum foil was used to cover the top of each pot before spraying.

4.2.3 Analysis of soybean plants

After the harvesting the plants in the phenological stages R2 (86 d), and R5 (98 d) to determine the activity of nitrogenase enzyme, where the plants were separated in leaves, stem, nodules and roots. The material was placed in labeled paper bags and dried in an oven at 65 °C for 72 hours and then weighted on a precision scale. The dried material was ground in a Wiley mill, passed through a sieve with mesh of 1 mm and sent to the laboratory for measurement of the concentrations of macro and micronutrients, according to the method described by Malavolta et al. (1997). The micro- and macronutrients, except for N, were determined using 0.25g of dried plant/nodule material. Samples were digested in acid solution (65% HNO_3 and 30% H_2O_2) in a digester block. Concentrations of the nutrients were determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (Spectro Cirus, Germany).

The plants harvested in the final stage (R7 [122 d]- reproductive phenological stage) were separated into: leaves, stem, grains, roots and nodules for further analysis of nutrient concentration, ureids and natural ^{15}N abundance ($\delta^{15}N$ ‰). The analysis non-destructive, as urease activity and nitrate reductase was performed in the third or fourth leaf from the tip of the main stem with petiole when plants reached the phenological stages V6 (55 d) and R2.

4.2.4 Determination of the Dualex index

Dualex is a portable meter used to estimate in real time and in a non-destructive way, the granted and combined indices of flavanols chlorophyll in the leaves (Cerovic and Masdoumier 2012). The equipment emits a beam of light of 375 nm (absorbed by the polyphenols) and a reference beam at 650 nm (red region), which penetrates the epidermis of the leaf (GOULAS; CEROVIC, 2004; CARTELAT et al., 2005).

The Dualex is a new-generation polyphenol and chlorophyll meter that measures the leaf chlorophyll index (Chl), the favanol index (Flav). Nitrogen balance index (NBI) is the ratio between Chl and Flav, corresponding to LChl corrected by dry leaf mass per unit area.

Depending on the specie, and the appropriate calibration, the N concentration in the leaf can be estimated indirectly with the equipment, due to the negative correlation between the content of phenolic compounds, and of N in the leaf, and the positive correlation with the content of chlorophyll and NBI. Therefore, Dualex has been used to estimate N concentration in the leaf in different species (TREMBLAY et al., 2010).

Non-destructive quantification of the polyphenols relative content was performed using Dualex (Force A, Orsay, France). The measures were done using the third leaf from the tip of the main stem with petiole in the phenological stages 15 days after foliar Ni application and R7.

4.2.5 Determination of N-total and BNF quantification (natural abundance of 15N)

The BNF was quantified by the technique of ^{15}N natural abundance (TRIVELIN et al., 1984; SHEARER; KOHL, 1988). This methodology is based on the fact that generally the N in the soil is slightly richer in the isotope ^{15}N in comparison with the N_2 in the air (SHEARER; KOHL, 1988; CADISCH et al., 2000). The N from the air contains about 0.3663% ^{15}N and the rest (99.6337) is ^{14}N (BODDEY et al., 2001). Due to the isotopic discrimination that occurs during the transformations of nitrogen in the soil-plant system, both may present ^{15}N values that are slightly higher than found in the atmosphere. These variations are extremely small, so that each unit of delta ^{15}N is considered to have natural abundance divided by one thousand, i.e, 0.0003663 atom% of ^{15}N in excess (SHEARER; KOHL, 1988; CADISCH et al., 2000). Plants able to obtain most of the N necessary for their nutrition will have $\delta\ ^{15}N$ values very near zero, since the largest part of the N will come from the air, which is the standard of the technique and contains 0.3663% ^{15}N, meaning zero excess units of $\delta\ ^{15}N$ (SHEARER; KOHL, 1988; CADISCH et al., 2000; BODDEY et al., 2001). Non-fixing N species (control plants) grown in the same soil will have higher $\delta\ ^{15}N$ values, near those in the soil, since all or most of the N necessary for their development will be derived from the soil. Like other isotopic techniques, this one depends on the basic premise that fixing and non-fixing plants grown in the same soil, absorb N with the same marking with ^{15}N (SHEARER; KOHL, 1988; BODDEY et al., 2001; GUIMARÃES et al., 2008). This limitation was overcome by selecting reference species with root development and N demand similar to soybean. The proportion of N in the plants that can fix N_2 from the air by the BNF process was calculated by the equation of Shearer and Kohl (1986).

$$\%BFN = 100 \times (\delta^{15}N \text{ reference} - \delta^{15}N \text{ soybean}) / (\delta^{15}N \text{ reference} - B)$$

Where,

%BFN= percentage of N obtained from BFN in the soybean plants;

$\delta^{15}N$ reference= natural abundance of ^{15}N in the reference plant (non-fixer of N);

$\delta^{15}N$ soybean= natural abundance of ^{15}N in soybean plants;

B= fraction contribution of ^{15}N in relation to ^{14}N by the soybean plants in absorbing N from the soil. In this present work, we used the average value of -1.85 ‰ based on the date obtained by Guimaraes et al. (2008). Subsamples of dried and ground material sampled were analysed for %N and $\delta^{15}N$ in automated mass spectrometer coupled to an ANCA-GSL N analyzer (Sercon Co.,UK). The total N concentrations and $^{15}N/\ ^{14}N$ isotope ratio were calculated according to the method of Barrie et al (1995).

4.2.6 Determination of urease activity

The urease activity was analyzed *in vivo* in different phenological stages V4, 15 days after Ni application and R2 by an adaptation of the method described by Hogan et al. (1983). The plant tissues were collected and placed in plastic bags and then in polystyrene foam boxes to maintain the temperature and enzyme activity low. The plant material (200 mg of green leaves, cut into "slices" with width of 1mm) were placed in a medium of 8 mL of NaH_2PO_4 buffer urea at pH 7.4 and incubated for 3 h at 30°C, protected from light in aluminium foil and kept under constant agitation. In a test tube containing 0.5 mL of the extract obtained after incubation, 2.5 mL of Reagent 1 (phenol 0.1 mol L^{-1}, sodium nitroprusside – 50 mg) and 2.5 mL of Reagent II (NaOH 0.125 mol L^{-1}, $Na_2HPO_4.12H_2O$ 0.15 mol L^{-1}, NaOCl – 3% Cl_2) were added. The tubes were closed with stoppers to avoid loss of NH_3 and placed in a water bath at 37°C for 35 min. After this interval, the reaction was measured by colorimetry in a spectrophotometer at 625 nm. The urease activity was determined by the quantity of ammonium (NH_4^+) produced, and the values were compared with a standard curve, previously determined using NH_4Cl. The results obtained were expressed in µmol NH_4^+ g^{-1} FW h^{-1}. The spectrophotometric analyses were performed with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

4.2.7 Determination of nitrate reductase

The nitrate reductase was assayed *in vivo* in different phenological stages V4, 15 days after Ni application and R2 by an adaptation of the method described by Mulder et al. (1959). The plant tissues were collected and placed in plastic bags and then in polystyrene foam boxes

to maintain the temperature and enzyme activity low. The plant material (200 mg of green leaves, cut into “slices” with width of 1mm) were placed in test tubes containing 4 ml of potassium nitrate. The buffer contains nitrate (NO_3^-) for the enzyme nitrate reductase, present in the leaf, converter NO_3^- in NO_2^-. Subsequently, the samples were incubated in a water bath at 37°C for 2 hours, protect from the light with an aluminum foil that covered the tubes, in addition, they were kept under constant agitation, every 5 minutes. After the incubation, an aliquot of 1 ml of the plant extract was removed and to stop the reactions, 1 ml of sulfanilic acid was added, waited 5 to 10 minutes, following the application of 1 ml of the alpha naphthylamine solution, stirred, then added 1 ml more of sodium acetate. The measure was performed on a spectrophotometer at 540 nm. The enzyme was determined by the amount of NO_2^- produced, which was compared with the values obtained in a standard NO_2^- curve, previously prepared with sodium nitrite. The spectrophotometric analyses were performed with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

4.2.8 Determination of nitrogenase enzyme

To determine the nitrogenase enzyme, we used a method indirect by the acetylene reduction assay (ARA) (BODDEY et al., 2001; LAVRES et al., 2016). The plants were collected in the phenological stage R2 and R6 and the roots containing nodules were carefully separated from the soil, after which the nodules were placed in hermetically sealed flasks. Subsequently, a needle and syringe were used to withdraw about 10% of the gas phase from each flask (equivalent to a volume of 1mL) and the same amount of acetylene gas, was injected in the flask. Due to the large variations of the ideal incubation time with acetylene gas described in the literature, we chose 1h, so try avoid problems of conversion of the acetylene gas into ethylene by the composition of the stoppers utilized to seal flasks. Then, 1 mL of the gas phase was withdrawn with a needle and syringe with capacity of 2.5 ml and injected in a gas chromatograph (Thermo Scientific Finnigan Trace^{TM} GC 2000 model), with two Porapack N columns (BODDEY et al., 2001). This measure the concentration of ethylene gas formed in the flaks.

The value of acetylene reduction were obtained in ppm (mg/mL) and were transformed to micromole per hours of incubation (µmol L^{-1}), following the equation bellow:

AN = (V x UCG x At x F) / (i x h x number of plants) = UCG x 0,0430443,

Where,

AN = activity of nitrogenase in mmol de C_2H_4 h^{-1} $plant^{-1}$; V = volume the flask = 50 mL; UCG = area produced; At = atenuation = 16; F calibration factor = 1.0761 x 10^{-5} mmol de C_2H_4 per UCG produced; i = volume of gas injected into the chromatograph = 1 mL; h = time of incubation = 1 h and number of plants = 2.

4.2.9 Determination of ammonia concentration

The ammonia concentration was determined in an extract containing 1.0 g of stem soybean at the end of the soybean cycle (R7) material in 10 mL of solution (60% [v/v] CH_3OH; 25% [v/v] $CHCl_3$. The extract was centrifuged at 13200 rpm for 5 minutes, and the supernatant was collected. The ammonia concentration was quantified according to the method (McCULLOUGH, 1967). For this, a 150 µL aliquot of the extract was added to 2.0 mL of a colorimetric solution. This solution was prepared using a 1:1 ratio of phenol reagent (2.5 g C_6H_5OH and 12.5 $Na_2Fe(CN)_5NO$ in 250 mL) to phosphate reagent (1.25 g NaOH, 13.4 g NaH_2PO_4, and 2.5 mL 5% NaClO in 250 mL). Samples were incubated at 37°C for 1 h. Ammonia concentration was then determined by colorimetry (color intensity) at 630 nm absorbance. The results are expressed a µmol g^{-1} FW.

4.2.10 Determination of nitrate

The nitrate was determined in soybean stems at the end of the soybean cycle (R7) by method described by (CATALDO et al., 1975). An aliquot of 0.1 mL was removed from the water-soluble phase extract of MCW, and 0.4 ml of 5% salicylic acid in sulfuric acid (w/v) was added. After 20 minutes at room temperature , 9.5 mL of 2 N NaOH was slowly added. After cooling to room temperature, spectrophotometer measures were obtained at 410 nm absorbance. The concentration of nitrate was determined using a standard curve of sodium nitrate solution. The results are expressed a µmol g^{-1} FW.

4.2.11 Determination of ureides

The ureides was determined in soybean stems at the end of the soybean cycle (R7) by the total of allantoin + allantoic acid in soybean stems, the method of Vogels and Van Der Drift (1970) was used. The analyze involves in two phases. First phase, 250 µL of MCW extract supernatant, 250 µL of 0.5 M NaOH, and 1 drop of phenylhydrazine were heated in an oven at 100°C for 8 minutes and then cooled to room temperature. In this step (alkaline hydrolysis),

allantoin is hydrolyzed to allantoic acid. The phase two, 250 µL of of 0.65 N HCl was added and heated again at 100℃ for 4 minutes (acid hydrolysis); this is when the hydrolysis of allantoic acid to glyoxylate occurs. The assay was cooled to room temperature, and then 250 µL of 0.4 m phosphate buffer pH 7.0 and 250 µL 0.33% phenylhydrazine solution were added. After 5 minutes at room temperature, the assay was incubated in an ice bath for 5 minutes. Next, 1.25 mL of previously frozen concentrated HCl and 250 µL of 1.65% potassium ferrocyanide solution were added. The tubes were removed from the ice bath and homogenized in a vortex. After 15 minutes at room temperature, measures obtained using a spectrophotometer at 535 nm absorbance. The concentration of ureides was calculated based on the standard curve of allantoin solution. The results are expressed as µmol g^{-1} FW.

4.2.12 Dry weight and grains productivity

In all harvests at the phenological stages R2, R6, and R7 the plants were harvested and separated in: leaves, stem, roots and nodules. The material was placed in labeled paper bags and dried in an oven at 65 ℃ for 72 hours and then weighted on a precision scale to obtain the dry weight. To determine grain production, after the drying, the grains were weighted on a precision scale.

4.2.13 Number of nodules and nodular mass

In each treatment in the different phenological stages the nodules were detached to determine the total nodules, and then these nodules were dried in an oven at 65 ℃ for 72 hours, and then weighted on a precision scale to obtain the dry weight.

4.2.14 Statistical analysis

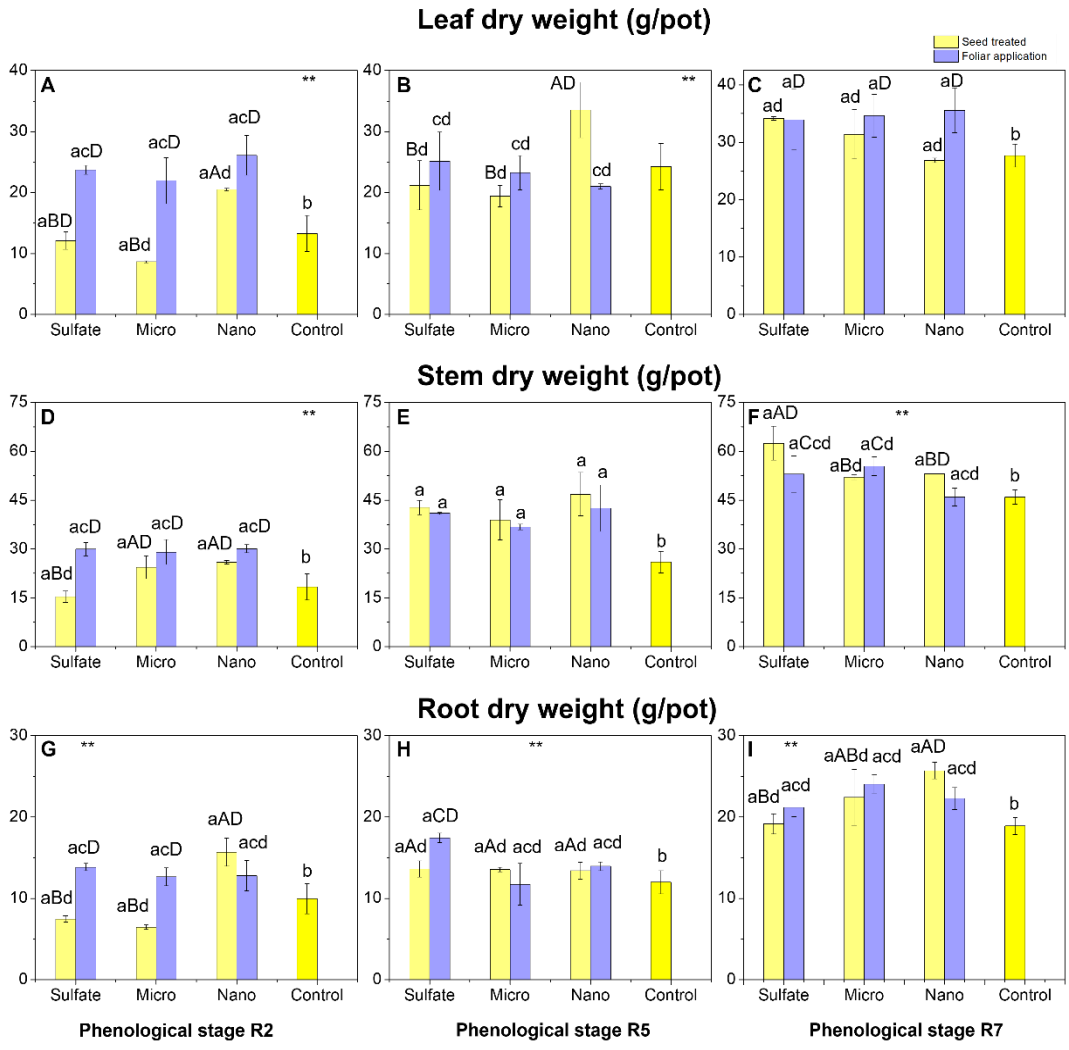
Data analysis was performed with R Development Core team (version 4.0.2) (2015). The data were submitted to analysis of variance by the F-test. When the effect was significant, we applied the Tukey test to compare the effects. The control treatment was used as an additional treatment to execute the statistical analysis. In all analysis, the level of significance was considered to be 5%.

4.3 Results

4.3.1 Development of soybean plants

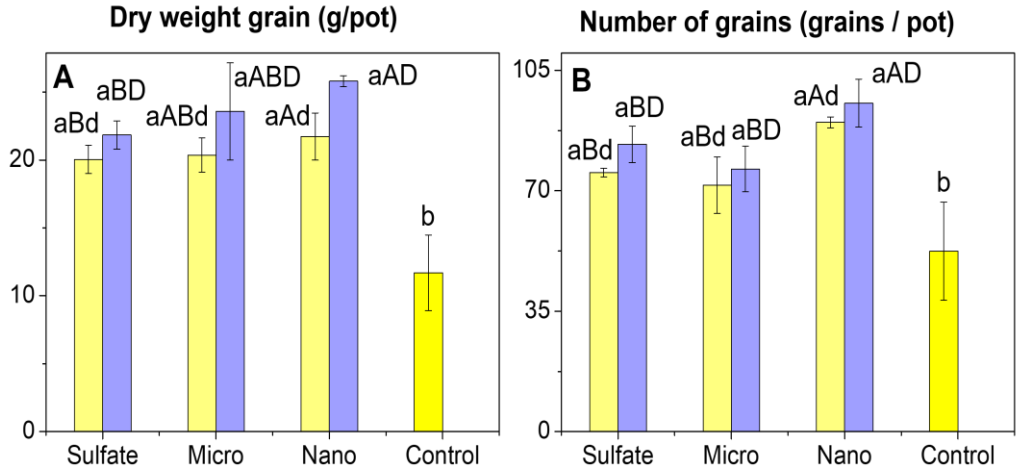
We detected significant interactions between nickel (Ni) sources and the different applications of Ni: Ni-treated seeds and Ni-foliar application (Ni sources x Ni application). The application of Ni-NPs positively affected the dry weight of leaves, stem and roots of soybean (Figure 1). We observed that in the phenogical stage R2 (FEHR et al., 1971) the foliar-applied Ni presented a boost in plant growth compared to the treatments that received Ni only by seed (c.a. of 31%), increasing the dry weight by 73% (leaves), 33% (roots), and 35% (stem), compared to the plants that did not receive foliar Ni application. Particularly, the application of Ni-NPs in seeds (without Ni foliar application) also lead to an increase in the dry weight of soybean tissues (leaves, stem and roots). Ni-NPs applied only in seeds showed an increase of dry weight of leaves by 69% and 37%, compared to the Ni sulfate and Ni micrometric, respectively. The roots and stem showed a similar pattern, with the Ni-NPs treatment presented an augment by 2.10-fold and 2.43-fold compared to the Ni sulfate and Ni micro, respectively, in the roots and 1.7- fold in the stem compared to the Ni sulfate. The control treatment presented less development compared to soybean plants that received Ni. We also observed that in the phenogical stage R5 and R7 the statistical difference between Ni-NPs, Ni micrometric, and Ni sulfate was smaller compared to the phenogical stage R2, even for the plants that received Ni either by foliar application or seed.

Figure 1 - Dry weight of soybean tissues: leaf, stem and roots. Plants were growth with different Ni sources ($Ni(SO)_4$, $Ni(OH)_2$ micrometric and $Ni(OH)_2$ nanometric and forms of Ni application (via seed and foliar) at the phenogical stages R2, R5 and R7 (A, B, C, D, E, F, G, H, and I). Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B** compares Ni sources when there was no interaction or compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source



At the phenogical reproductive stage of fruit and seed ripening (R7; FEHR et al., 1971), we also collected and recorded the grains yield and then we observed that the foliar Ni application, regardless of the type of Ni sources, increased the dry weight and the number of the grains of soybeans (Figure 2). Particularly, the Ni-NPs-based treatment exhibit higher results of dry weight and number of grains than the Ni sulfate and Ni micrometric treatment. Particularly, the foliar application with Ni-NPs increased 1.18-fold compared to the plants that received Ni by seeds. The additional treatment (control) had a massive difference of the dry weight among the treatments that received Ni, a decrease of 54% (Ni-NPs), 50% (Ni micrometric), and 42% (Ni sulfate) compared to the plants that received Ni via foliar. The number of grains followed the same trend.

Figure 2 - Effect of different Ni sources ($Ni(SO)_4$, $Ni(OH)_2$ micrometric and $Ni(OH)_2$ nanometric and forms of Ni application (via seed and foliar) on dry weight and number of grains in soybean at phenogical stage R7. Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B** compares Ni sources when there was no interaction; letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source.



4.3.2 Nickel assimilation

Nickel content in soybean tissues increased in both treatments: treated seeds and foliar-applied Ni compared to the control (Table 1). In leaves at the phenological stage R2, the sulfate treatment had an increase of Ni concentration by 1.72-fold and 2.26-fold in the seed treated and Ni foliar application, respectively, compared to the control. Whereas, Ni-NPs showed an augment of Ni concentration by 39% and 54%, respectively, in seed treated and Ni foliar application, compared to the control. Noticeably, similar trends were found in the nodules, by which seed dressing with sulfate increased Ni-nodule content by 1.28-fold in plants at the phenological stage R2, and 1.63-fold in plants that received Ni supplementary foliar application, compared to the control. While in plants at the phenological stage R5, sulfate-seeds dressing increased Ni-nodules content by 86% in comparison with plants nodules from the control treatment. In the phenogical stage R7 the plants nodules of all treatments that received Ni in seeds showed a decrease in Ni content compared to the control, as well as the roots in the seed dressing treatments with Ni-NPs in the stage R7, whose Ni concentration was lower in relation to the control, with values of 8.08 mg kg $^{-1}$ of Ni in the treatment and 15.06 mg kg $^{-1}$ in the control treatment. On the other hand, the other soybean tissues (stem, leaves, and root) in all phenological stages maintained the Ni content higher than the control treatment. Mainly the plants that received Ni by foliar application.

Table 1 - Nickel concentration in soybean plant tissues and Ni concentrations in grains (mg kg^{-1})

R2					
		Ni concentration (mg kg^{-1})			
Ni source	Application	**Plant tissues**			
		Root	**Nodule**	**Leaf**	**Stem**
Sulfate	**Seed**	7.97aBd	25.23aAD	4.16aAd	1.98aABd
	Foliar	15.90acD	32.02aAd	5.45aCD	1.79acd
Micro	**Seed**	20.62aAd	19.11aBD	4.60aAD	2.48aAD
	Foliar	18.67acd	21.67aBd	2.49afd	1.61acd
Nano	**Seed**	14.84aEd	18.59aBD	3.35aBd	1.86aBd
	Foliar	17.30acd	22.66aBd	3.73acd	1.77acd
Control	-	11.69b	19.61b	2.41b	0.59b
Application x Source		**	-	**	**
CV(%)		11.28	11.75	8.34	14.21

R5					
Ni source	Application	Ni concentration (mg kg^{-1}) Plant tissues			
		Root	**Nodule**	**Leaf**	**Stem**
Sulfate	**Seed**	11.21aAd	18.96aAD	2.17aBd	1.09aBd
	Foliar	14.28acD	14.89acd	3.28aCD	1.57aCD
Micro	**Seed**	10.16aABd	15.41aBd	2.93aAd	0.90aBd
	Foliar	15.46acD	19.27acD	2.66acd	1.09acd
Nano	**Seed**	8.93aBd	18.33aAd	1.33aEd	1.94aAD
	Foliar	17.24aCD	21.96aCD	2.59acD	1.49aCd
Control	-	12.65a	10.18b	1.30b	1.00b
Application x Source		**	**	**	**
CV(%)		6.44	5.64	11.43	8.85

R7						
Ni source	Application	Ni concentration (mg kg^{-1}) Plant tissues				
		Root	**Nodule**	**Leaf**	**Stem**	**Grain**
Sulfate	**Seed**	18.11aAD	21.32aAd	1.27aBd	0.80aBd	4.80aAd
	Foliar	15.76acd	16.80acd	1.65acD	1.46aCD	4.63acd
Micro	**Seed**	18.94aAd	23.70aAd	2.18aAD	1.13aAd	4.95aAd
	Foliar	21.69aCD	30.15aCd	2.26aCD	0.98acd	6.10aCD
Nano	**Seed**	8.08aBd	25.61aAd	1.33aBd	0.82aBd	3.81aBd
	Foliar	23.10aCD	31.30aCD	2.51aCD	0.89acd	5.22aCcD
Control		15.06b	28.47a	1.07b	0.51b	2.76b
Application x Source		**	**	**	**	**
CV(%)		7.02	14.55	6.91	13.08	11.16

*Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B-E** compares Ni sources when there was no interaction; letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source.*

We also determined the Ni concentrations in the grains at the phenological stage R7. All treatments showed an increase of Ni content compared to the control (additional treatment). The most remarkable result was observed in the foliar Ni application of micro and nano Ni

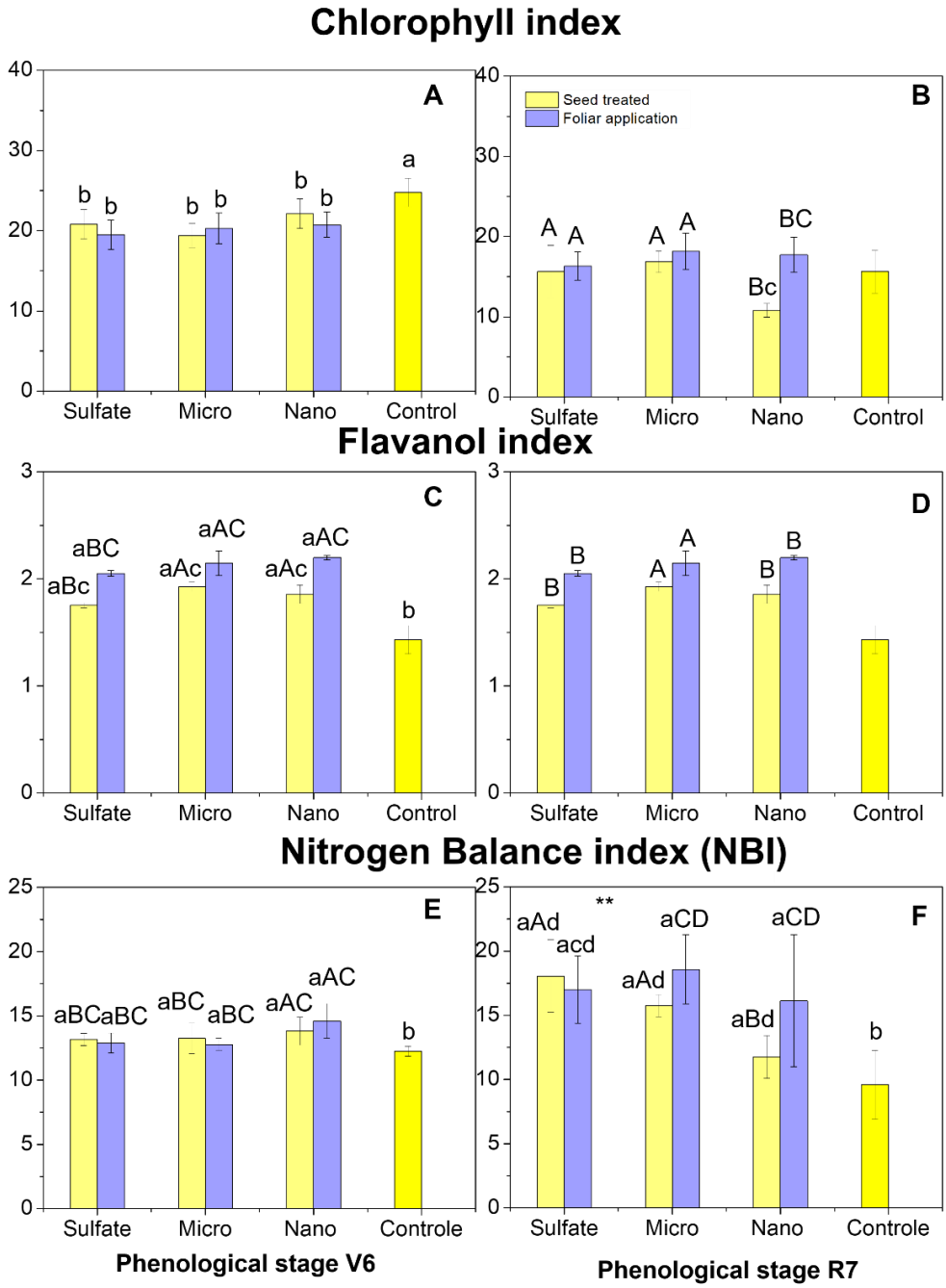
hydroxide sources, in which had an augment of Ni concentration by 2.21- fold and 1.89-fold related to the control, respectively. While Ni sulfate treatments, it was not observed differences between Ni-seed treatment and foliar–applied Ni for Ni content in the grains. However, treated seeds with Ni-NPs presented the lowest Ni content regarding to the other sources (Ni sulfate and micro), around 3.81 mg kg^{-1} of Ni concentration, while the other treatments had an average of 4.95 mg kg^{-1} of Ni micrometric and 4.80 mg kg^{-1} of Ni sulfate (Table 1). Plants from the control treatment, without any Ni application, had Ni concentration of 2.76 mg kg^{-1}.

4.3.3 Physiological parameters

The reading measurements by Dualex® were performed after 15 days of foliar application and at the phenological stage R7. We observed that the parameter Chlorophyll index (Chl) after 15 days of Ni foliar application showed no statistical difference (Figure 3A) among the treatments that received Ni either by seed or by leaf. However, plants leaf form the control treatment showed a higher Chl index compared to other treatments (Ni sulfate, micro, and nano). Additionally, there was statistical difference between Ni sulfate and Ni micrometric in relation to Ni-NPs treatment (Figure 3B), having an increase of 1.11-fold, and 1.22-fold in the Chl index, respectively. The impact of foliar Ni application in the Chl index was observed only in plants at the stage R7 (Figure 3B) in the Ni-NPs treatment, in which it showed a 64% increase compared to the Ni-NPs applied to the seeds. The Flavanol index measured 15 days after of Ni foliar application showed positive effect as compared to the plants that had Ni supplied by the seed dressing (Figure 3C). The Ni supply via leaf by Ni sulfate, Ni micrometric, and Ni-NPs increased by 1.17, 1.11, and 1.18-fold, respectively, in relation to plants that received Ni only via seed. At stage R7, only Ni micrometric treatment showed better results compared to the Ni sulfate and Ni-NPs treatments.

The nitrogen balance index (NBI) revealed significant interactions between the Ni source and the different applications of Ni: treated seeds and foliar-applied Ni at the stage R7. The Ni-NPs applied via leaf had an increase of 1.37- fold compared to the treatment that received Ni only via seed for NBI. Also, we observed that foliar Ni application had an effect on the NBI index, unlike at the stage 15 days after foliar application, which showed no difference between the form of Ni application (Figure 3E), only statistical difference among Ni sources, where Ni-NPs and Ni micrometric had a higher NBI index compared to the Ni sulfate treatment.

Figure 3 - Effect of different Ni sources ($Ni(SO)_4$, $Ni(OH)_2$ micrometric and $Ni(OH)_2$ nanometric and forms of Ni application (via seed and foliar) on physiological parameters: Chlorophyll, Flavanol and Nitrogen balance index (NBI) of soybean leaves 15 days after foliar Ni application and at stage phenogical R7. Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B** compares Ni sources when there was no interaction or compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source



4.3.4 Urease activity and nitrate reductase

The urease activity and nitrate reductase (NR) were evaluated *in vivo* twice: 15 days after Ni-foliar application (at 55 d - V6 after germination) and at the phenological stage R2 (86 d). The urease activity (Figure 4A; B) was increased by Ni supply either by seed or by leaves. Therefore, as expected, the control treatment had the lowest urease activity. Noticeably, regardless of the Ni sources, the foliar Ni application revealed a boost on the urease activity in both periods. However, 15 days after foliar Ni application, the urease activity was higher than that observed at phenological stage R2. The Ni-foliar application increased urease activity by 40, 58 and 15% in plants grown in the treatments with Ni sulfate, Ni micrometric and Ni-NPs, respectively, compared to plants from the treatment that only received Ni by seed (Figure 4A). Predominantly, the plants supplied with Ni sulfate and Ni micrometric treatments showed an improved urease activity than soybean that received Ni-NPs treatment, right after 15 days before applying Ni to leaves. Conversely, urease activity in the leaves taken at the R2 phenological stage on plants subjected to the Ni-NPs treatment showed higher values compared to plants treated with Ni sulfate and Ni hydroxide micrometric, showing an augment of 1.16-fold compared to the Ni-seed sulfate treatment.

The NR activity (Figure 4C; D) on plants at the first stage evaluated on 15th day after foliar Ni application – corresponding to phenological stage of V4) showed no significant difference among the forms of Ni supply, although only effect to Ni sources. Soybean plants under Ni hydroxide micrometric treatment, regardless of the forms of Ni supply, presented the highest NR activity followed by Ni sulfate followed by Ni-NPs. The treatment based on Ni-NPs decreased the NR activity in the leaves by 40 and 52%, respectively, compared to the Ni sulfate and Ni micrometric treatment, 15 days after Ni application on the leaves. Otherwise, at the phenological stage R2 the plants treated with Ni-NPs, mainly those which received Ni via foliar, had the highest NR activity compared to the other treatments, on average of 3.5 and 1.16-fold in relation to the Ni sulfate and Ni micrometric treatments, respectively.

In addition, it is possible to see on Figure 5 the difference among treatments after four (4) days of Ni foliar application, which corresponding to phenological stage of V4 in relation to the plants that received Ni only via seed. We observed that the foliar Ni application improved the plant growth, characterized by the greener color of the leaves and also with more biomass (Figure 5).

Figure 4 - Effect of different Ni sources ($Ni(SO)_4$, $Ni(OH)_2$ micrometric and $Ni(OH)_2$ nanometric and forms of Ni application (via seed and foliar) on urease and nitrate reductase activity in soybean leaves 15 days after foliar Ni application and at stage phenogical R2. Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a**-**b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B-E** compares Ni sources when there was no interaction; letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source

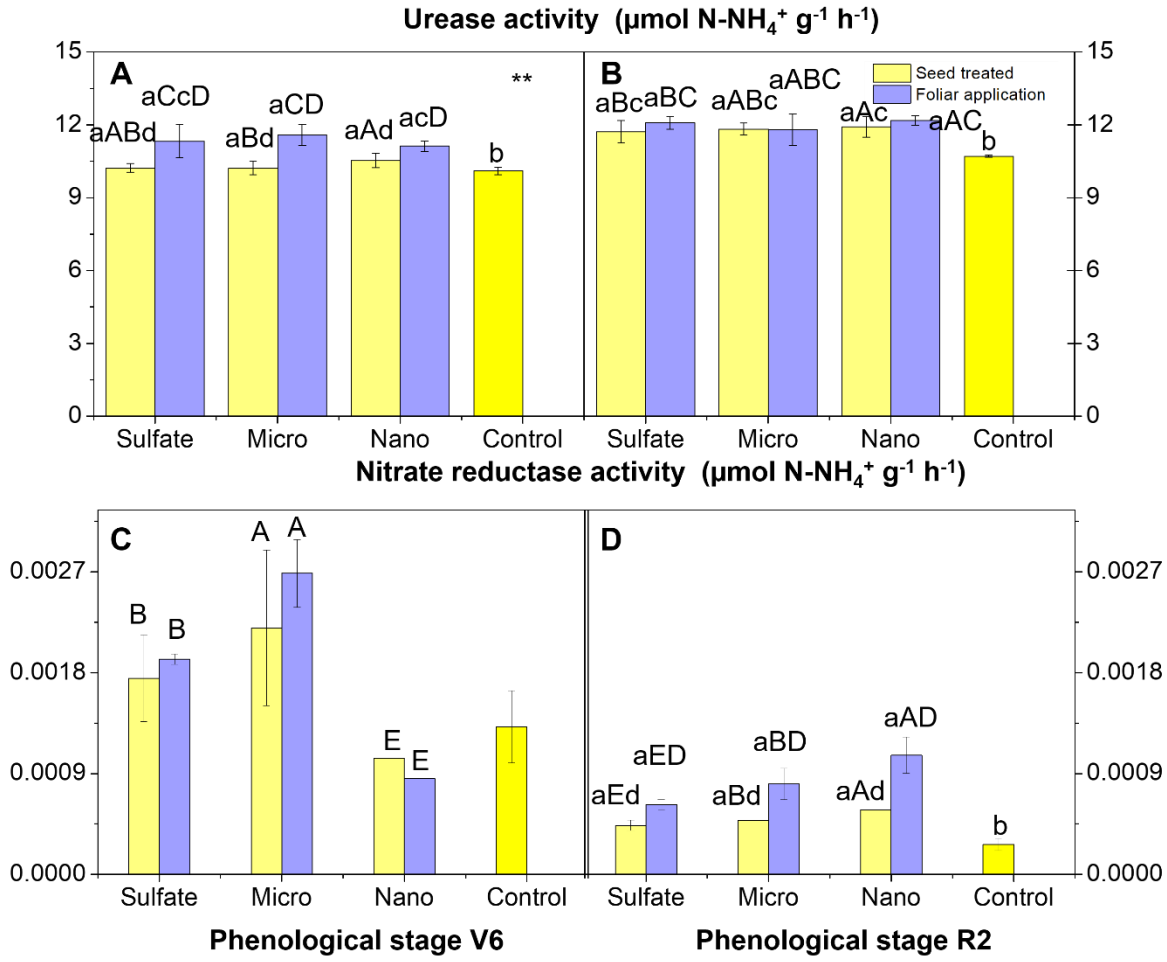
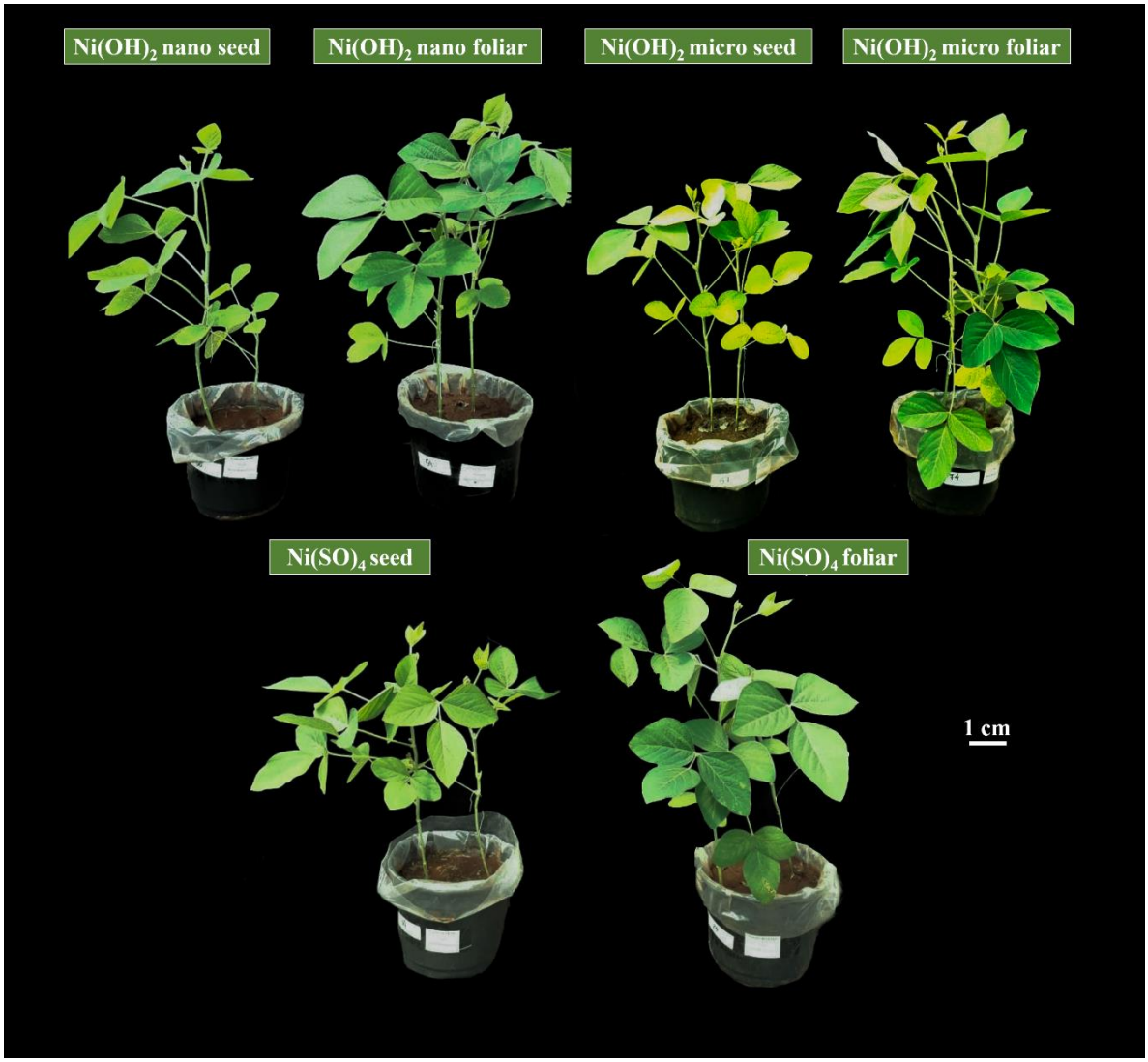


Figure 5 - Visual aspects of soybean growth at vegetative phenogical stage 4 days after foliar Ni application (phenological stage of V6). Legend: seed – plants growth treated with Ni via seed; foliar - plants growth treated with Ni via foliar



4.3.5 Biological nitrogen fixation changes caused by Ni application

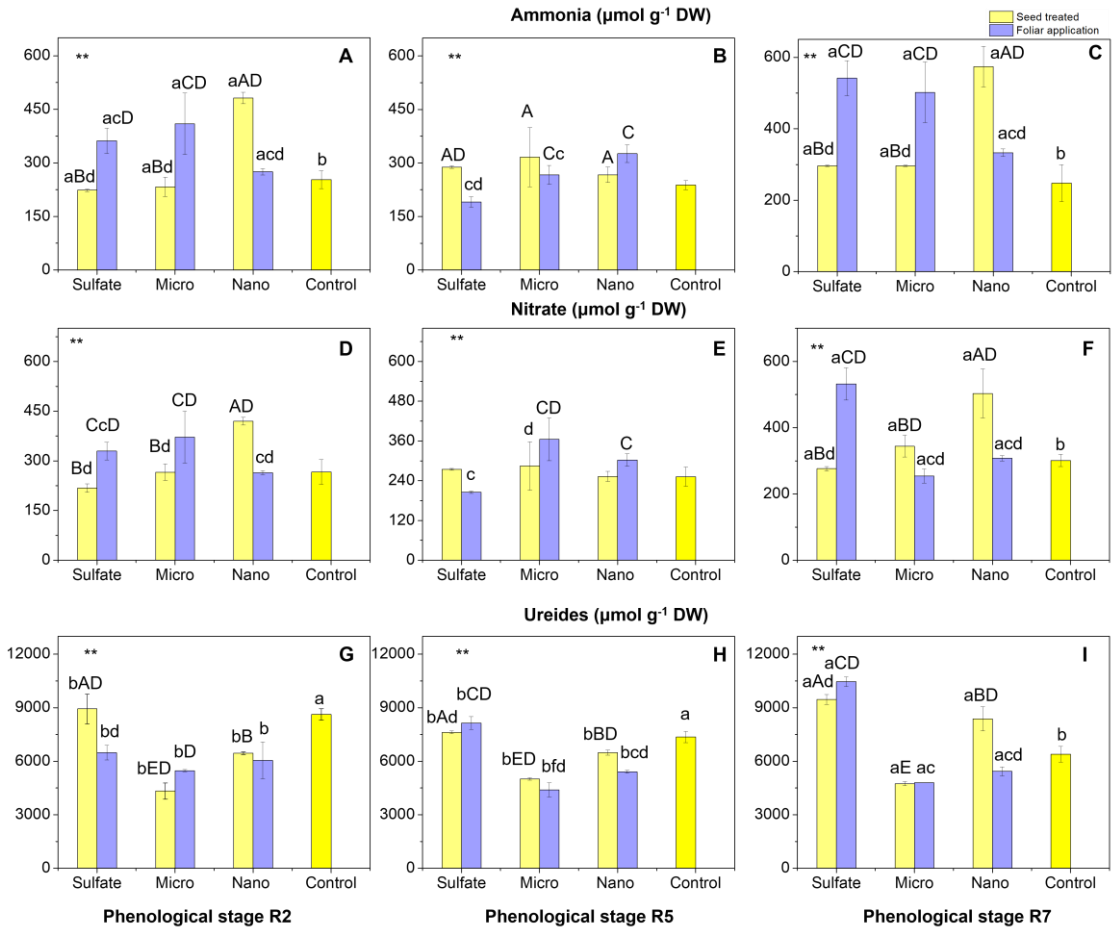
The results obtained for ammonia concentration, a product of the urease activity, in stem indicated a beneficial effect of Ni supply (Figure 6A-C). This effect was more pronounced at the R2 phenological stage in plants that received foliar Ni application, except to the Ni-NPs, whose Ni foliar application showed a decline in ammonia concentration, as well as urease and NR activities. Foliar application of Ni-NPs reduced ammonia content by 32% compared to the plant supplied with Ni by seed (Ni seed treatment), which had an average of 409.2 μmol g^{-1} DW of ammonia concentration (Figure 6A). However, the Ni-NPs applied via foliar only have positive effects at the stage R5, which showed highest concentration of ammonia compared to

Ni sulfate and Ni micrometric treatments applied by leaf, corresponding to an increase of 1.7- fold for the Ni sulfate and 1.22-fold for Ni micrometric. Foliar Ni fertilization also improved ammonia content on Ni sulfate and Ni micrometric in the phenological stage R7, except for the treatment of foliar Ni-NPs application, which showed a lower ammonia content compared to plant supplied by Ni-NPs per seed, which had a higher ammonia content .

Likewise, the nitrate concentration (Figure 6D-F) also had a similar trend, which the concentration of nitrate in the leaves was higher by applying foliar Ni, except for the application of Ni-NPs. The results obtained from nitrate concentration showed a value of 218 (Ni Sulfate), 266 (Ni micrometric), and 420 μmol g^{-1} DW (Ni-NPs) in plants that were Ni-supplied via seed at the phenological stageR2; and values of 330 (Ni sulfate), 371 (Ni micrometric), and 264 μmol g^{-1} DW (Ni-NPs) in plants supplied with Ni via foliar application. In the phenological stage R5 nitrate concentration in plants tissue were 275 (Ni sulfate), 285 (Ni micrometric), and 253 μmol g^{-1} DW (Ni-NPs) in plants that were supplied Ni via seed, whereas 205 (Ni sulfate), 365 (Ni micrometric), and 302 μmol g^{-1} DW (Ni-NPs) in plants supplied Ni via foliar. In addition, at the phenological stage R7 nitrate concentrations were 276 (Ni sulfate), 344 (Ni micrometric) and 503 (Ni-NPs) μmol g^{-1} DW in plants supplied with Ni via seed, and 532 (Ni sulfate), 254 (Ni micrometric), and 308 (Ni-NPs) μmol g^{-1} DW in plants that were Ni-supplied via leaves.

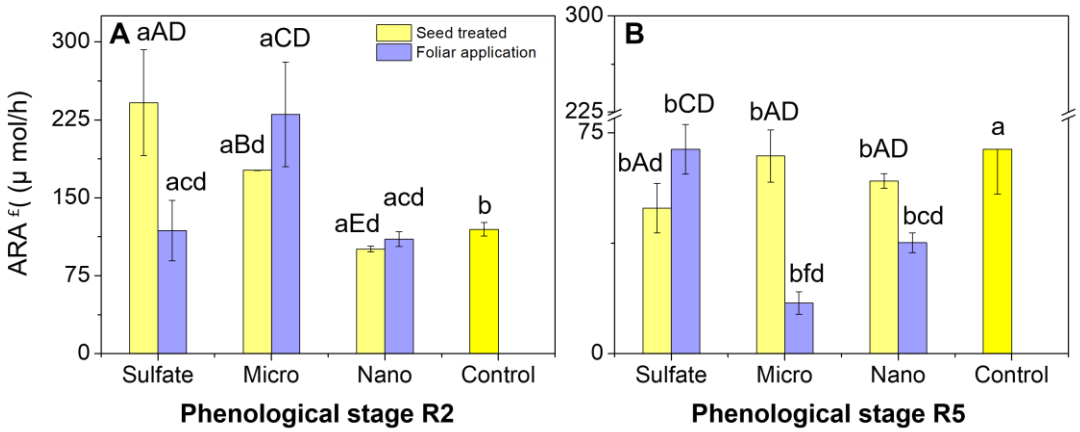
The concentration of total ureides (allantoin + allantoic acid) was upregulated by Ni sources and forms of Ni application (Figure 6G-I). However, the control treatment (without any Ni application) showed a higher concentration of ureides. However, at the last phenological stage (R7), the plants that received Ni presented better results than the control treatment. In general, foliar Ni application did not increase the ureides concentration, like the plants treated by Ni only via seed. The ureides concentration at the phenological stage R7 showed value of 7623 (Ni sulfate), 5006 (Ni micrometric), 6481 (Ni-NPs) and 7358 (control) μmol g^{-1} DW in plants that received Ni by seed, whereas values of 8144 (Ni sulfate), 4397 (Ni micrometric) and 5408 μmol g^{-1} DW (Ni-NPs) in plants that received supplementary Ni foliar-application.

Figure 6 - Effect of different Ni sources ($Ni(SO)_4$, $Ni(OH)_2$ micrometric and $Ni(OH)_2$ nanometric and forms of Ni application (via seed and foliar) on ammonia, nitrate and ureides concentration in soybean stems at phenogical stage R2, R5 and R7. Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B-E-F** compares Ni sources when there was no interaction; Letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source



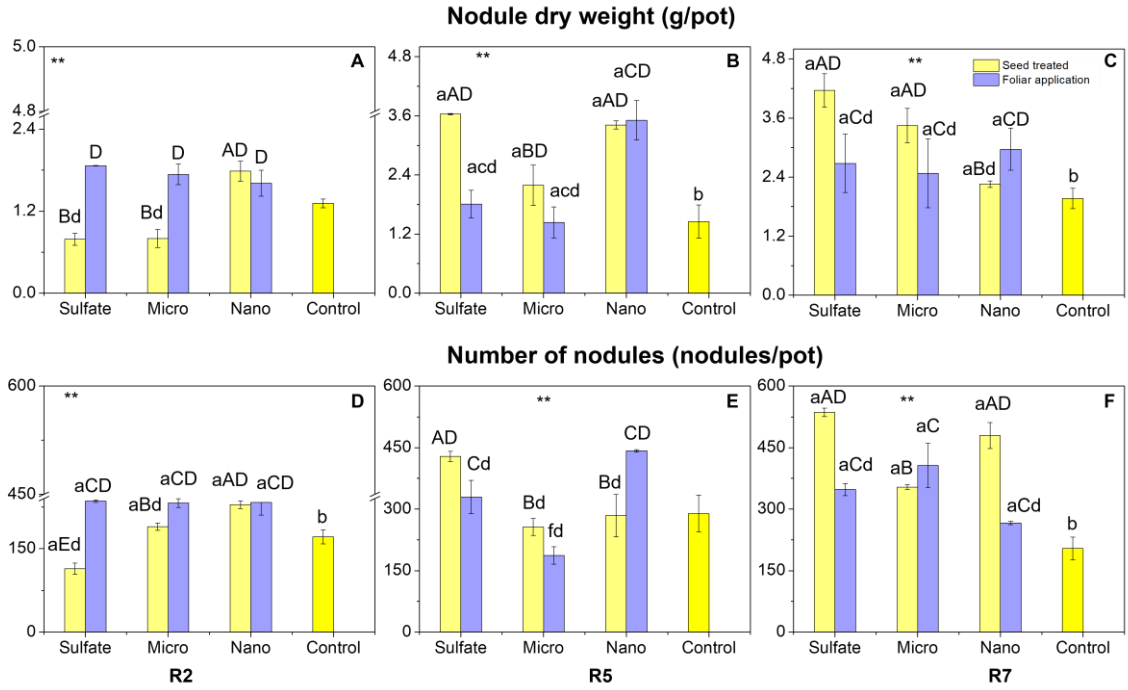
The maximum activity of nitrogenase (Figure 7), expressed on reducing acetylene (ARA), was recorded in plants treated with Ni sulfate via seed at the phenological stage R2, which had increased 2.01- fold its enzyme activity compared to plants from control treatment, followed by Ni micrometric foliar application with an increase of 1.92-fold in relation to the control. The Ni-NPs application in seeds decreased the nitrogenase activity of ca. of 16% and 7% relative to the control and Ni-NPs applied via leaf, respectively. In general, it was observed a remarkable reduction (ca. of 69 %) in ARA activity at the R5 phenological stage relative to R2 phenological stage.

Figure 7 - Effect of different Ni sources ($Ni(SO)_4$, $Ni(OH)_2$ micrometric and $Ni(OH)_2$ nanometric and forms of Ni application (via seed and foliar) on nitrogenase activity (by reduction acetylene) at stage phenogical R2 and R5. Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B-E** compares Ni sources when there was no interaction; Letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C and f** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source



Other parameters used to estimate biological N_2 fixation were the number and dry weight of nodules (Figure 8). The plants from Ni-NPs seeds treatment showed an augment of number of nodules of 2.0-fold compared to Ni sulfate treatment, 1.20-fold compared to Ni micrometric treatment and 1.33-fold compared to control treatment, at R2 phenological stage, whereas the dry weight of nodules also increased by Ni-NPs seeds treatment. Plants that received supplementary Ni application via foliar had the maximum response for both number and dry weight of nodules per plant at phenological stage R2 in plants treated with Ni sulfate (Figure 8A; D). For both number and dry weight of nodules increased by augmenting Ni supply to plant via leaf spray. However, at the phenological stage R5, Ni-NPs applied via foliar had a remarkable increase compared to application of Ni micrometric and sulfate, an average of 442 nodules per plant and 3.5 g dry weight of nodules. While, plant treated with Ni sulfate achieved 329 nodules per plant and 1.81 g of dry weight of nodules, respectively (Figure 8B; E). Whereas, at phenological stage R7 plants treated with Ni sulfate, regardless of the source or form of Ni application, had the best responses; ca of 20% higher than plants treated with Ni micrometric, Ni-NPs and control treatment in relation to number and dry weight of nodules (Figure 8C; F).

Figure 8 - Effect of different Ni sources ($Ni(SO)_4$, $Ni(OH)_2$ micrometric and $Ni(OH)_2$ nanometric and forms of Ni application (via seed and foliar) on dry weight and number of nodules in soybean at phenogical stage R2, R5 and R7. Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B** compares Ni sources when there was no interaction; letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C and f** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source



The BNF evaluated by the technique of natural abundance of $^{15}N‰$ (Table 2;3), by using the abundance of δ^{15} N‰ in the aerial parts and grains of soybean at R2 (86 d), R5 (98 d) and R7 (122 d) reproductive stage, as well as in the non-fixing plants (used as controls) showed values with respective δ^{15} N‰ for *Oriza sativa* of 3.5‰. This specie (non-fixers of N) was grown in the same soil and climate conditions as the soybean plants. In the aerial part of soybean plants there were significant difference and interaction between the factors. The values of δ^{15} N‰ ranged of -1.82 (Ni Sulfate) to -2.03 (Ni micrometric) in plants with Ni supply via foliar and plants treated with Ni-NPs via foliar had the value of -0.92. While, the BNF varied from 75.79 (Ni sulfate) to 100 % (Ni micrometric) at R2 phenological stage and Ni-NPs via foliar had 93.43% of BNF. Notably, these results indicated that the most of N accumulated in the aerial parts of soybean come from BNF from foliar Ni fertilization.

Whereas, plants from the control treatment showed a BFN ca. of 88.10%. At the R5 phenogical stage there were not significant difference among the treatments, only to the additional treatments (control), which had less BNF% in relation to the treatments that received Ni. The evaluation of BFN% at R7 phenological stage following the same trend of the period R5, which the additional treatment (control) showed less BNF (ca of 72%), whereas the all treatments that received Ni had 100% of BNF, i.e., N accumulated in the aerial parts come from BNF. The analysis of total nitrogen concentration in plants tissue showed the higher results at R2 phenological stage R2 in plants that had supplied with Ni-NPs in seeds, while at phenological stage R5 the greater results was in plants applied with Ni sulfate via foliar, an average of 15% higher than Ni micrometric and NI-NPs treatment. Foliar application of Ni had positive effect in the concentration of N-total, determined at phenological stage R7, an increase of 15% compared to plant that received Ni by seed. Clearly, fertilization of Ni regardless of the sources and Ni forms application improved the δ^{15} N‰, BNF and total nitrogen in the aerial parts of soybean. The analysis of the natural abundance of δ^{15} N‰ in the grains indicated that ca of 83% of the N accumulated in the grains came from BNF, while in the control treatment the N fixation rate was only 69% (Table 3).

Table 2 - Natural abundance of δ ^{15}N‰, estimation of BNF (%), and N-total (g kg^{-1}) in the soybean plant (aerial parts) under Ni application by seed and foliar at stage phenogical R2, R5 and R7.

		R2		
Ni source	Application	δ15N‰	BNF (%)	N-total (g kg^{-1})
			Aerial parts	
Sulfate	**Seed**	-0.48 aAd	75.79 aBc	39.88 aA
	Foliar	-1.82 acD	99.48 aBC	41.74 ac
Micro	**Seed**	-1.67 aBd	100.00 aAc	38.30 aA
	Foliar	-2.03 acD	100.00 aAC	33.24 aCc
Nano	**Seed**	-0.62 aAd	74.35 aBc	44.77 aAd
	Foliar	-0.92 aCD	93.43 aBC	34.58 acd
Control	-	-1.08 a	88.10 a	38.02 a
Application x Source		*	ns	*
CV(%)		20.85	6.86	7.32

R5				
Ni source	Application	δ15N‰	BNF (%)	N-total
			Aerial parts	
Sulfate	**Seed**	-1.58307 a	95.01 a	37.59
	Foliar	-1.75979 a	98.31 a	38.11 C
Micro	**Seed**	-1.62102 a	95.75 a	34.85 D
	Foliar	-1.89826 a	100.00 a	26.77 cd
Nano	**Seed**	-1.49076 a	97.15 a	30.75
	Foliar	-1.86217 a	100.00 a	36.95 C
Control	-	-0.59 b	76.61 b	3.21
Application x Source		ns	ns	*
CV(%)		16.32	6.44	7.95
R7				
Ni source	Application	δ15N‰	BNF (%)	N-total
			Aerial parts	
Sulfate	**Seed**	-2.35 a	100.00 a	29.02 ac
	Foliar	-1.94 a	100.00 a	33.22 aC
Micro	**Seed**	-2.16 a	100.00 a	28.72 ac
	Foliar	-2.23 a	100.00 a	28.23 aC
Nano	**Seed**	-2.12 a	100.00 a	27.75 ac
	Foliar	-2.07 a	100.00 a	32.41 aC
Control	-	-0.35 b	71.99 b	26.10 b
Application x Source		ns	ns	ns
CV(%)		9.4	5.5	5.3

*Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application); ns means not-significant. Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B-E** compares Ni sources when there was no interaction; letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source.*

Table 3 - Natural abundance of δ ^{15}N‰, estimation of BNF (%), and N-total (%) in the soybean plant (grain) under Ni application by seed and foliar at phenological stage R7

Ni source	Application	δ15N‰	BNF (%)	N-total
			Grains	
Sulfate	**Seed**	-0.31	81.54 ABd	48.61 aBd
	Foliar	-0.56	86.92 aCd	56.77 aCD
Micro	**Seed**	-0.20	79.13 ABd	55.10 aA
	Foliar	-0.61	88.05 aCD	53.97 aCc
Nano	**Seed**	-0.76	91.12 aAD	52.68 aA
	Foliar	-0.07	76.53 acd	52.57 ac
Control	-	0.25 b	69.54 b	45.30 b
Application x Source		*	*	*
CV(%)		56.79	4.72	2.42

*Different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** means that there was interaction between the factors (Ni source x Ni application). Letters lowercase (**a-b**) compares additional treatment (control) among the treatments that received Ni; letter uppercase **A-B-E** compares Ni sources when there was no interaction; letter **A-B** compares Ni source in relation to Ni application only by seed (when there was interaction). Letter **C** (lower and uppercase) compares the forms of Ni application when there was no interaction or if there was interaction compares Ni sources in relation to foliar Ni application. Letter **D** (lower and uppercase) compares forms of Ni application in the same source.*

4.4 Discussion

Few early reports as related to augmented efficiency of biologic nitrogen fixation (BNF) in legumes, including soybean plants, by supplying of cobalt (Co), molybdenum (Mo), nickel (Ni) and *Bradyrhizobium* strains seed treatment were published (HUNGRIA et al., 2005; 2006; CAMPO et al., 2009; LAVRES et al., 2016). Even though there are many studies describing to the importance of plants nutritional status of nitrogen (N), sulfur (S), iron (Fe), Co, and Mo supply affecting symbiotic nitrogen fixation, the actual evidences on either soybean seed treatment or foliar supplementary application with nickel (Ni), notably for Ni-sources including nano fertilizers, and its effect on BNF are still scarce. Therefore, the purpose of the present research was to demonstrate that Ni supply to soybean plants by seed treatment and foliar

applied Ni can increase the symbiotic nitrogen fixation effectiveness, directly by the ^{15}N natural abundance method ($\delta^{15}N‰$), evaluation of nodulation, ureides compounds, $N\text{-}NH_3$, $N\text{-}NO_3^-$, concentration of N in the grains and leaves, and by measurement of urease and nitrate reductase activities, as well as indirectly by nitrogenase (N-ase) activity.

Seed treatment (ST) includes the technique of applying materials to coat the seed surface and is thus called seed coating (SC). SC can be performed through deposition of nutrients, aiming to provide suitable conditions for plant nutrition, especially in situations in which the availability of nutrients in the soil is low or when they are present in unavailable forms to plants (FAROOQ et al., 2012; ULLAH et al., 2020). Foliar fertilization is an essential tool to increase efficiency of productive management of crops (FERNÁNDEZ et al., 2013). The decision to spray foliar fertilizers are determined by the magnitude of the financial risk associated with the failure to correct a deficiency of a nutrient and the perceived likelihood of the efficacy of the foliar fertilizer. Additionally to foliar fertilization, nanotechnology in agriculture is also a promising area (SUBRAMANIAN et al., 2015; DE LA ROSA et al., 2017; TRIPATHI et al., 2017; ZUVERZA-MENA et al., 2017).

The current study was conducted to assess how interaction between seed treated with Ni and foliar Ni supplementary application, specifically application of nanoparticles, could alter the growth of soybean plants. Although few studies have addressed the effect of oxide Ni nanoparticles (NiO-NPs) in barley, wheat and tomato (FAISAL et al., 2013; SOARES et al., 2016; SALEH et al., 2019), for the first time we investigated the effects of Ni nanoparticles ($Ni(OH)_2$) on physiological and biochemical responses of biological nitrogen fixation as well as of nitrogen metabolism in soybean plants.

Nickel is an essential element for plants; ordinarily, Ni is taken up by roots and then translocated to aerial parts (stem and leaves) in minimal quantities, not exceeding few micrograms per gram of dry mass in non-accumulators plants (YUSUF et al., 2011). Particularly, Ni is translocated along the plant conducting system (xylem and phloem), moving to the areas of highest metabolic activity, where it becomes concentrated (MISHRA; KAR, 1974). Soybean tissues: roots and nodules, showed the greatest Ni concentrations when compared with other organs (as leaves and stem), being 2.8 and 4.12-fold higher on average than other tissues, respectively at R2 phenological stage; 3.35 and 4.72-fold higher, respectively at R5 phenological stage , and 6.11, and 8.61-fold higher, respectively at R7 phenological stage R7.

Therefore, applied more Ni at the correct phenological stage phenological of the plant is known to increase the uptake and translocation of Ni in plants (PANDEY; GOPAL, 2010;

REIS et al., 2017).In the present study, specifically, foliar application of Ni caused increases in Ni concentration in both root and nodules (Table 1), corresponding to an augment of 16% and 17%, respectively, compared to plants that received Ni only by seed treatment at R2 phenological stage, and an increase of 35%, and 6%, respectively, at R5 stage phenological stage, and an increase by 25%, and 9%, respectively, at R7 phenological stage. This results suggest that Ni is preferentially accumulated in root tissues, a mechanism whereby plants act to tolerate Ni toxicity by limiting its root-to-shoot translocation to the photosynthetic organs (SOARES et al., 2018). Nickel uptake occurs both via passive diffusion and active transport, hence, this phenomenon explain higher concentrations of this micronutrient in underground organs (YUSUF et al., 2011). Higher concentration of Ni in root and nodule organs has also been verified in other studies (SEREGIN; KOZHEVNIKOVA, 2006; FREITAS et al., 2019). The fact of Ni is a structural component of hydrogenase enzyme can also elucidate the accumulation of Ni in the nodular tissues (KLUCAS et al., 1983; DALTON et al., 1985; STULTS et al., 1986; URETA et al., 2005; YUSUF et al., 2011). Particularly, roots and nodule of plants that received Ni-NPs foliar supplementary application revealed more Ni accumulation than those plants from seed Ni-treatment. Plants subjected to Ni-NPs application via seed had a decline of Ni content in nodules through phenological soybean development compared to Ni sulfate, by which differently of Ni-NPs applied via foliar treatment, in which plants increased Ni concentration in the tissues phenological during plants development.

For the plants shoot (stem and leaf), foliar application of Ni also increases Ni accumulation compared to the plants that only received Ni by seed treatment. Notably, plants treated with Ni-NPs not improved Ni concentration in relation to other sources. In line with the present result, similar study shows that root of barley grown under NiO-NPs had accumulated much higher Ni than shoot did (SALEH et al., 2019). Another work was also recorded for wheat and other plants treated with bulk Ni that NiO-NPs showed much higher Ni accumulation in roots and nodules (PANDEY; GOPAL, 2010; SOARES et al., 2016; URUÇ PARLAK, 2016; REIS et al., 2017). However, Ni concentration in leaves improved through phenological soybeans development, at the R2 phenological stage plants under Ni-NPs application, regardless of the form of Ni fertilization (by seed or leaves), revealed less Ni concentration compared to other Ni sources, mainly plants treated with Ni sulfate. In soybean leaves treated with Ni sulfate via foliar, Ni concentration had an average of 5.45 mg kg^{-1}, whereas, leaves with Ni-NPs via foliar had only an average of Ni concentration of 3.73 mg kg^{-1}. While, at the R7 phenological stage, soybean leaves with Ni-NPs showed increased Ni concentration in comparison to plants treated with Ni sulfate via foliar. This increase observed

during time course of the experiment – through plants development stages R2, R5 and R7 - after Ni-NPs application may be justify since nano-fertilizer foliar sprays have demonstrated to be suitable for field use because they can deliver the nutrient to plant progressively and in a more controlled manner than salt fertilizers (SUBRAMANIAN et al., 2015; KAH et al., 2018).

The foliar application of Ni proved to be a highly efficient alternative to enrich the concentration of this element also in the grains. The Ni-NPs and Ni micrometric applied in soybean plants via foliar spray had a greatest result in content of Ni in the grains (Table 1). Similar study was reported to soybean grains, in which obtained Ni concentrations of 0.04-8.32 mg kg^{-1} (KUTMAN et al., 2013) when Ni was sprayed on the leaves.

Higher Ni concentration in nodules indicates a higher requirement for this micronutrient by bacteria responsible to N_2 atmospheric fixation (WELCH, 1981). Then, in some legumes, mainly in soybean, Ni is essential for root nodule growth (SENGAR et al., 2008) and ideal effective of hydrogenase enzyme (YUSUF et al., 2011). The efficiency of BNF depends essentially on hydrogenase activity, which is responsible to the recycling of H_2, product formed by the process of N_2 reduction to ammonia by nitrogenase complex.

Forty percent of protein make up the dry weight of soybean plant, resulting in a high demand for N, and the majority, of this is supplied by BNF (ALVES et al., 2003; HUNGRIA et al., 2006; LUCA et al., 2014). This study suggests that foliar Ni application stimulated BNF and the effect was most pronounced in the aerial parts, it could be noticed that the highest BNF was achieved on plants that received Ni-NPs by foliar supplementary application revealed by the ^{15}N natural abundance method ($\delta\ ^{15}N‰$).

The higher % BFN was more pronounced at the R5 and R7 stage phenological, since the bacterium-plant symbiosis is better recognized, then the plants obtain most of N needed from BNF, since the delta ^{15}N values ($\delta^{15}N$ ‰) tend to approach zero, indicating that the N taken up and assimilated by plants comes from atmospheric air (GUIMARÃES et al., 2008; URQUIAGA et al., 2012; LAVRES et al., 2016). In this case, Ni employs a significant part in the BFN process and the foliar application of Ni increased the BNF, making a significant contribution to the supply of N.

The nitrogenase activity were measured by the technique of ARA (activity of reducing acetylene) in samples of nodules. In this present study, we observed a higher nitrogenase activity at the R2 stage phenological, once some studies confirmed that the nodulation peack is at phenological stage R2 (CAMARA, 2014). The supplying of Ni either by leaf or seed also was very responsive to increase of the nitrogenase activity. Lavres et al. (2016) also had similar results in soybean plants after application of Ni in seed treated with 0.45 mg kg^{-1} of Ni.

Remarkably, at the R2 phenological stage (FEHR et al., 1971) there were variable results, in which plants treated with Ni sulfate by seed improved the nitrogenase activity in comparison to plants that received Ni by leaf, whereas, Ni micrometric applied via foliar showed better results compared to plants that received Ni via seed and Ni-NPs had the lowest result compared to other sources. While, at the R5 phenological stage the results was opposite, foliar Ni sulfate application improved the nitrogenase activity and plants that received Ni micrometric and Ni nanometric by seed showed better result. This results may be explained in part due to the fact that nutritional requirement changes with the development of the plant and also by the fact of the difference in size and solubility of the particles from Ni sources, which have different rates of solubilization and delivery of Ni to plants. Yet, there is still little information on how the size and solubility of particles may impact plant growth.

Overall, the foliar fertilization of Ni, regardless of Ni source, enhanced the BNF in the soybean plants and the nitrogen-metabolism, improving the accumulation of N in the grains (ca. of 4%) and shoot (ca. of 9%). However, these results did not impact on indirect photosynthesis parameter: cholophyll, flavanol, and nitrogen balance index (Dualex) take on the leaves (Figure 3). Higher dose of nickel, which can cause toxicity symptoms, has been reported to inhibit the synthesis of photosynthetic pigments, replace the Mg ion chlorophyll molecule, induce damage in the thylakoid membrane, restrain electron transport chain, and inhibit the activity of ribulose-1.5-biphosphate carboxylase/oxygenase (rubisco), and other Calvin cycle enzymes (YUSUF et al., 2011; SRIVASTAVA et al., 2012; SOARES et al., 2016). We observed that Ni foliar fertilization inhibit the chlorophyll index, once the control treatment (without any Ni application) had better results than plants whose received Ni. Conversely, foliar Ni fertilization increases the NBI (nitrogen balance index), which is correlated to results regarding nitrogen metabolism in plants, such as nitrogenase and urease activities, N-content in the grains, as well as nitrogen isotope composition ($\delta^{15}N$ ‰) in the leaves and in the grains.

Other studies was reported in soybean and barley plants that Ni boosted the N concentration of the growing part of the shoot by up to 30% and 50% respectively (KUTMAN et al., 2013; KUMAR et al., 2018) . Nickel stimulates an increase of urease and nitrate reductase activity, promoting improved of N metabolism, and enhancing the remobilization of Ni from the old to new leaves (KUTMAN et al., 2013). Since, Ni is a constituent of urease, which is the enzyme responsible for the breakdown of urea into ammonia and carbon dioxide (DIXON et al., 1975), and therefore, responsible for the recycling of nitrogen in the plant (POLACCO et al., 2013; FABIANO et al., 2015). These results in this work suggests that foliar Ni fertilization increased urease activity ca. of 27% compared the plants that had supplied of Ni only by seed

treatment, regardless of the Ni source (Figure 4). To note, plant supplied with sources of Ni-NPs and Ni micrometric showed better results than Ni sulfate regardless of the type of Ni application. This may be explained due to their increased ability to penetrate the leaf than conventional Ni salts (ROSSI et al., 2019). The nitrate reductase activity also was improved by foliar Ni fertilization and specifically, Ni-NPs had the best results at the R2 stage phenological, probably due to that the peak of nodulation observed in this plants developmental stage (CÂMARA, 2014). Casarin (2019) also observed an augmenting in NR activity at the R2 phenological stage of soybean plants sprayed with ^{15}N-urea.

The importance of Ni as a micronutrient also ranges to the following steps in N assimilation. We observed that nodules showed higher ureides synthesis at stage phenological R7 when soybean were fertilized with Ni sulfate via foliar, by measuring its accumulation in the leaves (Figure 6). Utilization of ureide by soybean plants is directly involved with the breakdown of urea (TODD et al., 2006; WITTE, 2011; OHYAMA et al., 2017), because Ni fertilization leads to a higher synthesis of urease (POLACCO et al., 2013), and so may rise the quantity of ammonia in leaves (BAI et al., 2006; 2007). Additionally, soybean was studied confirming that urea and ammonia might be direct products of ureides degradation in urease pathway (TODD et al., 2006; POLACCO, 2014).

As far as we know, this is the first work that applied Ni based on nanoparticles ($Ni(OH)_2$) in soybean plants and also the first that showed the combination of Ni application by seed and leaves. Ni-NPs, in general, positively influenced soybean growth and physiology and demonstrated more favorable effects than conventional Ni salts ($Ni(SO)_4$), mainly via foliar application. Moreover, despite the high leaf Ni level measured in the treated plants at the end of the experiment, no toxicity effects were observed. These values are below the levels considered toxic to plants, which are >10 mg kg^{-1} in sensitive species, > 50 mg kg^{-1} in moderate tolerant species and >1000 mg kg^{-1} in Ni hyperaccumulator plants (SEREGIN; KOZHEVNIKOVA, 2006; CHEN et al., 2009; YUSUF et al., 2011). Rossi (2018) evaluating the effects of foliar application of zinc sulfate and zinc nanoparticles in coffee (*Coffee Arabica* L.) also had more positive impact on coffee growth to Zn-NP than Zn sulfate. Whereas, studying the effect of Nickel oxide nanoparticles in wheat, reduced whole plant growth, inhibited photosynthesis and increased the levels of antioxidants. Another study NiO-nanoparticels in tomato also exhibit potential to induce cell death, degenerated mitochondrial cristae and abundance in peroxisomes (FAISAL et al., 2013).

4.5 Conclusion

This study provides the first report regarding the effect of Ni-NPs fertilization by seed and leaves in soybean plants on growth, photosynthesis, and biological nitrogen fixation by ^{15}N abundance natural nitrogen ($\delta N^{15}‰$), ureides content, nitrogenase, urease and reductase nitrate activity. In general, foliar Ni fertilization, regardless of the Ni sources, led to significant improvements in several parameters in plants, as number and dry weight of nodules, roots, leaves, and shoot, BNF by ^{15}N abundance natural nitrogen ($\delta N^{15}‰$), Ni content and enzymes activity. Seed treatment with Ni also enhanced physiological and biochemical factors in soybean plants compared to the control (without any Ni application). Particularly, Ni-NPs positively influenced soybean growth, physiology and fixation of nitrogen and demonstrated more favorable effects than conventional Ni salts, mostly due to their increased ability to penetrate the leaf. Nanoparticle fertilizer represents a novel and efficient method of nutrient delivery to improve plant performance, which is of great importance for achieving more sustainable crop systems around the globe. Therefore, foliar fertilization with Ni-NPs in soybean plants represents a practical and effective strategy to ensure a better Ni nutrition.

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5 Synchrotron µXRF analysis of foliar-applied nickel in hydrated soybean leaves: shining light on *in situ* nickel leaf absorption and translocation

Abstract

Nickel is a component of the enzymes urease and hydrogenase. Nickel deficiency can occur in soybean grown on leached tropical soils that are low in nickel. The deficiency can be remedied by seed dressing or foliar application of nickel salts. It is important that any application of nickel results in safe levels of this element in soybean products for human consumption given the potential toxicity. In this study, soybean seeds with different nickel concentrations were produced in hydroponics. Nickel-depleted (<0.35 µg Ni per g) and nickel-sufficient (11.14 µg Ni g^{-1}) seeds were used in a nutrient solution experiment, where the plants were grown with or without nickel supply (0.85 µM Ni^{2+}) and with or without the cotyledons excised. The results were assessed to determine the effect of seed Ni concentration on urease activity and to establish whether an internal or external supply of Ni can compensate for low nickel within the seed. The results show that leaf nickel concentrations, shoot biomass and urease activity were augmented by increasing either internal (from cotyledon seed store) of external (in solution) nickel supply. Synchrotron micro-X-ray fluorescence analysis was used to examine the distribution and translocation of foliar-applied nickel in the soybean leaves. Within 15 min of application, nickel accumulated in the pedicles of the trichomes, then moved to the vascular bundles before dispersing further into tissues within 3 hours. Trichomes are hence an important pathway for foliar Ni absorption in soybean, but there are still major knowledge gaps in the physiological function of trichomes in taking up metal ions from foliar treatments.

Keywords: foliar nutrition, leaf ion absorption, nickel deficiency, soybean.

5.1 Introduction

Nickel (Ni) is a cofactor of the urease enzyme which activates two metalloenzymes that are directly involved in nitrogen metabolism (EC 3.5.1.5, urea amidohydrolase). Urease is responsible for the hydrolysis of urea and ureides, releasing two molecules of ammonia and one of carbon dioxide (DIXON et al., 1975; ESKEW et al., 1983; WITTE, 2011; POLACCO et al., 2013; OHYAMA et al., 2017; TODD et al., 2006). The first evidence for the essentiality of Ni in plants was reported in soybean (Glycine max [L.] Merril) in 1983 when grown in nutrient solutions in which urea was used as the N source. These plants accumulated toxic concentrations of urea in their leaf tips, but Ni addition prevented toxicity (ESKEW, 1983; SHIMADO; ANDO, 1980).

Nickel deficiency causes necrosis at the tip of the leaves due to disruption of ureide catabolism and affects other pathways such as amino acid metabolism and the citric acid cycle (BAI et al., 2006). Severe Ni deficiency has been reported in pecan (*Carya illinoinensis*

(Wangenh.) K. Koch), where symptoms of “mouse-ear” disease that can eventually kill the trees were reported (WOOD et al., 2004; 2006). Nickel deficiency has also been reported in River Birch (*Betula nigra* L.) when grown in low-Ni potting media (RUTER, 2005). Furthermore, Ni deficiency has been reported in other crop species when grown on low Ni soils, such as cowpea (*Vigna unguiculata* L. Walp), barley (*Hordeum vulgare*) and sunflower (*Helianthus annuus*) (BROWN et al., 1987; CHECKAI; NORVELL, 1992; GERENDÁS et al., 1997). Foliar application of FeEDTA to pecan induced severe Ni deficiency for trees growing on low Ni soils; at the pH of cytoplasm, Ni displaces Fe from FeEDTA, greatly reducing the availability of Ni for leaves for plant use (WOOD, 2013).

Soybean (*Glycine max* [L.] Merril) is one of the most important agricultural crops worldwide, being a major source of cattle feed, products for human consumption and vegetable oil (FAO, 2017). Soybean is often cultivated on leached tropical soils (especially in Brazil) low in Ni and with strong Ni absorption, with hidden Ni deficiency potentially occurring. Under such circumstances, plants do not reach their maximum growth potential despite a lack of visible deficiency symptoms (DABKOWSKA-NASKRET et al., 2014; FREITAS et al., 2019; JAWORSKA et al., 2013; LICHT et al., 2006; MORRISON et al., 2009; ROCA et al., 2008; RODAK et al., 2015). In soybean plants with insufficient Ni, urease activity decreases resulting in the accumulation of urea-related N compounds, causing toxicity and reduced plant growth (ESKEW et al., 1983; BROWN et al., 1990; KROGMEIER et al., 1991). Remediating Ni deficiency in soybean can be achieved by the application of Ni, either on the seeds or by foliar application (HOSSEINI; KHOSHGOFTARMANESH, 2013; LAVRES et al., 2016) or by soil Ni fertilization (MACEDO et al., 2016; 2020). Of particular interest in the present study is the foliar application of Ni, which has been shown to not only increase foliar Ni concentrations, but also to increase the foliar urease activity (OJEDA-BARRIOS et al., 2016). In addition, foliar application of Ni can prevent toxic symptoms caused by urea accumulation in leaf tissue, and glyphosate drift symptoms (ESKEW et al., 1984; KUTMAN et al., 2013; KUTMAN et al., 2014). Despite the importance of foliar fertilisation, including for Ni, there remains much uncertainty as to the underlying processes by which foliar-applied nutrients move across the leaf surface into the underlying plant tissue (FERNANDEZ et al., 2017; LI et al., 2017). Our lack of understanding regarding the physiological processes involved in the absorption of foliar-applied nutrients hinders efforts to increase the effectiveness of foliar micronutrient fertilizers (FERNANDEZ et al., 2013).

Synchrotron-based micro-X-ray fluorescence (μ-XRF) analysis is a powerful technique that allows for in situ analyses of the distribution of a wide-range of different elements in plants (KOPITTKE et al., 2018; VAN DER ENT et al., 2018). There are still only a limited number of studies that have examined changes in elemental distribution over time in living plants using X-ray elemental techniques at synchrotron facilities, with these having focussed on the toxic effects of Mn in soybean, cowpea and sunflower (BLAMEY et al., 2018; DOOLETTE et al., 2018). These previous experiments examining the foliar application of Zn fertilizers in sunflower have shown that trichomes were particularly important for foliar Zn absorption, with Zn preferentially accumulating within trichomes in ≤15 min, whereas the stomatal pathway did not appear to be important (LI et al., 2019).

The aim of the present study was to investigate the distribution and translocation of foliar-applied Ni in soybean plants. We first grew soybean to maturity in Ni-purified hydroponics culture to produce Ni-depleted seeds. Next, we then examined the lateral distribution of foliar applied Ni (as $NiSO_4$ solution) in situ within hydrated intact soybean leaves using synchrotron-based μ-XRF analysis. Finally, a hydroponics dosing experiment was undertaken to study the effect of seed Ni store (using Ni-sufficient and Ni-deficient seeds) on urease activity and biomass production and to establish whether the internal supply (by excising cotyledons) or external supply (Ni treatment in solution) of Ni could compensate for a low concentration of Ni within the seed.

5.2 Materials and Methods

5.2.1 Overall experimental design

In total, three different experiments and were undertaken. The first hydroponics experiment generated the Ni-depleted seed stock. The second experiment used plants grown from Ni-sufficient seeds to examine Ni distribution within plant tissues following foliar application of Ni fertilizers. The third experiment examined the effect of seed Ni concentration, seed Ni internal supply and external supply of Ni on biomass, tissue elemental concentrations and urease activity. This third experiment used the original Ni-sufficient seeds (11.14 μg Ni per g) and Ni-depleted seed stock (<0.35 μg Ni per g) to grow plants which were then dosed with or without Ni at 0.85 μM in solution and on which cotyledons were either kept attached or excised.

5.2.2 Experiment 1: Hydroponics culture to generate Ni-depleted seeds

Soybean plants were grown in nutrient solution at The University of Queensland (St Lucia, Australia) from October 2019 to February 2020. Nickel-sufficient seeds (*Glycine max* (L.) Merr. cv Bunya – containing 11.14 µg Ni per g) were germinated on a moistened fine perlite/vermiculite mixture at 26°C. After one week, corresponding to vegetative phenological stage of cotyledons, the seedlings were transferred to four 30 L containers containing nutrient solution. The lids of the containers had four holes in which the seedlings were inserted in foam baskets, with one seedling per hole. The cotyledons were excised upon transfer in order to remove the Ni-store in the seed.

The nutrient solution was continuously aerated and kept at room temperature (22°C) with a relative air humidity of 60–70 RH%, with light provided using high-intensity full-spectrum LED lighting (Black Dog LED, PhytoMAX-2 400, 365–750 nm, photon flux density of 1500 µmol m^{-2} s^{-1}) for 12 h d^{-1}. The nutrient solution was composed of: K (2.5 mM as KNO3), Ca (2.5 mM as $Ca(NO3)_2$), P (1 mM as K2HPO4), Mg (2 mM as MgSO4), Cl (1 µM as KCl), B (2 µM as H3BO3), Mn (1 µM as MnSO4), Zn (2 µM ZnSO4), Cu (3 µM CuSO4), Mo (4 µM as Na2MoO4). The Fe-source was Fe-diethylenetriaminepentaacetic acid (FeDTPA, 40 µM Fe) as earlier tests with Fe-hydroxybenzyl ethylenediamine (FeHBED) showed that Fe uptake was limited, even at 100 µM Fe. Nutrient solutions were fully changed twice a week and the nutrient solution pH was maintained at 5.8 by daily additions of 0.01 M KOH or 0.01 M HNO_3 as required. In order to achieve Ni-limiting conditions in the nutrient solutions, high-purity (AR trace grade) chemical reagents were used, and the solutions were purified as follows: the macro-element concentrate was purified using Chelex 100 resin (Bio Rad iminodiacetate type resin) to remove polyvalent metal ions ($Fe2/3^{+}$, Mn_2^{+}, Ni_2^{+}, Cu_2^{+}, Zn_2^{+}). The micro-nutrient concentrate was purified using a Ni-specific resin (Eichrom Nickel Resin based on dimethylglyoxime) to selectively remove Ni_2^{+} ions. Measured concentrations of Ni in the nutrient solution were <0.001 mg L^{-1} as determined using inductively coupled plasma atomic emission spectroscopy analysis (ICP-AES). The plants were grown to complete physiological maturity after 110 d (corresponding to reproductive phenological stage of fruit and seed ripening; R7 - FEHR et al., 1971). The Ni-depleted seeds were harvested when plants senesced completely, with these Ni-depleted seeds found to contain <0.35 µg Ni per g, compared to the value of 11.14 µg Ni per g for the original Ni-sufficient seeds.

5.2.3 Scanning Electron Microscopy (SEM)

Plant specimens at the R1 stage (42 d) from Exp #1 were freeze-dried for SEM analysis by rapid freezing against a solid metal block cooled by liquid nitrogen (196°C). The cooled block with samples was then loaded into the freeze-drying machine (Thermoline), vacuum-pumped and set to -85°C. Freeze-drying then progressed slowly (by changing the set temperature with 5°C increments) over the course of three days until room temperature was reached. The specimens were then sputter-coated with carbon (~25 nm) and mounted on stubs. The samples were imaged using a Hitachi SU3500 instrument. Imaging was performed at 100–1000× magnification at 4–5 kV with secondary electron (SE) returns.

5.2.4 Experiment 2: Synchrotron-based µ-XRF analysis

This experiment aimed to examine the distribution of Ni within the plant tissue after foliar Ni application treatments. Plants were grown from (original) Ni-sufficient seeds in Ni-purified nutrient solution, as described for Exp #1, until they reached 14-days old – equivalent to phenological stage V3. The plants were then transported to the Australian Synchrotron (Clayton, Australia) where they continued to be grown under the same conditions as at The University of Queensland. The Ni foliar fertilizers were prepared at two concentrations (50 and 100 mg L^{-1} Ni), from NiSO4.6H2O. The pH values of the solutions were not adjusted, being pH 5.4 for the 50 mg L-1 treatment and pH 5.1 for the 100 mg L^{-1} treatment. The solution also contained 0.5 µL of Silwet® L-77 (trisiloxane-based adjuvant) per 10 mL of solution as a non-ionic surfactant/wetting agent. We first performed a time series experiment to investigate the Ni absorption process using intact, hydrated young leaves (14 d old) for which $NiSO_4$ (aq) foliar application was used. Specifically, we investigated several foliar application times: 0 (control), 45 min and 3 h for 50 mg L^{-1} Ni, and 0 (control), 15 min, 25 min, 45 min and 3 h for 100 mg L^{-1} Ni. For each of these treatments, two 5-µL droplets of the appropriate $NiSO_4$ solution were applied to the adaxial surface of the soybean leaves. The whole leaf (still attached to the plant) was then placed inside a closed Petri dish (the dish had a hole in the side wall to allow the petiole to pass through) with moistened filter paper in the bottom of the dish to maintain humidity. The temperature within the Petri dish was 30°C and the relative humidity increased rapidly to >98% RH. The LED lighting was switched on during the incubation period and the droplets remained as a liquid (i.e. did not dry out) during the experimental period. After incubation for the appropriate length of time, the leaves were excised and rinsed using tap water (Figure Suppl. 1 -Appendix). For each specimen, the rinsed leaf was carefully blotted

dry and mounted on a sample holder between two stretched layers of Ultralene film (4 μm thick each). Samples were then immediately analysed using μ-XRF at the X-ray fluorescence microscopy (XFM) beamline of the Australian Synchrotron.

The XFM beamline, with its 384-element Maia detector system in a backscatter geometry, was used to collect X-ray fluorescence emitted by the specimen (PATERSON et al., 2011). An incident energy of 12.9 keV with a total photon flux of approximately 1.5 × 109 photons s-1 was used. First a 'survey scan' was used to obtain a comparatively rapid map of the leaf from which the area for a detailed scan could be identified. After the survey scan, an area entirely underneath where the Ni droplet had been applied was selected and a detailed scan was conducted. The detailed scans were typically approx. 4 × 5 mm in size, used a step size of 10 μm, had a dwell of 5 ms, and took approx. 75 min to complete. The possibility of radiation-induced damage in μ-XRF analysis (especially in fresh hydrated samples) is an important consideration and radiation dose limits for μ-XRF analysis in hydrated plant tissues dose-limits are 4.1 kGy before damage occurs (JONES et al., 2020). The synchrotron μ-XRF event stream was analysed using the Dynamic Analysis method (RYAN; JAMIESON, 1993; RYAN, 2000) as implemented in GeoPIXE (RYAN et al., 1990; 1995).

5.2.5 Experiment 3: Nickel dosing to plants grown from Ni-depleted and Ni-sufficient seeds

We used two types of seeds: (i) Ni-depleted seeds produced as described earlier; and (ii) the original Ni-sufficient seeds that were used at the starting material. The experiment consisted of a total of eight treatments, with two types of seeds (Ni-depleted and Ni-sufficient seeds), two solution Ni concentrations (0 and 0.85 μM Ni, supplied as $NiSO_4.6H_2O$), and two cotyledon treatments (cotyledons attached, or cotyledons removed after germination). This experiment aimed to examine the effects of Ni dosing on plants grown from either Ni-sufficient or Ni-depleted seeds and the effect of cotyledon Ni-store removal. Each treatment condition (two types of Ni seed stock × cotyledon attached or removed × 0 or 0.85 μM Ni) had four biological replicates. Free metal ion activities (-log10 values) were determined using GEOCHEM-EZ software of the full nutrient solution formulation in the 0.85 μM Ni treatment and the $pNi_2^+ = 11.4$. The two types of seeds were placed on moistened fine perlite/vermiculite mixture for one week. The seedlings were then transferred to 20 L containers filled with nutrient solution, with the lids having six holes for each replicate. The nutrient solution was the same as outlined previously, with solutions changed once per week and being maintained at pH 5.8. The soybean plants were harvested at phenological stage V4 (fourth trifoliate;

FEHR et al., 1971). The third expanded leaf (from the top) was collected for analysis of urease activity (as described in detail below). At the end of the experiment, roots, tap root, stem, young and old leaves were separately collected for acid digestion and ICP-AES. Wet and dry weights of the various plant fractions were also determined.

5.2.6 Urease activity measurements

The urease activity was analysed in vivo twice (20 d – V3 and 27 d – V4 after germination) by an adaptation of the method described by Hogan et al. (1983). The plant tissues from Exp #3 (200 mg of fresh leaves, cut into slices with a width 1 mm) were placed in a medium of 8 mL of NaH2PO4 buffer urea at pH 7.4 and incubated for 3 h at 30°C, protected from light in aluminium foil and kept under constant agitation. In a test tube containing 0.5 mL of the extract obtained after incubation, 2.5 mL of Reagent 1 (phenol 0.1 mol L^{-1}, sodium nitroprusside – 50 mg) and 2.5 mL of Reagent II (NaOH 0.125 mol L^{-1}, $Na2HPO_4.12H_2O$ 0.15 mol L-1, NaOCl – 3% Cl2) were added. The tubes were closed with stoppers to avoid loss of NH3 and placed in a water bath at 37°C for 35 min. After this interval, the reaction was measured by colorimetry in a spectrophotometer at 625 nm. The urease activity was determined by the quantity of ammonium (NH_4^+) produced, and the values were compared with a standard curve, previously determined using NH_4Cl. The results obtained were expressed in µmol NH_4^+ g^{-1} FW h^{-1}. The spectrophotometric analyses were performed with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

5.2.7 Elemental analysis of plant tissues

All combinations of seed stock × Ni solution concentration × Ni foliar treatments from Exp #3 were used for the elemental analysis. In addition, seeds, cotyledons and unripe pods from Exp #1 were analysed. Parts of the plant material samples (old leaves, young leaves, tap root and stem from each treatment) were oven dried at 60°C for 3 d. Plant tissue samples were ground to a fine powder in an impact mill at 15 000 rpm (IKA Tube Mill 100 control with disposable titanium blades) and weighed to 100 ± 5 mg in 6 mL polypropylene tubes. These samples were pre-digested using 2 mL AR-grade HNO3 (70%) for 24 h before being digested in a block heater (Thermo Scientific™ digital dry bath) for a 2-h programme (1 h at 70°C followed by 1 h at 125°C) and diluted to 10 mL with ultrapure water (Millipore 18.2 MΩ·cm at 25°C) before analysis with ICP-AES with a Thermo Scientific iCAP 7400 instrument for macro-elements (Na, Mg, Al, P, S, K, Ca) and trace-elements (Cr, Mn, Fe, Co, Ni, Cu, Zn) in

radial and axial modes depending on the element and expected analyte concentration. In-line internal addition standardization using yttrium was used to compensate for matrix-based interferences. Quality controls included matrix blanks, certified reference material (Sigma-Aldrich Periodic table mix 1 for ICP TraceCERT®, 33 elements, 10 mg L^{-1} in HNO3), Standard Reference Material (NIST Apple 1515 digested with HNO3), and internal reference materials. The limit of detection for Ni (on the basis of 100 mg of sample in 10 mL acid digestion solution) was 0.35 µg g^{-1} and values below this were considered as not quantifiable and not used.

5.2.8 Statistical analysis

Data analysis was performed with R Development Core team (version 4.0.2) (2015). The data were submitted to analysis of variance by the F-test. When the effect was significant, we applied the Tukey test to compare the effects. In all analysis, the level of significance was considered to be 5%. Nickel ionic activity in solution was modelled using GEOCHEM-EZ software (JON et al., 2010).

5.3 Results

5.3.1 Nickel depletion in hydroponics culture

First, plants were grown to maturity (phenological stage R7-110 d) under Ni-limiting conditions in hydroponics in order to generate Ni deficient seeds for the subsequent Ni dosing experiment (Exp #3). The mature plants in Exp #1 displayed mild Ni deficiency symptoms, including foliar chlorosis, especially at the leaf tips and margins, and some seed pods were infertile (Figure Suppl. 2- Appendix). The original seed stock contained 11.14 µg g-1 Ni, whereas the Ni seeds produced in Exp #1 contained <0.35 µg g-1 Ni (Table 1). We examined the leaves from plants grown in Exp #1 using SEM, with is important for understanding the foliar absorption of Ni fertilizers. Two types of trichomes were found on the adaxial surface of soybean (Figure 1): non-glandular trichomes (multicellular, thick walled and non-secretory) and glandular trichomes (multicellular and secretory) (FRANCESCHI; GIAQUINTA, 1983; HEALY et al., 2009). The glandular trichomes (Figure 1b) are comparatively short structures that are highly vacuolated. They typically consisted of an epidermal cell and around four or five stalk cells with one small cell on the apex. The external morphology of the glandular trichomes had a series of strong linear cells separated by periclinal cross walls. The non-glandular trichomes (Figure 1c) are empty, comprising of a multicellular base and one long stalk cell.

Figure 1 - Scanning electron microscopy (SEM) images of trichomes on the adaxial surface of soybean leaves at the R1 stage from plants originating from Exp #1. Different types of trichomes: non-glandular trichomes (NGT) and glandular trichomes (GT)

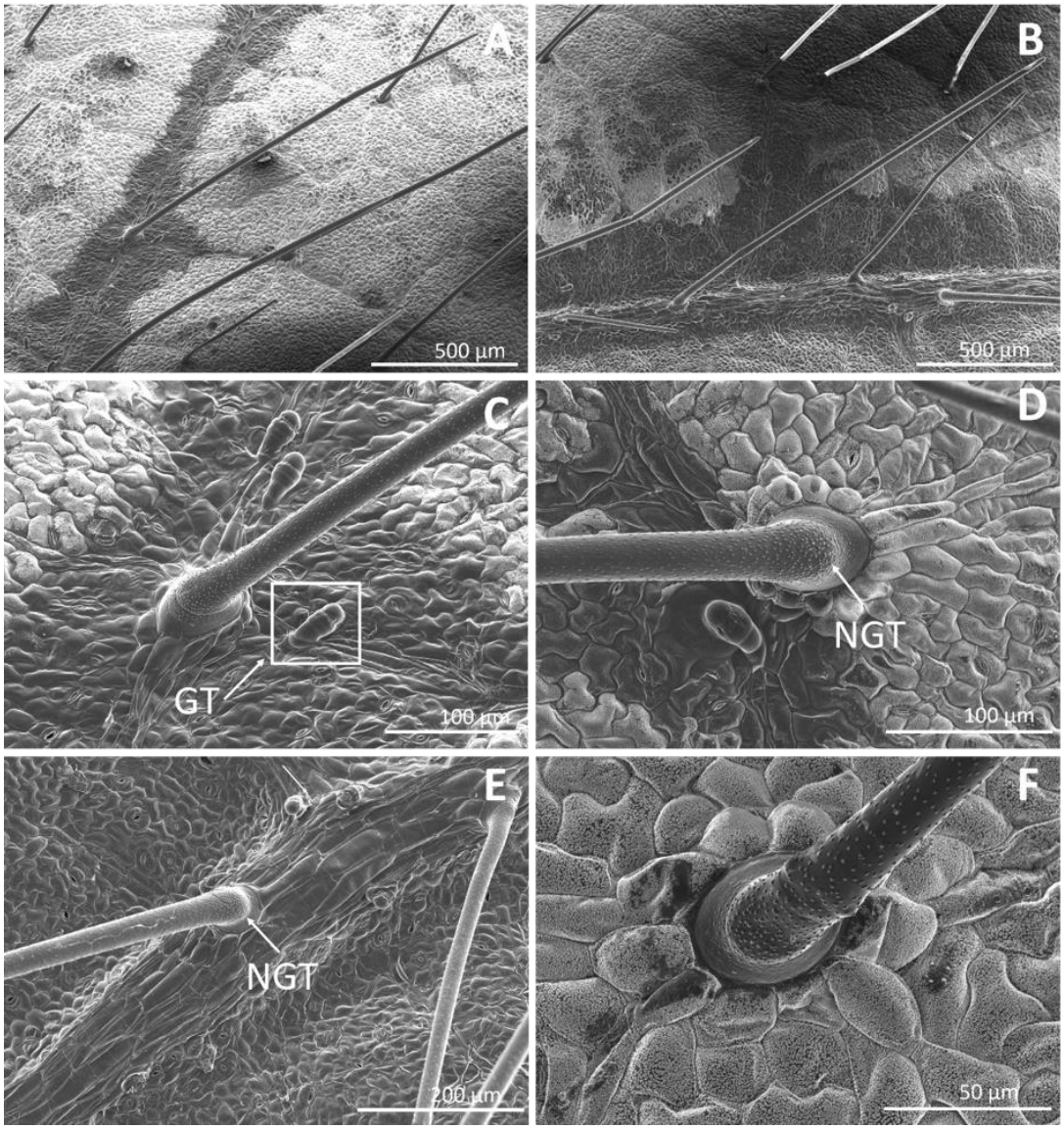


Table 1 - Elemental concentrations in different plants tissues in soybean plants grown in hydroponic solution culture (Exp #1), except for cotyledons which originate plants grow from Ni-sufficient seed stock from #Exp 3. Macronutrients – mg g^{-1} (Ca, Mg, K, P) and micronutrients – µg g^{-1} (Ni, Cu, Mn, Zn, Fe)

	Parts of plant				
	Seeds		**Cotyledons**		**Unripe pods**
	Ni depleted seeds	**Ni sufficient seeds**	**Ni depleted seeds**	**Ni sufficient seeds**	**Ni depleted seeds**
Ni	<0.35	11.1	<0.35	5.32	<0.35
Cu	7.30	13.9	8.51	16.4	8.71
Mn	133	97.4	187	212	188
Zn	71.0	56.1	70.3	65.20	89.8
Fe	69.6	86.7	71.8	124	75.5
Ca	3.02	2.07	5.27	9.72	12.3
Mg	3.24	2.48	4.24	4.97	4.51
K	23.1	19.8	23.3	31.3	44.9
P	6.16	5.06	7.93	6.24	5.74

5.3.2 Synchrotron µXRF analysis of soybean seeds

The spatial distribution of elements in whole soybean seeds (original stock with 11.14 µg g^{-1} bulk Ni) was analysed by synchrotron µXRF. Suppl. Figure 3 (Apeendix) shows the distribution of Ca, K, Fe, Zn, Mn and Ni in the seeds. Calcium is localised around the seed coat (with on average of 1.5 µg g^{-1}), with hotspots in parts of the hilum (0.3 µg g^{-1}). Potassium was localised in the endosperm of the seed and was very low in the radicle (0.2 µg g^{-1}), while there were some regions of the seed coat with higher prevailing K concentrations (1.4 µg g^{-1}) (Suppl. Figure 3 - Appendix). Similar to Ca, Fe is localised in the seed coat (0.15 µg g^{-1}) and in hotspots at the tip of the radicle (0.5 µg g^{-1}). Zinc has a more even distribution and is uniformly localised throughout the seed (in the endosperm and seed coat), however in the radicle Zn is more enriched up to 250 µg g^{-1} (Suppl. Figure 3- Appendix). Prevailing Mn concentrations were extremely low, although there are some hotspots at the margin of the radicle (up to 200 µg g^{-1}), while other parts of the seed have approximately 50 µg g^{-1} Mn. The distribution of Ni is mainly localised in the seed coat, being very low throughout seed, with an average of 40 to 20 µg g^{-1}. However, there are areas of hotspots in the seed coat, where the concentration of Ni is around 80 µg g^{-1}.

5.3.3 Synchrotron µXRF analysis of non-nickel treated whole leaves

We examined the elemental distribution of the elements K, Ca, Fe, Mn, Ni and Zn in the leaves of soybean from #Exp 2 (non-Ni treated whole leaves) (Figures 2; 3). The distribution of K in the leaves is mainly in the veins (Figures 2; 3), where the highest concentrations are around 0.6 G/Lin the veins, whilst in other parts of the leaf the concentration is 0.2 Wt%. Zinc is evenly distributed in the leaves with about 20 µg g^{-1}, but Zn was notably lower in the veins. Iron had a distribution similar to that of Zn, however Fe concentrations in the leaf are up to ~200 µg g^{-1}. In contrast, Mn is localized mainly in the trichome bases and also the trichome stalks (Figure 2), with up to 100 µg g^{-1} locally. In other areas of the leaf, Mn is lower than 20 µg g^{-1}. Since no Ni was applied, the concentration of Ni in the leaves was extremely low, typically under 5 µg g^{-1} (Figures 2; 3).

Figure 2 - Synchrotron µ-XRF elemental maps showing the distribution of elements (K, Ca, Fe, Zn, Mn, Ni) in non-Ni treated control soybean leaf. The concentrations are in g/Lfor K and Ca and µg g^{-1} for Fe, Mn, Ni, and Zn with brighter colors corresponding to higher prevailing elemental concentrations

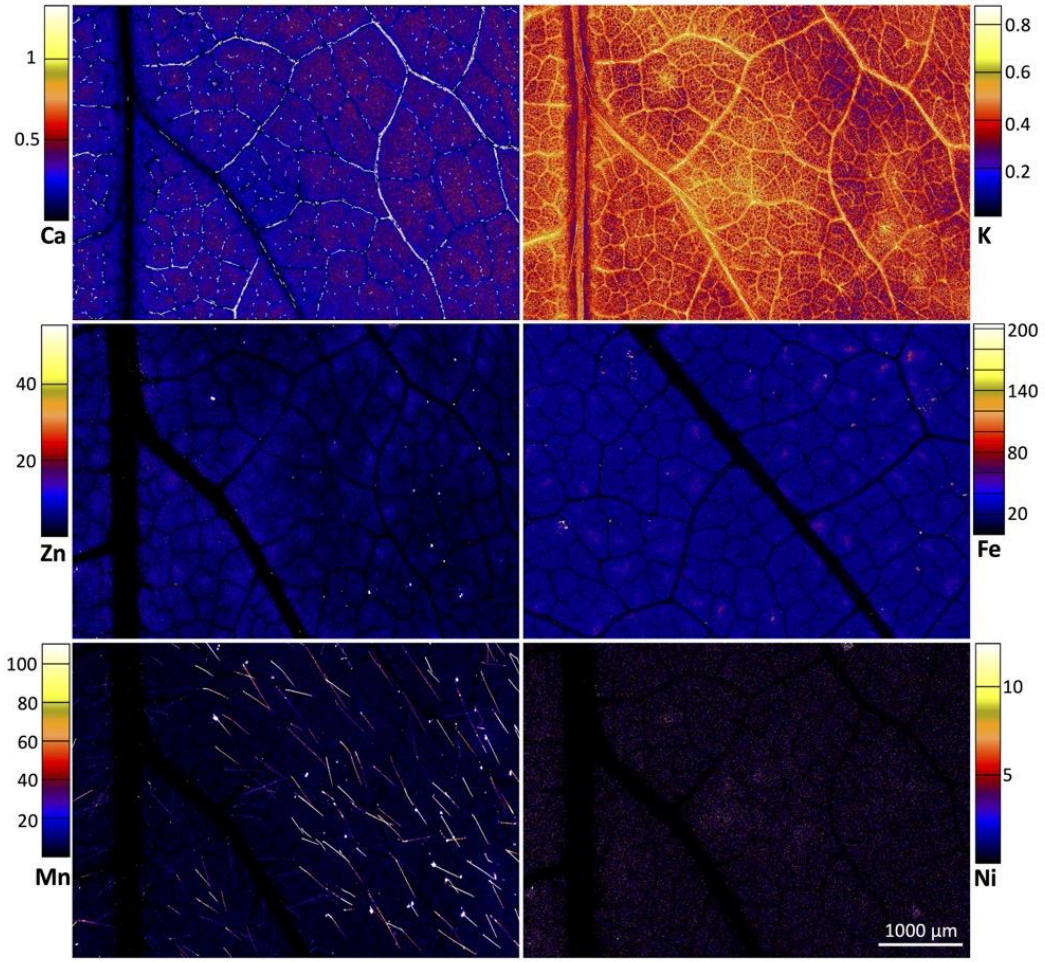
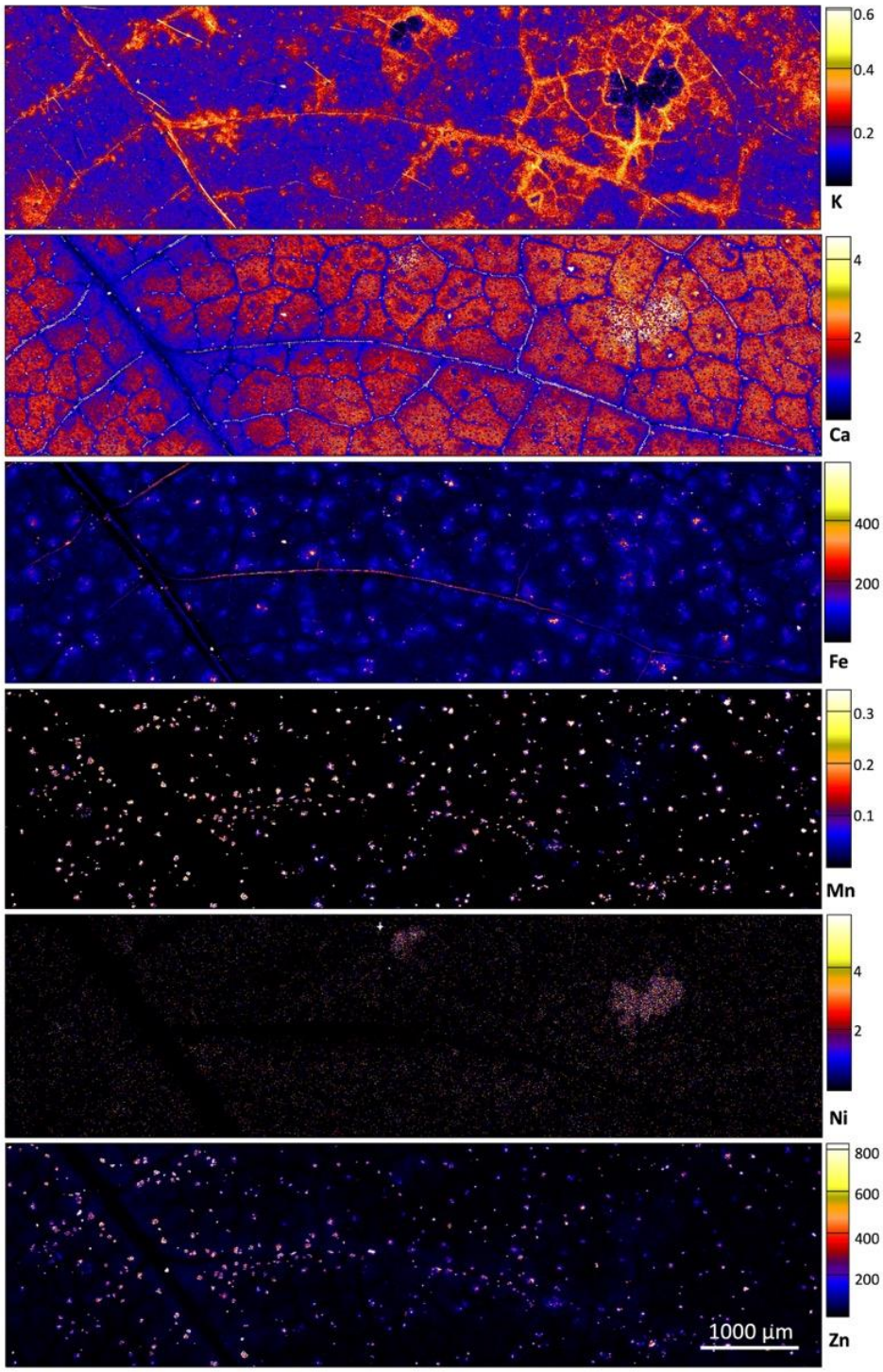


Figure 3- Synchrotron µ-XRF elemental maps showing the distribution of elements (K, Ca, Fe, Mn, Ni, Zn) in non-Ni treated control soybean leaf. The concentrations are in g/L for K and Ca and µg g^{-1} for Fe, Mn, Ni, and Zn with brighter colours corresponding to higher prevailing elemental concentrations



5.3.4 Synchrotron µXRF analysis of uptake and redistribution of foliar applied nickel

Next, the spatial-temporal distribution of Ni (and other elements) were analysed in situ within fresh hydrated soybean leaf tissues at different stages from Exp #2 following exposure to foliar applied nickel treatment at 100 and 50 mg L^{-1} in 5 µL droplets (as $NiSO_4$ solution) after 15 min and after 3 hr. Rapid survey scans were used for the selection of areas of interest, and these showed that within 15 min the Ni had already moved across the leaf surface and was present inside the leaf tissues, mainly around the trichomes (Figure 4) and tissue Ni concentrations increased as the exposure time increased. After 15 min, Ni was observed to accumulate in the pedicles of the trichomes with some Ni moving toward the rest of the leaf through the veins; movement continued in the 3-hr study. Nickel and Mn were both strongly concentrated in pedicles and trichome bases, respectively. Figure 4 shows the distribution of Ni and Mn in a hydrated leaf and panels show trichomes after 15- and 25-min Ni exposure to 100 mg L^{-1} of $NiSO_4$ solution, Ni was clearly present in the pedicles of the trichome, whilst Mn was localised in the bases of the trichomes. Figure 4 shows foliar application of Ni at 45 min and 3 hr exposure to $NiSO_4$ which suggests that the Ni that had rapidly translocated away from the site of application. Then, after 3 hr, the extent of accumulation increased further, and within interveinal tissues high Ni concentrations were found to accumulate in the apoplast. The overall patterns are very similar after application of 50 mg L-1 Ni (Figure 5) compared to 100 mg L-1 Ni. Detailed scans were then used to more closely examine Ni distribution around trichomes after foliar application. In Figure 5 shows high concentrations of Ni accumulated in the pedicles of trichomes suggesting that the pathway of Ni is entry though the trichomes. Using tri-colour maps of Ca-Ni-Mn (Figure 6) it is possible to see how Ni and Mn concentrate near the trichomes, whereas Ca is more widespread throughout the tissues. Zinc follows a similar trend as Mn, locating mainly in the trichome bases (Figure 6). Detailed µ-XRF scans (Figure 6 and Figure Suppl. 4 [Appendix]) of leaf surfaces after foliar application of Ni on soybean between 25 and 45 min showed that 15 min after foliar application, Ni was present only in the pedicles of the trichomes, whilst Mn was concentrated only in the bases of trichomes.

Figure 4 - Synchrotron µ-XRF elemental maps showing the distribution of Ni and Mn after foliar application of Ni at 15, 25, 45 min and 3 hr time intervals. The concentrations are in g/Lfor Mn in the second panel and µg g^{-1} for Ni and Mn in all other panels with brighter colors corresponding to higher prevailing elemental concentrations

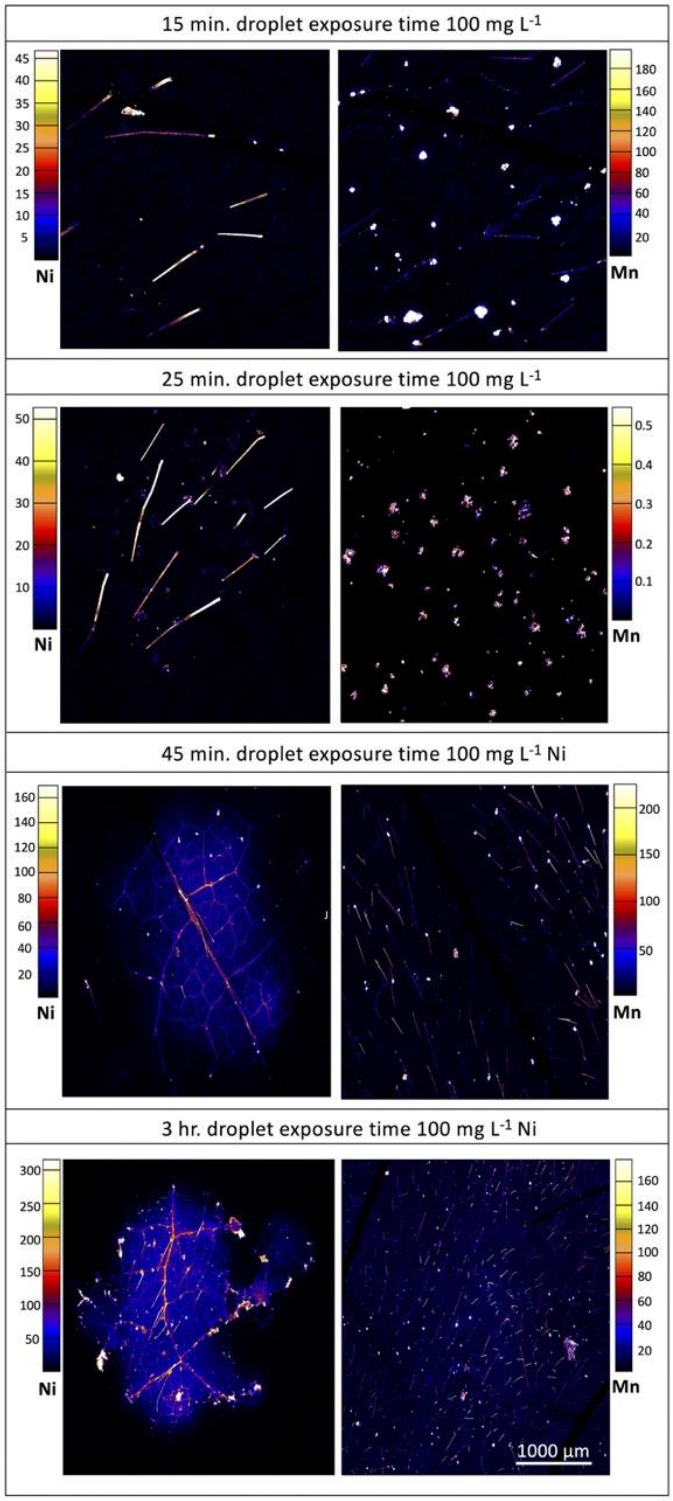


Figure 5 - Synchrotron µ-XRF elemental maps showing the distribution of Ni and Mn. Images show areas where droplets of 50 mg L^{-1} (as $NiSO_4$) were deposited on the leaves of soybean at 45 min and 3 hr time intervals. The concentrations are in µg g^{-1} with brighter colours corresponding to higher prevailing elemental concentrations

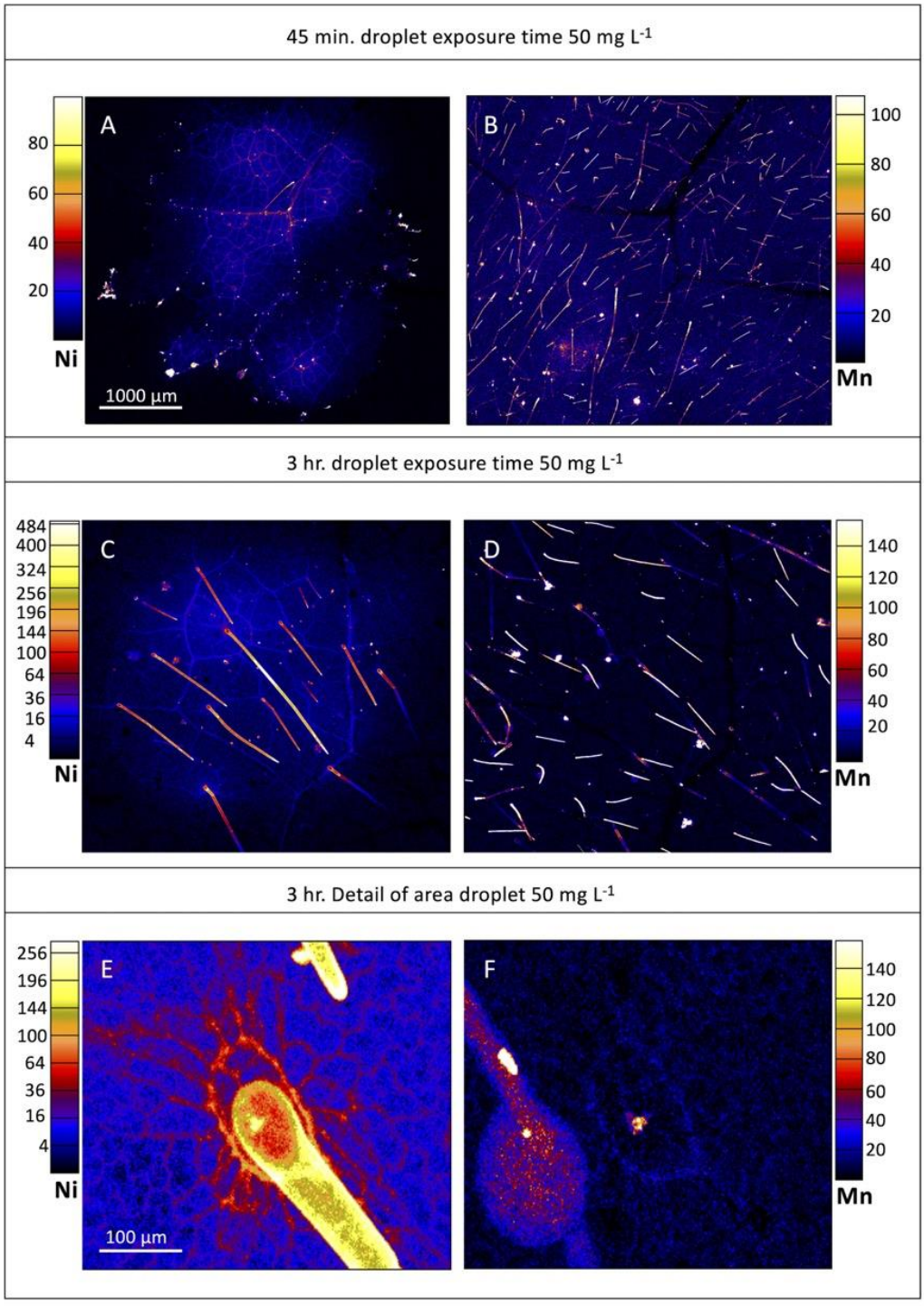
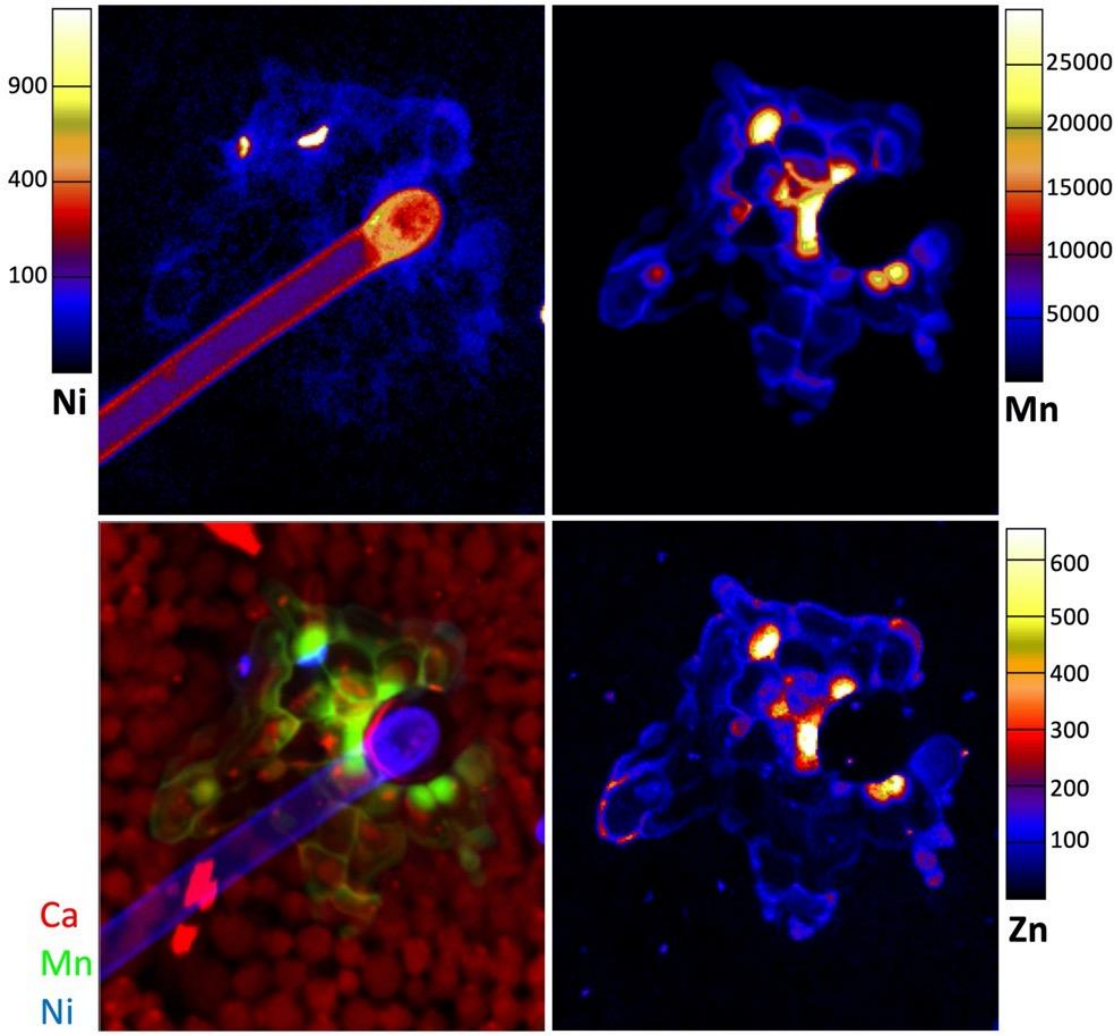


Figure 6 - Synchrotron µ-XRF elemental maps showing the distribution of Ni, Mn and Zn. The tricolour image shows Ca in red, Mn in green and Ni in blue. Images show detail of trichomes at foliar Ni application doses 50 mg Ni L^{-1} (3 hr) and 100 mg Ni L^{-1} (45 min), respectively. The concentrations are in µg g^{-1} with brighter colors corresponding to higher prevailing elemental concentrations



5.3.5 Effect of Ni supply on tissue concentrations and urease activity

Plants were cultured in (Ni-purified) hydroponics solution using the original and low Ni seeds for a period of three weeks (V4 phenological stage) (Exp #3). Nickel was supplied in solution at 0 (control) or 0.85 µM Ni, and at the conclusion of the experiment, root and shoot biomass were determined, urease activity measured, and elemental concentrations determined in the different plant parts. The Ni concentration in soybean tissues was compared with the plant fractions with the cotyledon either removed or in the plants that maintained the cotyledons without Ni application (control) (Figure 7). The presence of the cotyledons even though they contained very low Ni (<0.35 µg g-1 of Ni – table 1) was sufficient to increase the

Ni concentration in soybean tissues compared to the plants that had their cotyledons removed, except for the roots, in which the plants without cotyledon revealed an augment of 3.0 fold of Ni concentration compared to the plants with cotyledon (Figure 7c). The seeds obtained from plants grown from Ni sufficient seeds stock contained 5.32 µg g-1 Ni (Table 1), whereas the plants with cotyledons had a higher increase of Ni concentration compared to plants with removed cotyledons. There were significant interactions between the two factors (seed and cotyledons) in specific parts of soybean plant (stems, roots and whole plant).

It is noteworthy that for all plant parts, the average tissue Ni concentration was higher when the plants were grown in solutions containing 0.85 µM Ni compared to the control with 0 µM Ni (Figure 7). The Ni doses raised the Ni concentration in the Ni depleted seed stock by 3.12-fold with the cotyledons attached and 3.13-fold in the absence of the cotyledons in the whole plant. The plants grown from Ni sufficient seed stock the Ni doses increased Ni concentration by 3.78- fold with cotyledon and 7.12-fold without the cotyledons in the whole plant. Analysing plants that received Ni grown from Ni sufficient seed stock, with cotyledons attached had a higher concentration of Ni than those grown without cotyledons (Figure 7), an average by 1.18-fold in the whole plant (stem, roots, old and young leaves) (Figure 7 l). Plants grown from Ni depleted seed stock with cotyledons attached also had a higher Ni concentration by 1.09-fold compared to plants with the cotyledon removed in the whole plant. In the young leaves of soybean from plants grown without cotyledons had an augmentation of the Ni concentration compared to those with cotyledon, by 1.61-fold in the leaves (Figure 7 g).

Figure 7 - Box plot of Nickel concentrations in different parts of the soybean plants grown from two types of seeds (Ni-depleted seeds and Ni-sufficient seed stock), with or without cotyledon removed and two Ni treatments: control (black) and 0.85 µM Ni (red) in different parts of soybean plant: **(a)** stem; **(b)** roots; **(c)** old leaves; **(d)** young leaves; **(e)** all leaves and **(f)** whole plant. Boxes with different letters indicate significant differences according to least difference (Tukey) test P <0.05. ** indicates that there was interaction between the factors according to least difference. Letter a-b (lowercase) compares both types of seed regarding absence of cotyledon; letters **A-B** (uppercase) compares both types of seed regarding presence of cotyledon; letter **C** compares the presence or absence of cotyledon inside the same type of seed and letter **D** (lowercase and uppercase) compares significant difference between the seed when did not have interaction between the factors

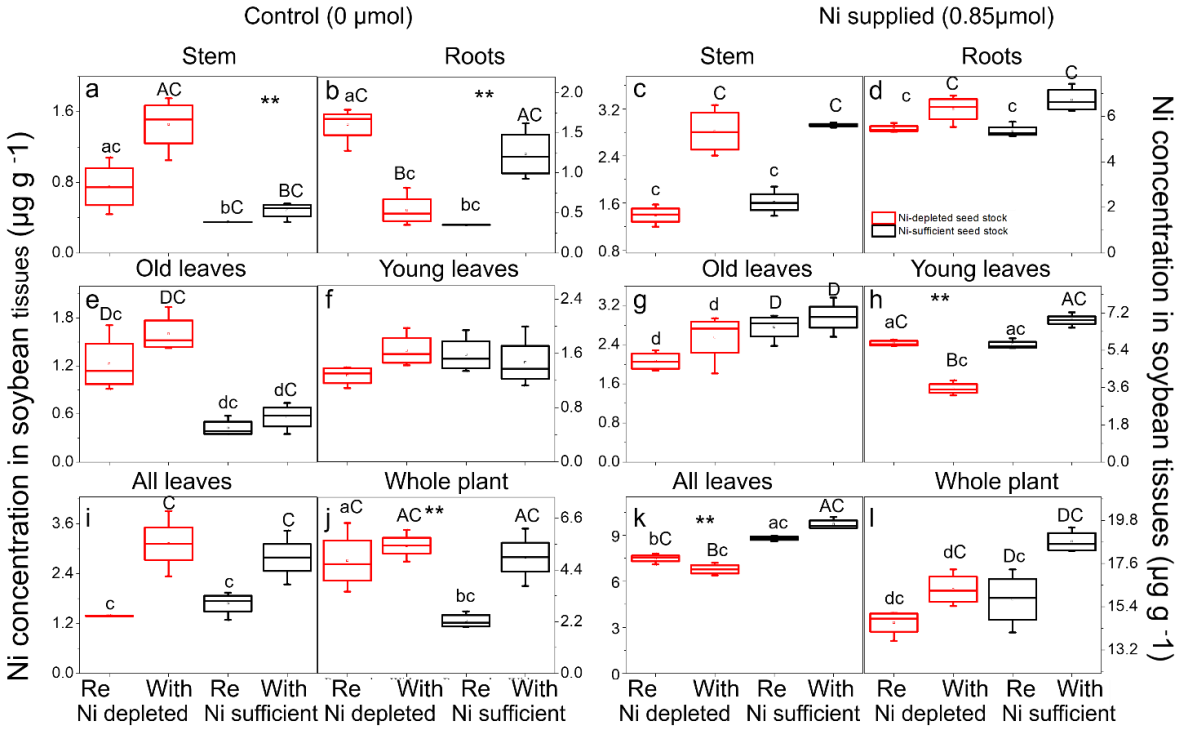
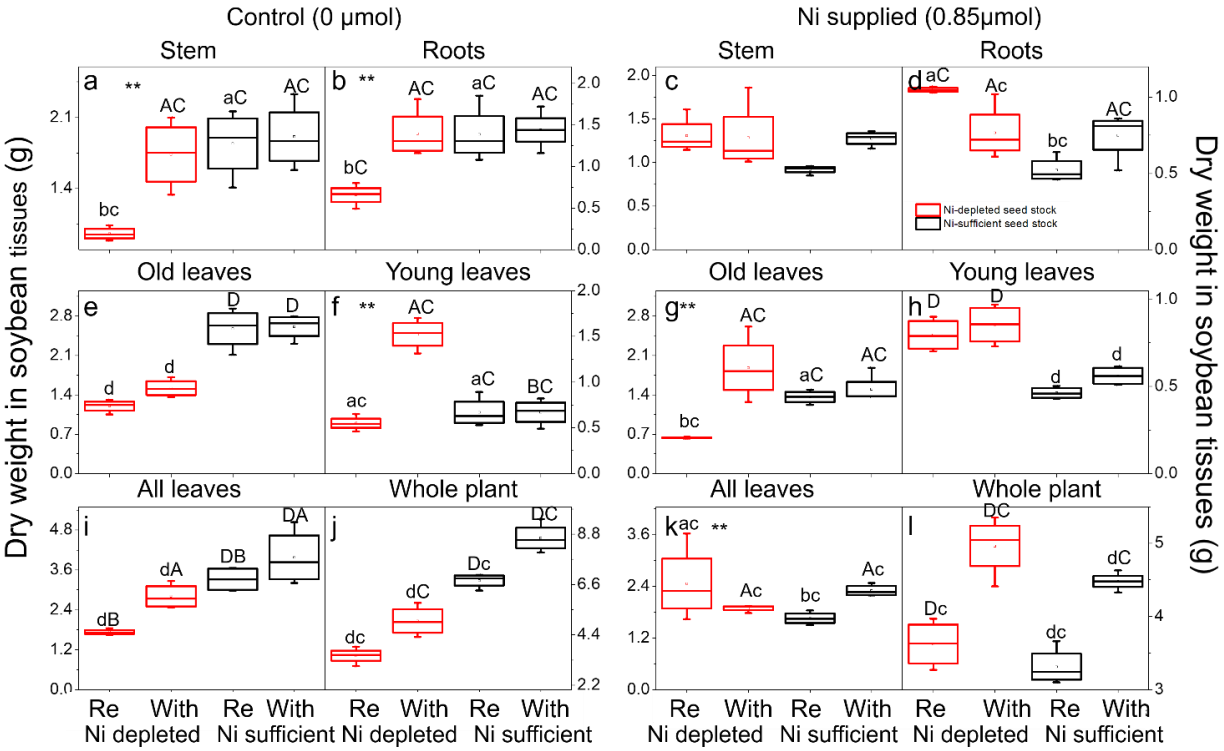


Figure 8 shows the biomass in different soybean parts: stem, roots, old leaves, and young leaves. The soybean plants grown from Ni sufficient seed stock in solution without Ni (0 µM), and those that kept the cotyledon showed a greater increase of biomass compared to other treatments (Figure 8k). The plants from Ni-depleted seed stock, whose cotyledon was removed, had the lowest biomass compared to the plants with cotyledons attached, an average by 31% lower than plants with the cotyledons attached. Thus, the seedling development was affected by the presence of cotyledons even without Ni treatment. The Ni treatment (Figure 8) also resulted in an increase of the biomass in the soybean tissues compared to the plants with cotyledons removed. Additionally, Ni depleted seed stock that received Ni had an augmentation compared to plants from Ni sufficient seed stock that in the Ni treatment, an average by 9.2% higher than the plants grown from Ni sufficient seed stock (Figure 8k). Finally, the soybean plant fractions of both types of seeds that received Ni supply had an average of 31% decrease in biomass compared to the plants did not receive the Ni treatment.

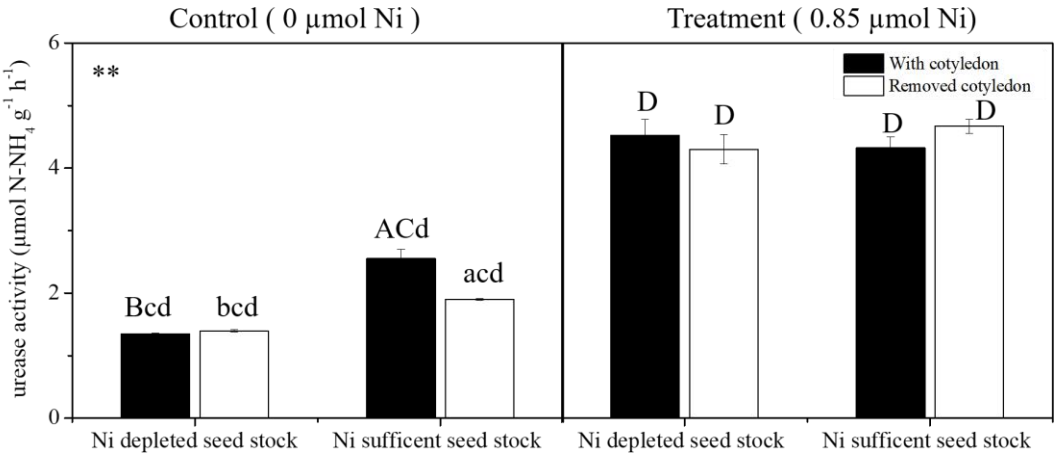
Figure 8 - Box plot of dry weight (g) of soybean parts: stem; roots; old leaves; young leaves; all leaves and whole plant. Plants were grown from two types of seeds (Ni-depleted seeds and Ni-sufficient seed stock), with or cotyledon removed and two Ni treatments: control 0 µM Ni (black) and 0.85 µM Ni (red). Boxes with different letters indicate significant differences according to least difference (Tukey) test P <0.05. Letter a-b (lowercase) compares both types of seed regarding absence of cotyledon; letters **A-B** (uppercase)compares both types of seed regarding presence of cotyledon; letter **C** compares the presence or absence of cotyledon inside the same type of seed and letter **D** (lowercase and uppercase) compares significant difference between the seed when did not have interaction between the factors. ** indicates that there was interaction between the factors according to least difference



5.3.6 Urease activity in nickel treated plants

The urease activity (Figure 9) in soybean was influenced by the different seed types (Ni depleted and Ni sufficient) as well as the Ni treatment (0 and 0.85 µM Ni). The addition of 0.85 µM Ni to the solution increased urease activity in the plants grown from both the Ni depleted and Ni sufficient seed stock. In the plants grown from Ni depleted seeds, it increased from 1.3 at 0 µM Ni to 4.7 µ mol N NH4+g-1h-1 at 0.85 µM Ni. For plants grown from Ni sufficient seed stock, the urease activity tended to be slightly higher than in plants grown from Ni depleted seed stock, especially in the control (0 µM Ni).The presence of the cotyledons in the Ni sufficient seeds also increased the urease activity even without Ni supply, it increased from 1.9 (removed cotyledons) to 4.6 N $NH_4^+ g^{-1} h^{-1}$. Therefore, the urease activity was enhanced by higher levels of Ni in the plants.

Figure 9 - Urease activity in leaves from plants originating from Exp #3 (grown from Ni depleted seeds and Ni sufficient seeds), with two Ni treatments Ni (control - 0 and 0.85 µM Ni) after 27 days germination). Letters **a-b** (lowercase) compares both types of seed regarding absence of cotyledon; letters **A-B** (uppercase) compares both types of seed regarding presence of cotyledon; letter **C** compares the presence or absence of cotyledon inside the same type of seed and letter **D** (lowercase and uppercase) compares plants which received Ni supply (0.85 µMol) and plant did not receive (0 µMol). ** indicates that there was interaction between the factors according to least difference. All tests were according to least significant difference (Tukey) test P<0.05



5.3.7 Elemental concentrations in the treated plant tissues

When the Ni concentrations in the soybean tissues were compared with the concentrations of other macro-micro nutrients (Table 2), a higher concentration of Mn was found in all parts of the plant (old leaves, young leaves, stem and roots) in the plants grown from Ni depleted seeds, compared to plants grown from Ni sufficient seed stock. Conversely, plants raised from Ni sufficient seeds had higher Mn concentrations in the treatment which received 0.85 µmol of Ni compared to those that received no Ni. However, in all tissues of plants grown from Ni sufficient seed stock the average of Mn concentrations was 10.3% lower compared to the plants from Ni depleted seeds. No statistically significant difference in the Mn concentration was seen in the plants with or without cotyledons. The concentrations of Cu and Zn increased in soybean tissues following Ni application. In old leaves of plants which received solution of Ni there was an increase of 65% of Cu and 5% of Zn concentration, respectively. The concentrations of Ca and Mg did not follow a distinct pattern in the soybean tissues. However, the concentrations of Mg and Ca had a tendency to increase the concentrations as Ni concentration increase (Table 2).

Table 2. Effect of application of Ni on the concentration of macronutrients – mg g $^{-1}$ (Ca, K, Mg, P) and micronutrients – µg g $^{-1}$ (Cu, Zn, Mn, Fe) in soybean plants grown in hydroponic solution culture (Exp #3)

Seeds	Treatment	Cotyledon	Ca	Mg	K	P	Cu	Zn	Mn	Fe
Old leaves										
Ni depleted seeds	Control	Attached	32.7 ± 1.80 a	7.45 ± 0.30 a	39.4± 4.20 a	4.14 ± 0.50 b	5.29 ± 0.460 b	85.8 ± 7.05 b	399. ± 27.4 a	144. ± 3.16 a
		Removed	31.7 ± 2.10 a	7.37 ± 0.40 a	37.5± 2.50 a	4.65 ± 0.80 b	5.62 ± 0.720 b	91.5 ± 6.86 b	405± 25.6 a	152 ± 14.7 a
	Ni 0.85 µM	Attached	31.6 ± 4.30 a	6.71 ± 0.70 b	40.9 ± 2.60 b	4.65 ± 0.70 b	8.63 ± 1.06 a	94.7 ± 7.22 a	359 ± 48.0 b	156 ± 15.8 a
		Removed	29.7 ± 4.40 a	6.39 ± 0.10 b	40.3 ± 3.20 b	4.72 ± 0.60 b	8.09 ± 0.690 a	92.4 ± 10.0 a	356 ± 62.6 b	157 ± 27.1 a
Ni sufficient seeds	Control	Attached	25.0 ± 2.20 b	5.69 ± 0.40 c	34.5 ± 4.20 a	3.81 ± 0.80 b	6.39 ± 1.48 b	72.9 ± 12.0 d	265 ± 28.7 d	141 ± 19.3 a
		Removed	27.8 ± 3.50 b	5.96 ± 0.60 c	38.7 ± 4.10 a	4.04 ± 0.60 b	6.41 ± 0.540 b	83.0 ± 10.3 d	302 ± 33.5d	140 ± 19.8 a
	Ni 0.85 µM	Attached	28.1 ± 1.60 b	5.93 ± 0.40 ab	42.3 ± 5.60 c	5.03 ± 0.30 a	8.55 ± 0.720 a	89.6 ± 5.00 c	330. ± 16.7 c	135 ± 9.50 a
		Removed	29.0 ± 3.20 b	6.21 ± 0.50 ab	36.5 ± 1.90 c	5.41 ± 0.23 a	8.76 ± 0.490 a	88.0 ± 4.10 c	354 ± 23.6 c	143± 8.78 a

Young leaves

Ni depleted seeds	Control	Attached	14.9 ± 0.90 abc	5.47 ± 0.300 a	52.0 ± 4.70 b	6.72 ± 0.70 c	6.70 ± 0.750 b	86.1 ± 6.93 a	261 ± 19.1 AC	107 ± 10.0 a
		Removed	12.8 ± 0.60 abc	5.14 ± 0.190 a	56.1 ± 3.50 b	7.71 ± 0.60 c	7.80 ± 0.350 b	90.0 ± 3.23 a	253 ± 20.6 AC	115 ± 11.4 a
	Ni 0.85 µM	Attached	13.3 ± 2.10 abc	4.51 ± 0.62 b	45.8 ± 0.60 b	6.67 ± 0.90 b	9.24 ± 1.40 a	79.1 ± 13.0 a	275 ± 41.7 aC	102 ± 14.7 a
		Removed	12.9 ± 1.70 abc	4.82 ± 0.80 b	55.1± 4.50 b	7.87 ± 0.30 b	11.1 ± 1.03 a	91.7 ± 3.02 a	233 ± 9.64 aC	105 ± 8.36 a
Ni sufficient seeds	Control	Attached	10.7 ± 1.80 aBc	4.46 ± 0.50 c	46.3 ± 9.20 c	6.47. ± 1.40 c	7.48 ± 1.84 b	76.8 ± 9.42 a	186 ± 20.4 bc	95.2 ± 12.7 a
		Removed	11.3 ± 1.80 abc	4.29 ± 0.20 c	44.8 ± 3.10 c	6.19 ± 0.60 c	7.35 ± 1.11 b	77.2 ± 7.76 a	202 ± 29.9 bc	101 ± 21.2 a
	Ni 0.85 µM	Attached	15.5± 1.80 abC	5.02 ±0.30 ab	53.3 ± 2.80 a	8.13± 0.50 a	9.77 ± 0.84 a	86.1 ± 7.28 a	241 ± 13.9 Bc	105 ± 2.64 a
		Removed	11.8 ± 0.40 ac	4.91 ±0.30 ab	55.9 ± 3.10 a	8.67 ± 0.73 a	10.7 ± 1.15 a	86.7 ± 11.0 a	238 ± 22.8 Bc	104 ± 8.89 a

Stem

Ni depleted seeds	Control	Attached	13.7 ± 1.30 a	3.17 ± 0.30 a	66.2 ± 4.50b	3.96 ± 0.60 b	6.02 ± 0.83 b	77.6 ± 10.4 b	120. ± 13.7 a	57.6 ± 9.28 a
		Removed	13.2 ± 0.40 a	2.96 ± 0.40 a	64.1 ± 3.90 b	4.32± 0.50 b	7.39 ± 0.95 b	77 ± 3.25 b	120. ± 5.33 a	63.1 ± 8.02 a
	Ni 0.85 µM	Attached	14.1 ± 0.80 a	3.16 ± 0.50 a	70.3 ± 5.20 a	4.94± 0.60 b	12.6 ± 1.0 a	84.0 ± 6.93 a	112 ± 10.9 a	58.3 ± 3.15 a

		Removed	13.5 ± 2.10 a	3.10 ± 0.20 a	64.4 ± 6.00 a	4.55 ± 0.50 b	12.9 ± 0.86 a	81.9 ± 12.3 a	110. ± 13.8 a	65.7 ± 8.17 a
Ni sufficient seeds	Control	Attached	12.6 ± 1.10b	2.71 ± 0.30 a	63.0 ± 5.40 b	3.35 ± 1.0 b	7.39 ± 1.17 b	61.4 ± 6.18 c	87.7 ± 4.23 c	50.8 ± 4.47 a
		Removed	11.6 ± 0.60 b	2.80 ± 0.20 a	58. 8± 7.50 b	3.15 ± 0.50 b	7.49 ± 0.89 b	62.1 ± 5.27 c	91.5 ± 1.93 c	51.3 ± 3.97 a
	Ni 0.85 µM	Attached	13.5 ± 1.20 b	3.12 ± 0.00 a	67.6 ± 5.50 a	5.11 ± 0.90 a	11.9 ± 1.38 a	73.5 ± 12.9 c	110. ±13.9 b	58.3 ± 6.20 a
		Removed	12.9 ± 0.20 b	3.23 ± 0.50 a	68.3 ± 4.10 a	5.68 ± 1.10a	12.3 ± 1.17 a	69.5 ± 9.11 c	122 ± 16.2 b	62.6 ± 12.6 a

Roots

Ni depleted seeds	Control	Attached	4.95 ± 0.30 a	15.8 ± 2.10 b	63.4 ± 1.89 a	4.11 ± 0.20 b	8.46 ± 1.02 a	48.3 ± 3.72 a	1060 ± 247 b	366 ± 18. 5 b
		Removed	5.16 ± 0.30 a	16.3 ± 1.40 b	62.2 ± 4.40 a	4.32 ± 0.30 b	8.87 ± 0.06 a	49.0 ± 2.28 a	1070 ± 170.b	283 ± 21. 8 b
	Ni 0.85 µM	Attached	5.05 ± 0.50 a	18.1 ± 7.50 a	56.0 ± 3.60b	4.39 ± 0.20 ab	14.4 ± 1.31 b	46.9 ± 2.60 a	957 ± 80.6 ab	739 ± 84.9 b
		Removed	5.32 ± 0.30 a	17.5 ± 1.30 a	54.6 ± 7.30 b	4.18 ± 0.30 ab	14.1 ± 0.47 b	49.1 ± 3.04 a	1040± 213 ab	615 ± 131 b
Ni sufficient seeds	Control	Attached	4.19 ± 0.20 b	16.1 ± 1.60 b	47.0 ± 5.70 c	2.95 ± 0.11 c	7.91 ± 0.86 d	42.1 ± 4.93 b	805 ± 161 c	228 ± 23.6 b
		Removed	3.98 ± 0.20 b	16.9 ± 1.70 b	44.3 ± 2.70 c	2.96 ± 0.20 c	7.66 ± 0.80 d	40.4 ± 2.00 b	812 ± 130. c	260. ± 39.5 b

	Ni 0.85 µM	Attached	4.09 ± 0.40 b	17.7 ± 1.40 a	50.4± 7.60 c	4.16 ± 0.20 a	11.96 ± 1.03 c	41.4 ± 3.96 b	1170 ± 191 a	649± 87. 9 b
		Removed	4.32 ± 0.20 b	18.4 ± 2.10 a	48.9 ± 4.70 c	4.67 ± 0.70 a	14.0 ± 1.99 c	44.5 ± 3.66 b	1180 ± 256 a	967 ± 323 a

Values are means and standard deviations of four replicates

Different letters indicate significant differences according to least difference (Tukey) test P <0.05, showing that there was no interaction between the factors.

Different size of letters (lowercase and uppercase) indicates that there was interaction between the factors according to least difference (Tukey) test P <0.05.

5.4 Discussion

Soybeans grown in hydroponic solutions (Exp #3) from seed containing <0.35 µg Ni g^{-1} had a significant decrease in urease activity compared to plants grown from the original seed stock containing 11.1 µg Ni g^{-1}. The Ni concentrations in soybean tissues obtained from plants grown in Ni-sufficient nutrient solution was 3.67-fold higher than in tissues obtained from plants grown in Ni-deficient nutrient solution. The Ni concentrations varied from <0.35 to 7.42 µg g-1 in the roots, <0.35 to 3.26 µg g^{-1} in the stem, <0.35 to 4.33 µg g-1 in old leaves and 0.63 to 7.22 µg g-1 in young leaves. The relatively high dose rate of Ni supplied in this experiment did not have a major impact on Ca, Mg, Zn and Fe concentration in tissues. It is important to note that Ni has high phloem mobility in soybean (CATALDO et al., 1978; PAGE; FELLER, 2005; RIESEN; FELLER, 2005). Particularly, the young leaves had high Ni concentrations compared to another parts of plant, showing a strong requirement for Ni in metabolically highly active, meristematic tissues. In leaves of soybean suffering Ni phytotoxicity, older leaves contain much higher Ni concentrations.

Nickel supply by nutrient solution gave a distribution of Ni within the plant where more Ni was transported from the root to shoot and allocated to leaves and growing tissues (Table 2). The roots in direct contact with the nutrient solution with Ni had the highest Ni concentrations compared to other tissues, being 59.3% higher on average. This higher Ni concentration in the roots can be explained by the need for this element in the synthesis and activation of hydrogenase, an important enzyme in the process of absorption of N (DALTON et al., 1985; KLUCAS et al., 1983; STULTS et al., 1986; URETA et al., 2005; YUSUF et al., 2011). In plants treated with 0.85 µM of Ni, the concentrations of macronutrients (Ca, Mg, K and P) were enhanced by improved Ni status. It was also observed that more Fe was absorbed by roots following Ni application. Another study reported a similar result, where a supply of 10 µM of Ni augmented Fe concentration in roots in barley plants (RAHMAN et al., 2005). Nickel in the nutrient solution induced a significant increase in root Fe concentration of plants (BRUNE; DIETZ, 1995; RÖMHELD, 1991), since Ni displaces Fe from FeEDTA this produces Fe(OH)3 precipitates (CHANEY, 1988). A similar result has been reported by Freitas et al (2019) in soybean plants, which verified that Ni-supplied plants had an increase of Fe in the roots following Ni application. Furthermore, a decreased concentration of Fe in plant leaves has been reported, which is analogous with the finding in the present study in highly Ni-responsive soybean (PICCINI; MALAVOLTA, 1992). This phenomenon can be explained because Ni is able to suppress specific root-to-shoot Fe signalling, which results in a reduction

of transport of Fe to leaf tissues. Therefore, in this present study it was to necessary apply double the usual Fe concentration in solution to avoid inducing Fe deficiency. However, we believe that the quantity of Ni supplied had a negative effect in the development of soybean plants, where dry and fresh weight were lower in plants that received Ni fertilization. Thus, the results indicate Ni toxicity in the development of plants without visual symptoms, such as interveinal chlorosis (NAGAJYOTI et al., 2010; PRASAD et al., 2005).

In plants grown from Ni-sufficient seed stock which received Ni treatment, the urease activity was increased significantly in tissues which are involved in N storage and transport (GERENDAS et al., 1998; WITTE, 2011). We observed that soybean plants had increased urease activity when plants were treated with Ni and there was also an increase in the urease activity in plants from Ni sufficient seed stock compared to plants from Ni depleted seed stock without Ni application. Soybean with higher urease activity has the highest Ni concentrations in the tissues (POLACCO et al., 2013). This result corroborates Freitas et al. (2018) and Barcelos et al. (2017), which have shown that leaf urease activity was very responsive to Ni fertilization. The development of soybean plants were significantly enhanced by the presence of cotyledon. This means that cotyledon is an important storage of Ni to plants soybean. Previously, Cataldo et al. (1978) also observed in their study with soybeans that highest Ni concentration was found in the plants with cotyledon and the hull comparable on a dry weight basis.

A major function of the surface cuticle is to protect the leaf from excessive water loss by transpiration as well as to protect the leaf against excessive leaching of organic and inorganic solutes by rain, temperature control, and a role in defence against pathogens/diseases (KERSTIENS, 1996; RIEDERE; SCHEREIBER, 2001; MARSCHNER, 2012). However, there are still many gaps in understanding of the processes by which foliar applied nutrients move across the leaf surface in soybean, including the potential role of trichomes (FERNANDEZ et al., 2017; LI et al., 2018; 2019). Using synchrotron-based µXRF in fresh leaves of soybean after foliar Ni application with two concentrations we observed that after foliar Ni application, Ni was concentrated in the pedicles of trichomes, while Mn concentrated in the trichome bases. Manganese localisation in trichomes base has also been reported for sunflower (BLAMEY et al., 1986), pumpkin (IWASAKI; MATSUMURA, 1999) and cucumber (HORIGUCHI, 1987).

Synchrotron micro-X-ray fluorescence analysis of foliar applied Ni revealed that Ni moved quickly through the leaf surface, and it was observed in the pedicle of trichomes 15 min after foliar application (Figures 8; 9). Thus, the results showed that trichomes are a primary

pathway for the foliar uptake of Ni in soybean. Ni accumulated first within the trichomes, subsequently moved to the leaf cells and depending on the time after exposure started, the concentration of Ni increased in the interveinal tissues, especially in the apoplast. It has also been reported that in sunflower non-glandular trichomes (NGTs) are important for foliar Zn absorption (LI et al., 2019), but trichomes are not part of the primary pathway of foliar-applied Zn uptake in soybean and tomato (LI et al., 2018).

Total Ni concentrations in the shoot (young leaves, old leaves and stem) were lower when prevailing Mn concentrations were higher and vice versa. This suggests that an increased accumulation of Mn in roots may interfere with Ni uptake and storage. Plants grown from Ni depleted seeds had lower concentrations of Ni, but higher Mn concentrations, when compared to plants grown from Ni sufficient seeds, an average of 1.09-fold of Ni concentration in the treatment with highest Ni concentration. The most likely reason is that Mn competes with Ni at transport sites on the cell membrane for root uptake and possibly transport to the shoot (DEMIDCHIK et al., 2007).

Urease activity was correlated with Ni concentration, as expected, in plants grown from Ni sufficient seeds; and with Ni-dosed plants, Ni supply was positively correlated with urease activity. The period of Ni supply did not statistically affect urease activity; however, it was possible to observe that in the treatment with Ni sufficient seeds, the at the 27-d point there was a minor decrease compared to the 20-d point. Plants grown from Ni sufficient seeds which received 0.85 µM Ni in solution had a smaller shoot biomass compared to the treatment that did not receive Ni treatment. Whereas in the treatment of Ni depleted seeds, the plants which received Ni and did not have the cotyledons removed grew higher biomass compared to the treatment in which the cotyledons were removed and did not receive Ni. Therefore, the seed Ni level in the cotyledons has an important impact on seedling growth.

Distribution of Ni in leaves after foliar application observed using synchrotron-based µXRF showed that the absorption of Ni occurred mainly via trichomes, specifically via the pedicle. Thus, this study illustrates the primary pathway by which foliar applied Ni moves from the trichome pedicles toward the leaf surface and then to other plant tissues. The processes whereby foliar-applied nickel moves across the leaf surface and is translocated throughout the plant are not well understood (BICKFORD, 2016), and major knowledge gaps in the physiological function of trichomes in taking up metal ions from foliar dosing remain.

5.5 Conclusions

Urease activity was correlated with Ni, as expected, in plants grown from Ni sufficient seeds; and with Ni-dosed plants, Ni supply was positively correlated with the urease activity. The presence of cotyledon also had resulted an increase of urease activity even in plants without Ni supply. Therefore, the low Ni concentration in the seed (<0.35 µmol of Ni) was enough to increase the urease activity compared to plants that have the cotyledon removed. Soybean plants without Ni supplied (0 µmol of Ni) the Ni depleted seeds had a decrease of biomass compared to the Ni sufficient seeds. Conversely, the plants that received Ni supply (0.85 µmol of Ni) Ni depleted seeds had an increase of biomass compared to Ni sufficient. The seed Ni level in the cotyledons has an important impact on seedling growth. Therefore, these data supporting the evidence for the essentiality of Ni for higher plants and boosting plants growth. The Ni foliar application observed using synchrotron-based µXRF showed that the absorption of Ni occurred mainly by trichomes, specifically via the pedicle. Thus, this study illustrates the primary pathway by which foliar applied Ni moves from the trichome pedicles toward the leaf surface and then to other plant tissues.

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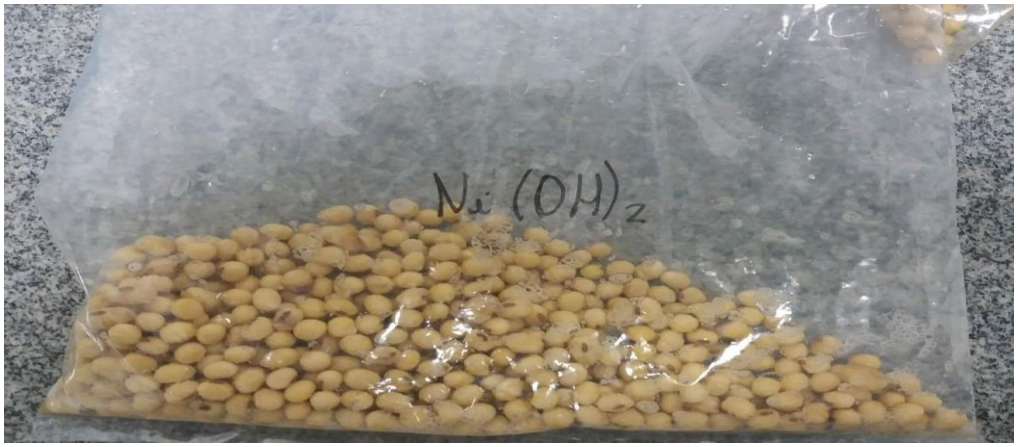
APPENDIX

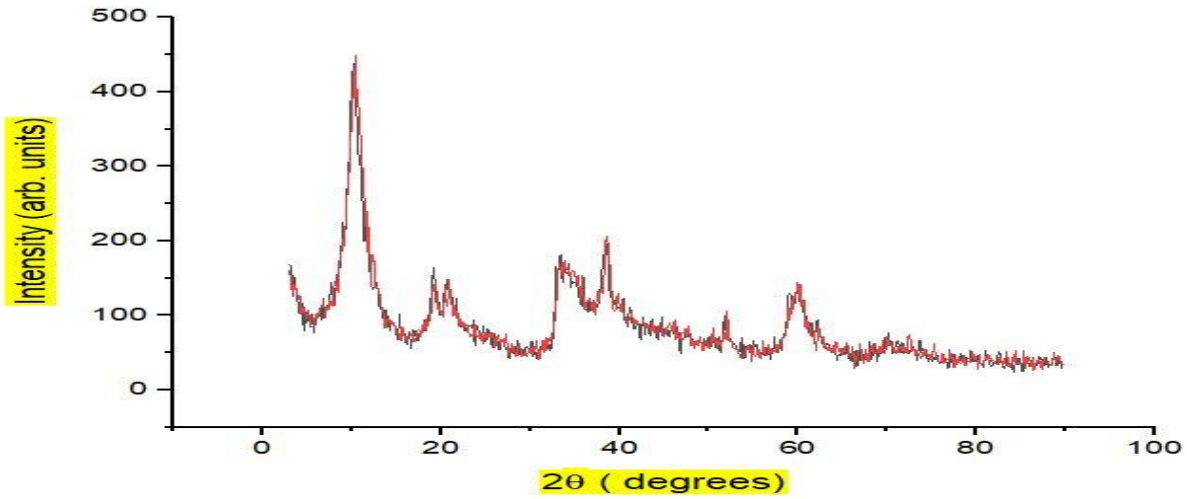
Material supplementary chapter 2: Do nickel hydroxide nano- microparticles or sulfate improve seed (*Glycine max)* germination? A multifacetated overview

Supplementary Figure 1. The seeds were soaked into the Ni based solution and dispersions, using the dose of 360 mg kg^{-1} of Ni, after put to dry

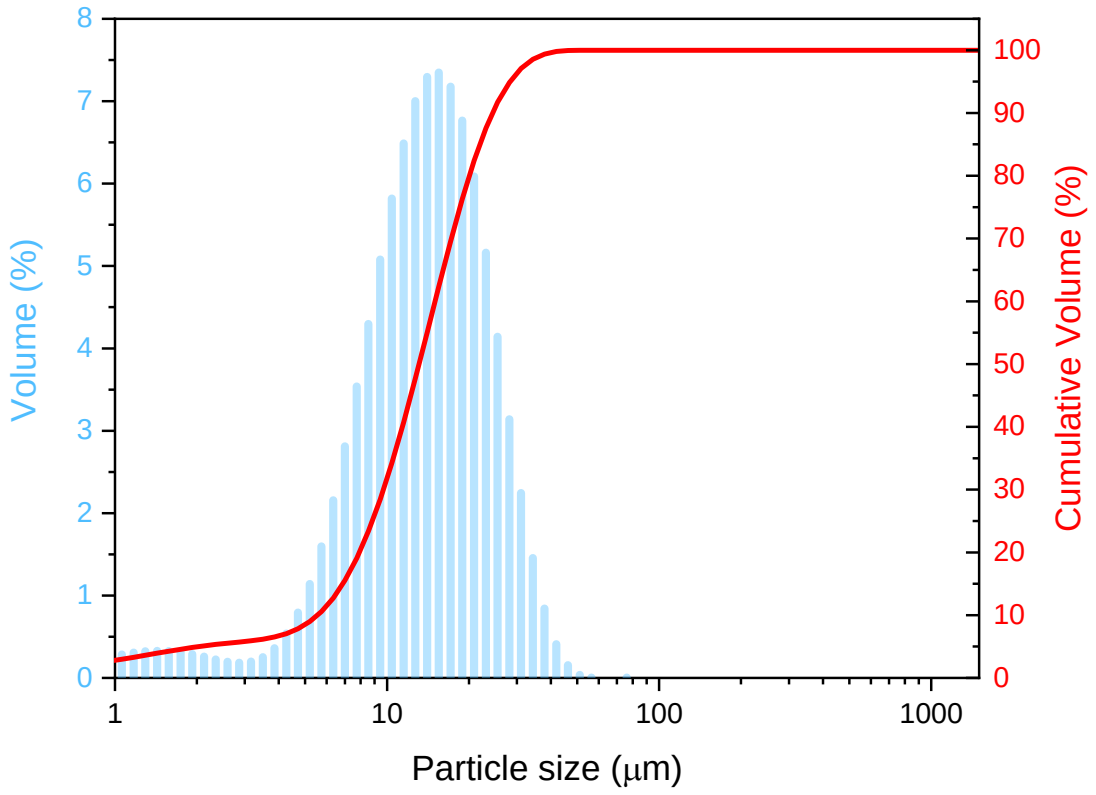


Supplementary Figure 2. Synthesis of nickel hydroxide by precipitation from Ammonia-induced precipitation. Ammonia solution (2 M, 100 mL) was added at the rate of mL min−1 to a nickel nitrate (1 M, 50 mL) solution placed in a thermostat. Separate precipitations were carried out at 4, 25 and 65 ◦C. The resultant slurries were aged in mother liquor for 18–28 h at the respective precipitation temperatures (Ramesh, 2006)

Supplementary Figure 3. The nickel hydroxid micrometric were characterized by XRD using either a JEOL Model JDX8P powder diffractometer



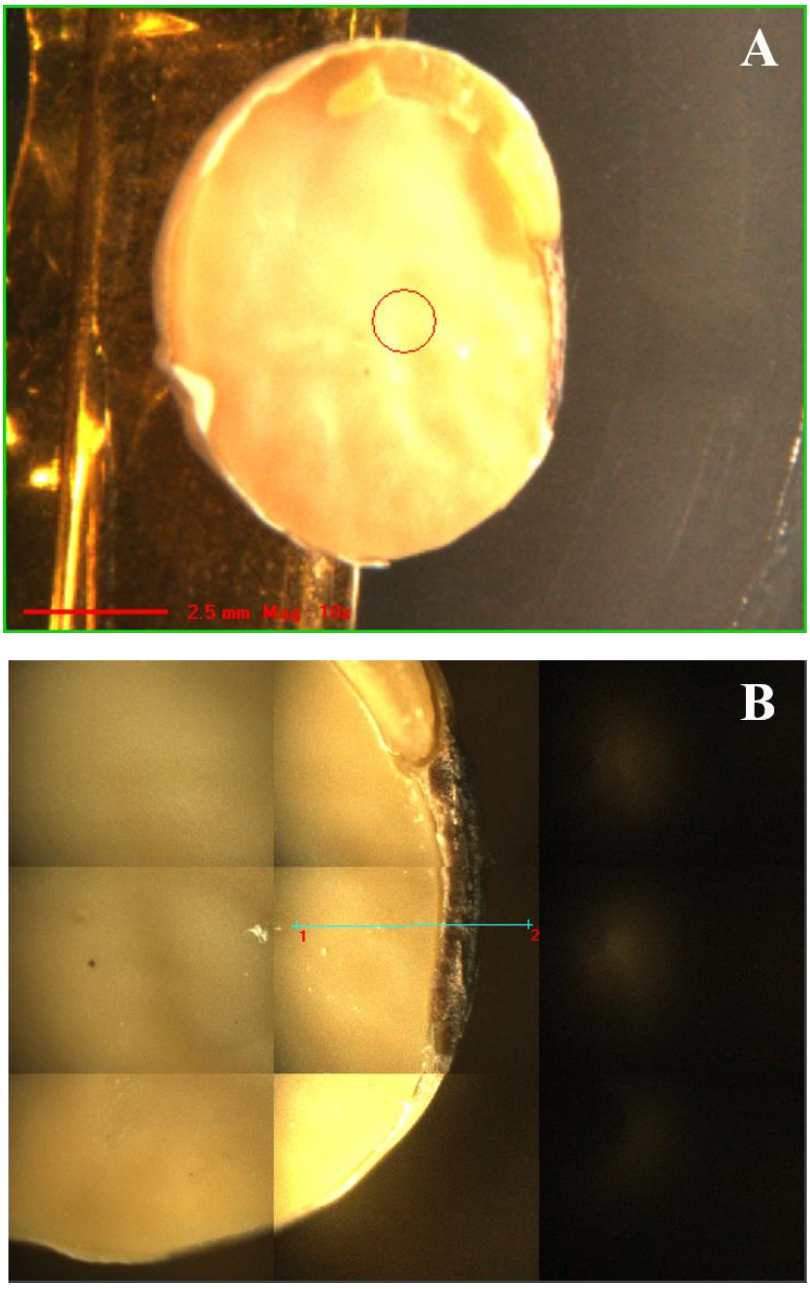
Supplementary Figure 4. Particle Size Analysis – $Ni(OH)_2$ by Analysette 22 MicroTec Plus, Fritsch



Dispersion in water

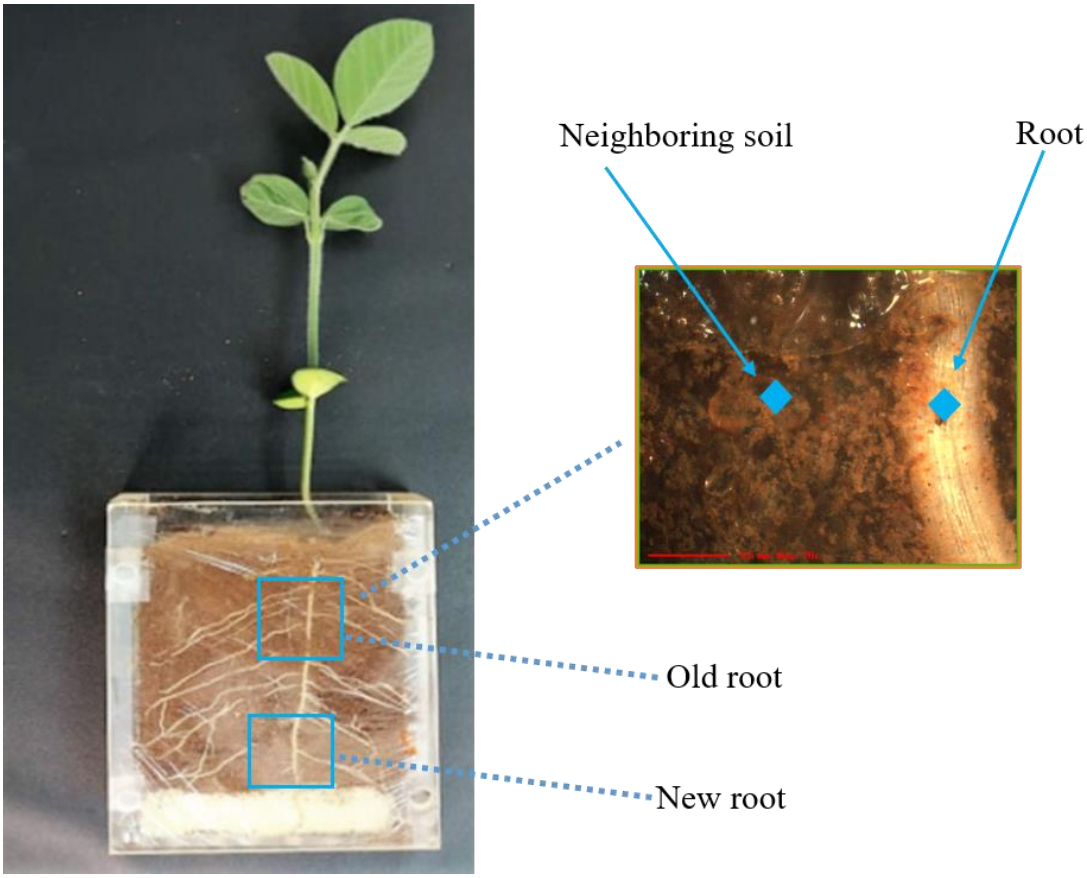
Cumulative Volume	Particle Size (µm)
10%	< 6
50%	< 13
90%	< 24
95%	< 28

Supplementary Figure 5. Seeds of soybean longitudinally sliced at the hilum region. (A) Overall vision; (B) Linescanned on the soybean seed

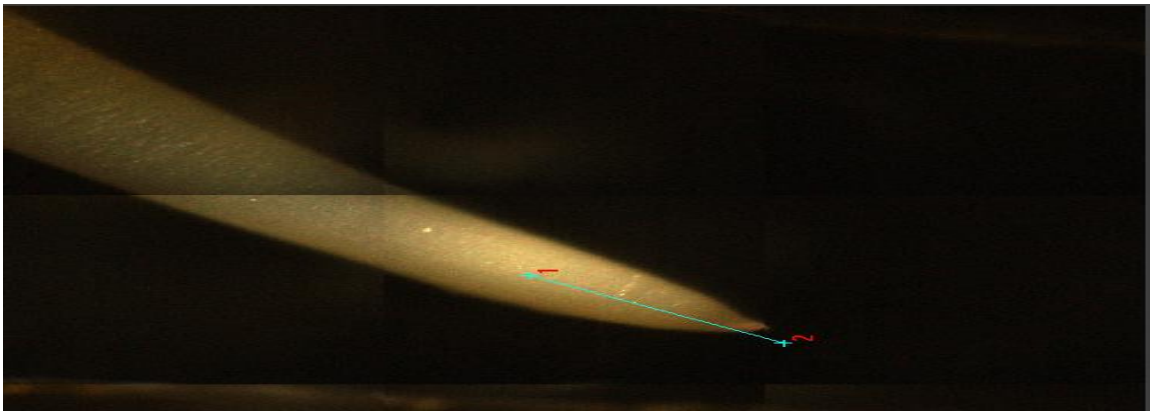


Supplementary Figure 6. Overall scheme show how was the experiment set up in rhizotron. The old and new roots were analyzed and the soil next to the roots were analyzed as well.

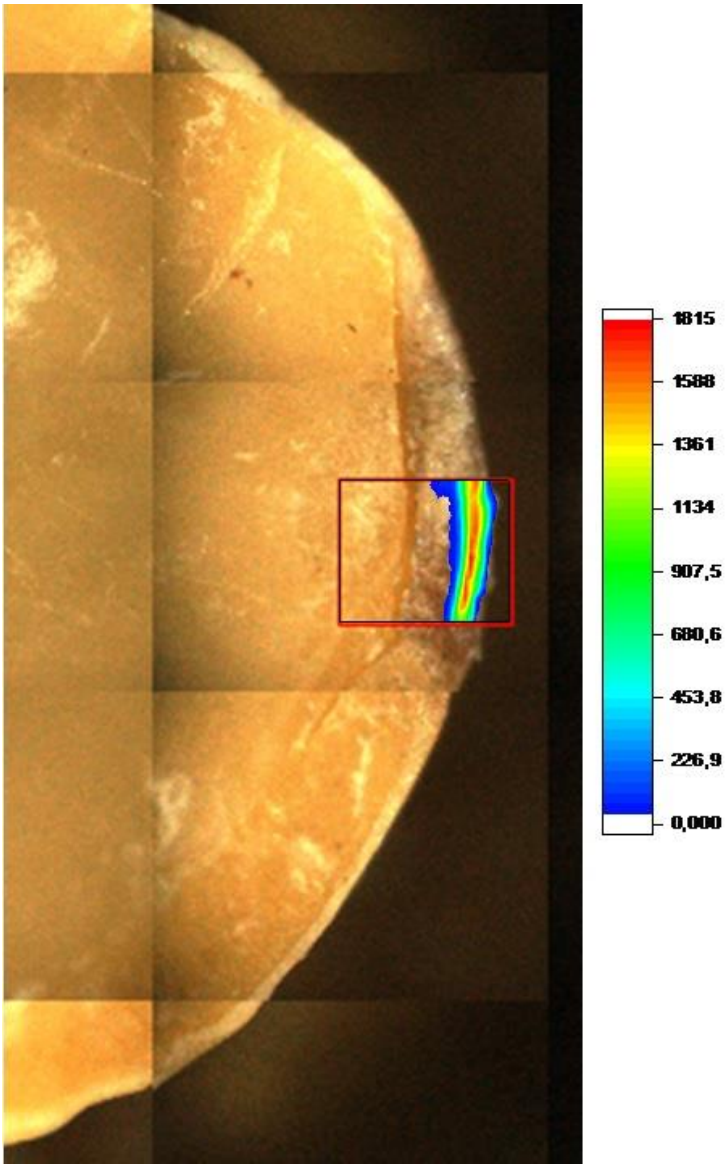
d



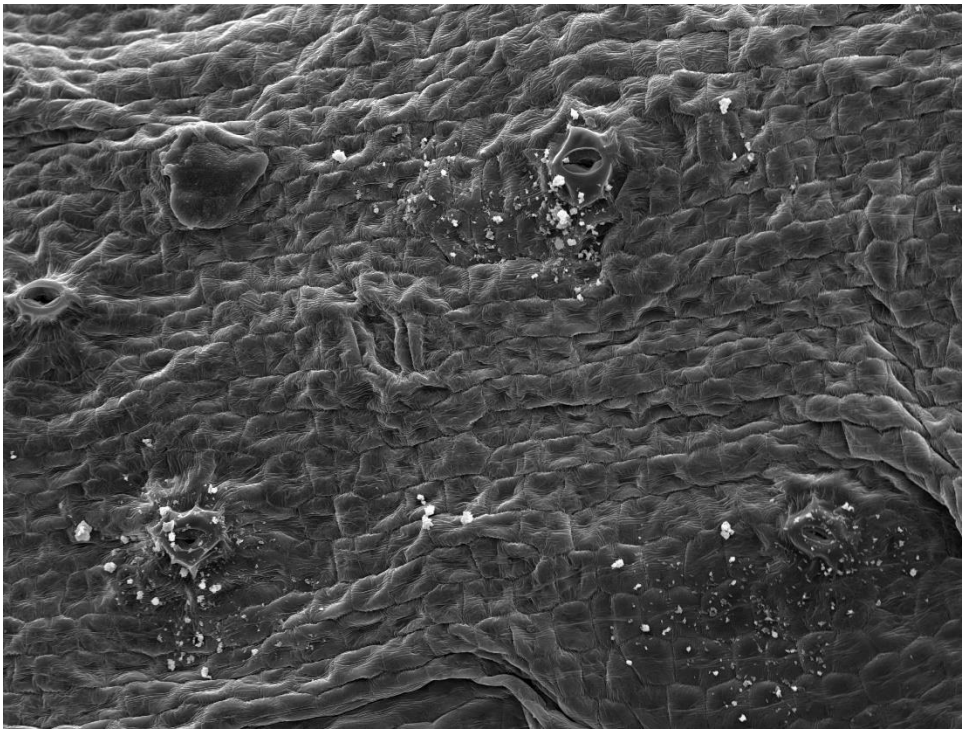
Supplementary Figure 7. Analyze by µ-XRF of tip of roots of soybean after 2 days of germination with different sources of Ni on the seed



Supplementary Figure 8. Mapping of seed treated with nickel sulfate – $NiSO_4$ by µ - XRF. The intense color indicate higher concentration of Ni and light colors indicate less Ni concentration. The most concentration of Ni is present in the hilum region



Supplementary Figure 9. Scanning electron microscopy of the tip of the root treated with Hydride nickel micrometric – $Ni(OH)_2$ – where it is possible to observe agglomerations of the source close to the stomata

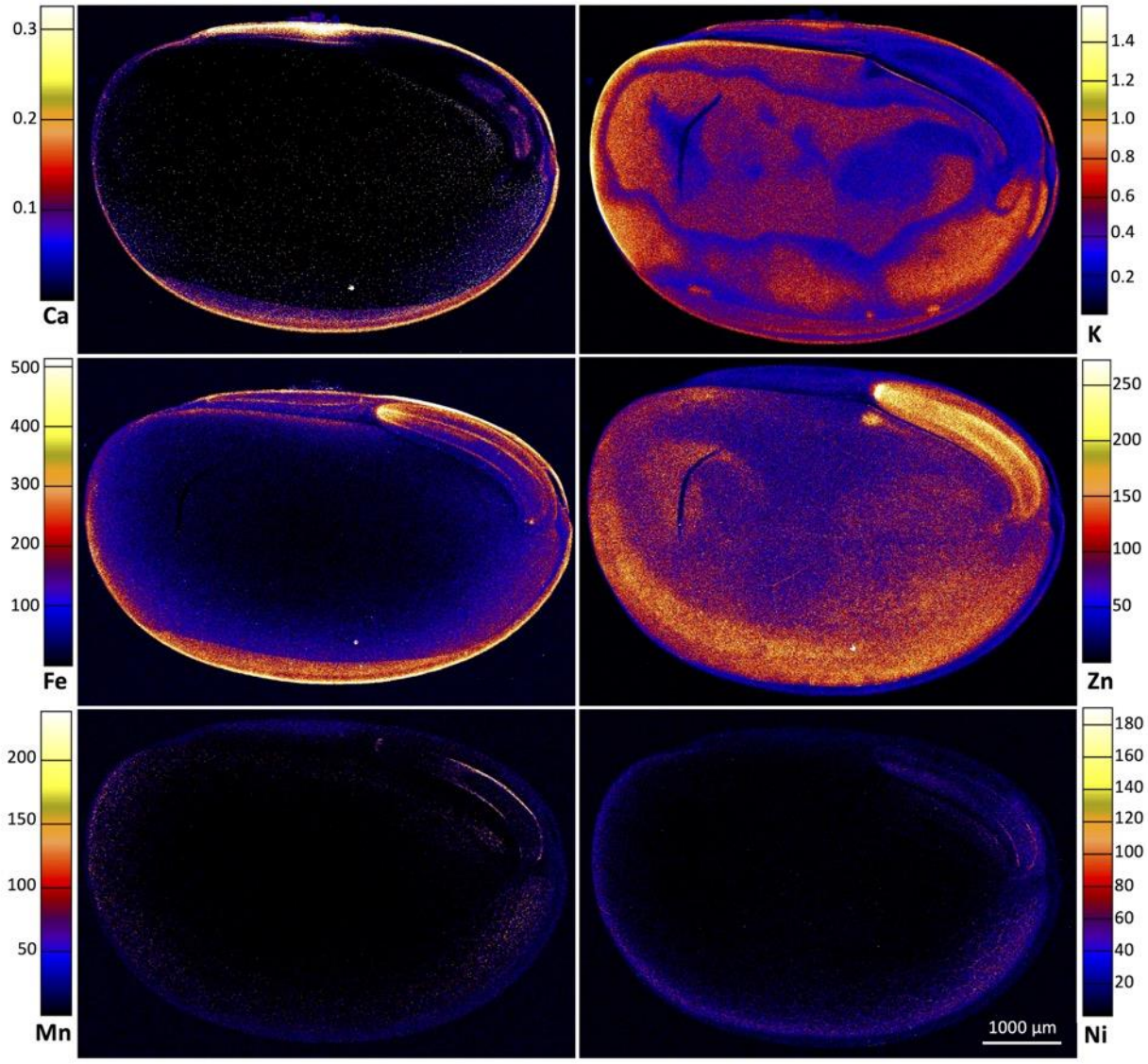


Material supplementary chapter 5: Synchrotron µXRF analysis of foliar-applied nickel in hydrated soybean leaves: shining light on *in situ* nickel leaf absorption and translocation.

Supplementary Figure 2. Images showing sample preparation. (A) Ni foliar application on young leaves soybean. (B-C) 5 µmol droplet on surface leaf soybean 100 mg Ni L^{-1}. (D) Droplet being washed with DI water after exposure time Ni sulphate. (E) Light micrographs showing more details of soybean leaf and trichomes. (F) Sample ready to be analysed at the XFM beamline

Supplementary Figure 2. Images showing mature plants in Exp #1 displayed mild Ni deficiency symptoms, including foliar chlorosis, especially at the leaf tips and margins, and some seed pods were infertile the soybean plant cultured in hydroponic solutions (A) health leaf (B) initial symptoms of necoriss lesions on leaflet tips (C)) advanced stage of necrosis in soybean leaf

Supplementary Figure 3. µ-XRF maps showing element distribution in soybean seed. The concentrations are in g/Lfor K and Ca and µg g^{-1} for Fe, Mn, Ni, and Zn with brighter colours corresponding to higher prevailing elemental concentrations



Supplementary Figure 4. Detailed µ-XRF scan showing trichomes on the leaf surfaces after foliar application of Ni on soybean (A-B). The color green indicates – Ni, blue – Mn and red- Ca. Figure (A) and (B) are the leaf surface after 25- and 45-min Ni foliar application, respectively