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Toxicity of copper hydroxide nanoparticles, bulk copper hydroxide, and ionic copper to alfalfa plants: A spectroscopic and gene expression study☆

Keni Cota-Ruiz [a, d], José A. Hernández-Viezcas [a, f], Armando Varela-Ramírez [b], Carolina Valdés [a], José A. Núñez-Gastélum [c], Alejandro Martínez-Martínez [c], Marcos Delgado-Rios [c], Jose R. Peralta-Videa [a, e, f], Jorge L. Gardea-Torresdey [a, e, f, *]

[a] Department of Chemistry and Biochemistry, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX, 79968, USA
[b] Border Biomedical Research Center, Department of Biological Sciences, University of Texas at El Paso, 500 West University Avenue, El Paso, TX, 79968, USA
[c] Departamento de Ciencias Químico Biológicas, Instituto de Ciencias Químico Biológicas, Universidad Autónoma de Ciudad Juárez, Anillo envolvente del PRONAF y Estocolmo s/n, Ciudad Juárez, Chihuahua, 32310, Mexico
[d] El Colegio de Chihuahua, Partido Díaz 4723 esquina con Anillo Envolvente del PRONAF, Ciudad Juárez, Chihuahua, 32310, Mexico
[e] Environmental Science and Engineering Ph.D. program, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX, 79968, USA
[f] UC Center for Environmental Implications of Nanotechnology (UC CEIN), The University of Texas at El Paso, 500 West University Avenue, El Paso, TX, 79968, USA

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ABSTRACT

Bulk Cu compounds such as $Cu(OH)_2$ are extensively used as pesticides in agriculture. Recent investigations suggest that Cu-based nanomaterials can replace bulk materials reducing the environmental impacts of Cu. In this study, stress responses of alfalfa (*Medicago sativa* L.) seedlings to $Cu(OH)_2$ nanoparticle or compounds were evaluated. Seeds were immersed in suspension/solutions of a $Cu(OH)_2$ nanoform, bulk $Cu(OH)_2$, $CuSO_4$, and $Cu(NO_3)_2$ at 25 and 75 mg/L. Six days later, the germination, seedling growth, and the physiological and biochemical responses of sprouts were evaluated. All Cu treatments significantly reduced root elongation (average = 63%). The ionic compounds at 25 and 75 mg/L caused a reduction in all elements analyzed (Ca, K, Mg, P, Zn, and Mn), excepting for S, Fe and Mo. The bulk-$Cu(OH)_2$ treatment reduced K (48%) and P (52%) at 75 mg/L, but increased Zn at 25 (18%) and 75 (21%) mg/L. The nano-$Cu(OH)_2$ reduced K (46%) and P (48%) at 75 mg/L, and also P (37%) at 25 mg/L, compared with control. Confocal microscopy images showed that all Cu compounds, at 75 mg/L, significantly reduced nitric oxide, concurring with the reduction in root growth. Nano $Cu(OH)_2$ at 25 mg/L upregulated the expression of the Cu/Zn superoxide dismutase gene (1.92-fold), while ionic treatments at 75 mg/L upregulated (~10-fold) metallothionein (MT) transcripts. Results demonstrated that nano and bulk $Cu(OH)_2$ compounds caused less physiological impairments in comparison to the ionic ones in alfalfa seedlings.

1. Introduction

Pesticides have been used to reduce crop losses caused by pests and diseases for a long time. Copper-based pesticides began to be used in late 1800 with the invention of the Bordeaux mixture, a combination of copper sulfate and calcium hydroxide, which is still in use (Oustriere et al., 2017). Over the years, other copper-based products including copper hydroxide ($Cu(OH)_2$), cuprous oxide (Cu_2O), and copper oxychloride ($Cu(OH)_2 \cdot CuCl_2$) have been extensively used in plant protection, leading to copper (Cu) accumulation in agricultural soils (Giannousi et al., 2013; Zuverza-Mena et al., 2015).

In addition to its use in agriculture, Cu is widely used in electricity, the automotive industry, and consumer products (Schipper

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* Corresponding author. Environmental Science and Engineering Ph.D. program, The University of Texas at El Paso, 500 West University Avenue, El Paso, TX, 79968, USA.
E-mail address: jgardea@utep.edu (J.L. Gardea-Torresdey).

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et al., 2018). This variety of applications generates a great discharge of Cu that may end in agricultural soils. According to the US Environmental Protection Agency, the land application of sewage sludge may contain up to 4300 mg Cu/kg (USEPA, 1994). Furthermore, with the increasing use of Cu-based nanoparticles (NPs) as catalysts (Gawande et al., 2016) and antimicrobials (Giannousi et al., 2013), crop plants are potentially exposed to high concentrations of different Cu forms, including nanoforms. It has been estimated that 15% of total Cu-based NPs produced, ends-up in soil (Keller and Lazareva, 2013).

Copper and other transition elements work as cofactors for important antioxidant enzymes such as superoxide dismutase (SOD) (Sunkar, 2006). They are also involved in biological roles including structural stabilization of nucleic acids and protein components (Mendoza-Cózatl et al., 2005; Zenk, 1996). However, at high concentrations, Cu causes impairments such as radicle growth shortening, photosynthesis inhibition, and reactive oxygen species (ROS) production (Gill et al., 2012; Mourato et al., 2009; Muccifora and Bellani, 2013; Wang et al., 2015). Additionally, an excess of Cu generates damages to proteins and lipids of the plasma membrane. This impacts the bi-layer permeability, producing imbalances in the concentration of elements and other small molecules (Quartacci et al., 2001).

Few studies have analyzed the effect of Cu-based NPs on seedlings at physiological and molecular levels. In *Oryza sativa*, CuO NPs at 0.5, 1.0 and 1 mM, caused oxidative damage and activation of the antioxidant system (Shaw and Hossain, 2013). Another study demonstrated that CuO NPs affected root growth, augmented ROS, and induced the expression of antioxidant genes in 14-day old green pea (*Pisum sativum* L.) seedlings (Nair and Chung, 2015). The foliar application of $Cu(OH)_2$ nanowires on two basil (*Ocimum basilicum* L.) varieties, differing in anthocyanin content, significantly reduced manganese (Mn) in leaves and roots of both varieties, while the interaction Cu products × variety showed an effect on essential oils and fatty acids (Tan et al., 2018).

The plant synthesis of the small signaling molecule nitric oxide (NO) is of great research interest. So far, two mechanisms have been documented regarding NO production: the reductive and the oxidative pathways (Moreau et al., 2010). The reductive mechanism involves the mitochondrial electron transport system and the multi-domain enzyme nitrate reductase (NR) (Kolbert et al., 2008; Moreau et al., 2010). Concerning the oxidative mechanism, recent evidence indicates that NO is synthesized from arginine by the enzyme nitric oxide synthase (NOS) (Barroso et al., 1999; Zhao et al., 2007). The accumulation of NO has been used as indicative of root growth and root hair formation (Anderson et al., 2017). Adams et al. (2017) proved that CuO NPs, at 10 mg/kg, increased NO in root hairs, which was associated with an increment in lateral surface area.

The performance of a plant is significantly driven by its development at early growth stages (Liao et al., 2004; Namuco et al., 2009). Disturbance in the physiological and biochemical machinery controlling the germination would affect the entire plant life (Miransari and Smith, 2014). Alfalfa (*Medicago sativa* L.) is considered an important plant because of its role in ecology, nutrimental value, and potentialities for heavy metal phytoremediation (Kabir et al., 2016). However, biochemical data of alfalfa seedlings under Cu-based nanomaterials (NMs) stress has not been previously reported. The data would be of great help to understand the behavior of alfalfa cultivated in Cu impacted soils. Thus, the objectives of this study were to assess and contrast the macro and micronutrient concentrations as well as the biochemical and molecular responses in alfalfa seedlings exposed to nano, bulk, and ionic Cu compounds. The inductively coupled plasma-optical emission spectroscopy (ICOES), fluorescence confocal microscopy, and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were used in this investigation to gain a deeper understanding on macro and microelements behavior, nitric oxide accumulation, and antioxidant gene expression, respectively, in alfalfa seedlings exposed to Cu compounds.

2. Methods

2.1. $Cu(OH)_2$ nanoparticle and chemicals

$Cu(OH)_2$ nanowire ($nCu(OH)_2$), bulk copper ($bCu(OH)_2$) (Kocide® 3000), ionic $CuSO_4$ (Spectrum Chemical®) and $Cu(NO_3)_2$ (Baker analyzed® A.C.S. reagent) were used in this study. Physicochemical properties of $nCu(OH)_2$ and $bCu(OH)_2$ have been previously described (Tan et al., 2018; Zuverza-Mena et al., 2015) and are shown in Table S1.

2.2. Plant cultivation and treatment

Alfalfa seeds (Mesa variety) were disinfected with 1% NaClO for 3 min, rinsed five times with Milli-Q water (MW), and air-dried for 3 h. The $nCu(OH)_2$ and the $bCu(OH)_2$ suspensions were sonicated in a water bath for 15 min at 23 °C (Crest Ultrasonics, Trenton, NJ Model 275 DA; 120 V, 3 A, 59/60 Hz). Subsequently, 35 seeds were placed in individual Petri dishes (100 mm × 15 mm) containing germination paper. The suspensions/solutions of the copper compounds were added. Treatments consisted of 25 and 75 mg/L of Cu from each compound and MW was used as a control (no Cu added). Each treatment was replicated five times. The dishes were covered with aluminum foil and settled for 6 day at 20 °C. Then, seedlings were removed from the Petri dishes, washed thoroughly with MW, measured from the crown to the root tip, and prepared for different analyses.

2.3. Elemental analysis

For the elemental analyses, the seedlings were oven dried for 72 h at 72 °C and digested (100 mg/sample) for 45 min at 115 °C with 4 mL of plasma pure HNO_3, using a DigiPREP MS digestion block (SCP Science, NY). Digests were adjusted to 50 mL with MW and analyzed for macro and micronutrients using an ICP-OES; Perkin-Elmer Optima 4300 DV. For quality control/quality assurance, blanks, spikes, and the reference material NIST-SRM1570a (spinach leaves) were read as samples. A multi-element standard solution was read every 10 samples. In addition, the recovery percentage and the detection limits for each analyzed element are shown in Table S2. The detection limit for the elements were determined by reading eight replicates of the blank. The mean, plus three standard deviations (μ ±3 SD) was in the range of 50 μg/L.

2.4. Microscopic analysis

The fluorescent dye 4-amino-5-methylamino-20, 70-difluorofluorescein diacetate (DAF-FM DA) was used as a dye to determine NO accumulation. DAF-FM DA diffuses inside living cells where it is deacetylated (DAF-FM) by endogenous esterases. DAF-FM increases fluorescence intensity by reacting with NO. The 6-days old root tips from different treatments were carefully washed with MW and incubated in DAF-FM DA 10 μM at 37 °C for 40 min. Then, roots were thoroughly washed with MW and tip segments of approximately 4 mm were cut for further confocal microscopy analysis. High-resolution digital fluorescence images were captured using an LSM 700 confocal microscope (Zeiss, New York, NY) by utilizing an excitation/emission wavelength of 495/515 nm. Unstained and untreated root samples were analyzed to eliminate the auto-fluorescence in fluorescently labeled tissues. At

least three independent experiments were performed for each treatment and representative images are depicted. To evaluate significant differences between all the experimental samples, regions of interest (ROIs) were delineated within captured images and the mean of fluorescence intensity signal was recorded. Images from each experiment were equally processed and analyzed using the ZEN 2009 software (Zeiss, New York, NY).

2.5. RT-qPCR

For gene expression analysis, total RNA was extracted using the PureLink™ Plant RNA Reagent (Invitrogen™), following the manufacturer instructions. The RNA was quantified in a NanoDrop spectrophotometer (Thermo Scientific). RNA quality and integrity were confirmed by 260/280 Abs ratio and by 1% agarose gel electrophoresis. RNA was treated with RNase-free DNAse I (Roche) at 37 °C for 10 min. A sample of 200 ng was reverse transcribed using the Enhanced Avian RT First Strand Synthesis Kit (Sigma). Final cDNA concentration was of 10 ng/μL regarding the initial RNA.

Quantitative PCR assays were done using a StepOnePlus™ Real-Time PCR. Target analyzed genes were: Cu/Zn superoxide dismutase (Cu/Zn SOD) gene (GenBank accession no. XM_003626314.3) and metallothionein (MT) gene (GenBank accession no. AF189766.1). Translation Initiation Factor IIA was used as reference gene (Guerriero et al., 2014). PCR reaction tubes (triplicates) contained iQ™ SYBR® Green Supermix (Biorad) and primers (800 nM) in 20 μL final volume. PCR conditions were as described: 40 cycles at 95 °C for 3 s and 60 °C 30 s, including a final melting curve program from 60 °C to 95 °C. Primers for Cu/Zn SOD were: SOD forward, 5′–CCTGAGGATGAGACTCGACA-3′ and SOD reverse, 5′-GAACAACAACAGCCCTTCCT-3'; as previously reported (Santos et al., 2013). Primers for TFIIA were: TFIIA forward, 5′-GGCATGTGACTCGAAATTGC-3′ and TFIIA reverse, 5′-ATGCTGGTTCCTGCAAGAAC-3′ (Guerriero et al., 2014). Primers for MT were designed based on Primer 3 Software (http://bioinfo.ut.ee/primer3-0.4.0/): MT forward, 5′-TGCAACAGCAGGTCTAGTGG-3′ and MT reverse, 5′-GGGTCACATGTGCAGCTATC-3'. PCR efficiencies were performed by running standard curves with cDNAs as templates. Data were analyzed following the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.6. Statistical analysis

The Shapiro-Wilk test was used to assess the normality of the data. When necessary, the data were normalized using the Box-Cox algorithm. One way ANOVA, followed by Tukey HSD test comparison with an error α at $p \leq 0.05$ were performed. The Bonferroni correction was applied for the multiple comparisons. Analyses were done using the statistical package OriginPro 2018.

3. Results and discussions

3.1. Copper absorption

The Cu concentration in seedlings is shown in Fig. 1. As expected, in all treatments, Cu was significantly higher, compared with control. In addition, Cu in root increased as the external Cu concentration increased. At 25 mg/L, ionic treatments had, in average, more Cu (7.14 ± 0.3 μg/mg DW (Dry Weight) mass), compared with $bCu(OH)_2$ (4.45 ± 0.33) and $nCu(OH)_2$ (4.32 ± 0.39) ($p \leq 0.01$). At 75 mg/L, roots of seedlings exposed to ionic compounds had 10.9 ± 0.49 and 10.65 ± 0.21 μg of Cu/mg DW, for $CuSO_4$ and $Cu(NO_3)_2$, respectively; while roots from $bCu(OH)_2$ and $nCu(OH)_2$ had 7.68 ± 0.26 and 6.95 ± 0.54, μg of Cu/mg DW, respectively.

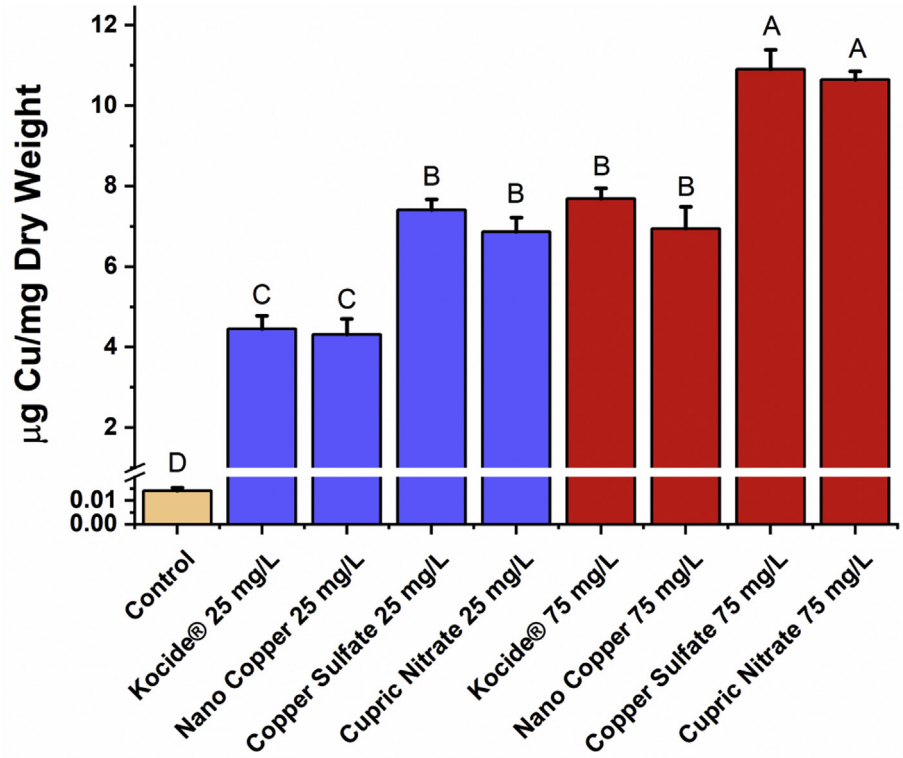

Fig. 1. Copper concentration in alfalfa seedlings exposed for 6 days to Kocide® ($bCu(OH)_2$), nanowire ($nCu(OH)_2$), copper sulfate ($CuSO_4$), and cupric nitrate ($Cu(NO_3)_2$) at 25 and 75 mg/L. MW was used as a control. Error bars are the standard deviation (SD) of five independent samples per treatment. Different letters indicate significant differences at $p \leq 0.05$.

Although some of the particles could remain on root surface after washing, the highest Cu concentration was found in plants exposed to ionic Cu. This suggest that the available Cu ions were effectively absorbed by the seedlings. Similar results were reported by Hong et al. (2015).

3.2. Effect of Cu NPs/compounds on root elongation

Treatment effects on root elongation is shown in Fig. S1. As seeing this figure, all treatments significantly reduced root elongation, compared with control ($p \leq 0.005$). Ionic compounds had more effects than particulate compounds. In average, at 25 and 75 mg/L, $CuSO_4$ and $Cu(NO_3)_2$ reduced root elongation by 63% and 68%, compared with $bCu(OH)_2$ and $nCu(OH)_2$, respectively. These results suggest that the reduction in root elongation was linked to the Cu availability. Excess Cu^{2+} ions possess high redox properties, which causes disruptions on many subcellular components, including DNA damage (Atha et al., 2012). Cu^{2+} interferes with primordial metabolic processes such as respiration and photosynthesis (Yruela, 2009). Thus, by affecting cellular respiration, availability of energy for root elongation may potentially be compromised. Additionally, as it will be discussed later on, ionic treatments greatly reduced Ca concentration. Since Ca works as a messenger to promote growth and development, impairments such as shortening in root growth, are associated with low Ca concentrations (Hepler, 2005). Our results are in agreement with a previous study on alfalfa where 40 mg/L of Cu^{2+} significantly reduced root growth by approximately 70% against control (Aydinalp and Marinova, 2009). In wheat seedlings, it was found that Cu treatments (in both nano and ionic forms) significantly reduced root length (Adams et al., 2017). In *Arabidopsis thaliana*, root elongation was severely reduced after 7 days of exposure to 50 μM $CuSO_4$ (8 ppm) (Lequeux et al., 2010). However, contrary to our results, the seedling growth in maize (*Zea mays* L.) was inhibited by nCu but not by bulk or ionic compounds (Wang et al., 2012). This corroborates that the toxicity of Cu depends on the compound, dose, and plant species (Tan et al., 2018).

3.3. Macro and micronutrient concentration

The effect of $bCu(OH)_2$, $nCu(OH)_2$, $CuSO_4$ and $Cu(NO_3)_2$ on macro (Ca, K, Mg, P, and S) and micro (Zn, Fe, Mo and Mn) element concentrations in alfalfa seedlings is shown in Fig. 2. Ionic treatments caused a significant reduction in all elements analyzed, except S, Fe, and Mo. On the other hand, $bCu(OH)_2$ and $nCu(OH)_2$, depending on their concentration in the medium, caused differential impacts on macronutrients and Zn concentration.

3.4. Macronutrient concentration

The Ca concentration in alfalfa seedlings was significantly reduced ($p \leq 0.005$) by ionic treatments, compared with control (2.10 μg Ca/mg DW) (Fig. 2A), while bulk and nano copper compound did not affect Ca. Calcium reduction (Fig. 2A), mainly in presence of ionic compounds, could be explained by the overproduction of hydroxyl radicals ($OH^{\cdot}$) near to the cell wall and within chloroplasts of plant cells due to Cu excess (Rodrigo-Moreno et al., 2013). Additionally, increased concentrations of hydrogen peroxide (H_2O_2) has demonstrated to affect Ca channel permeability in root cells of *Arabidopsis* (Demidchik et al., 2007). In our

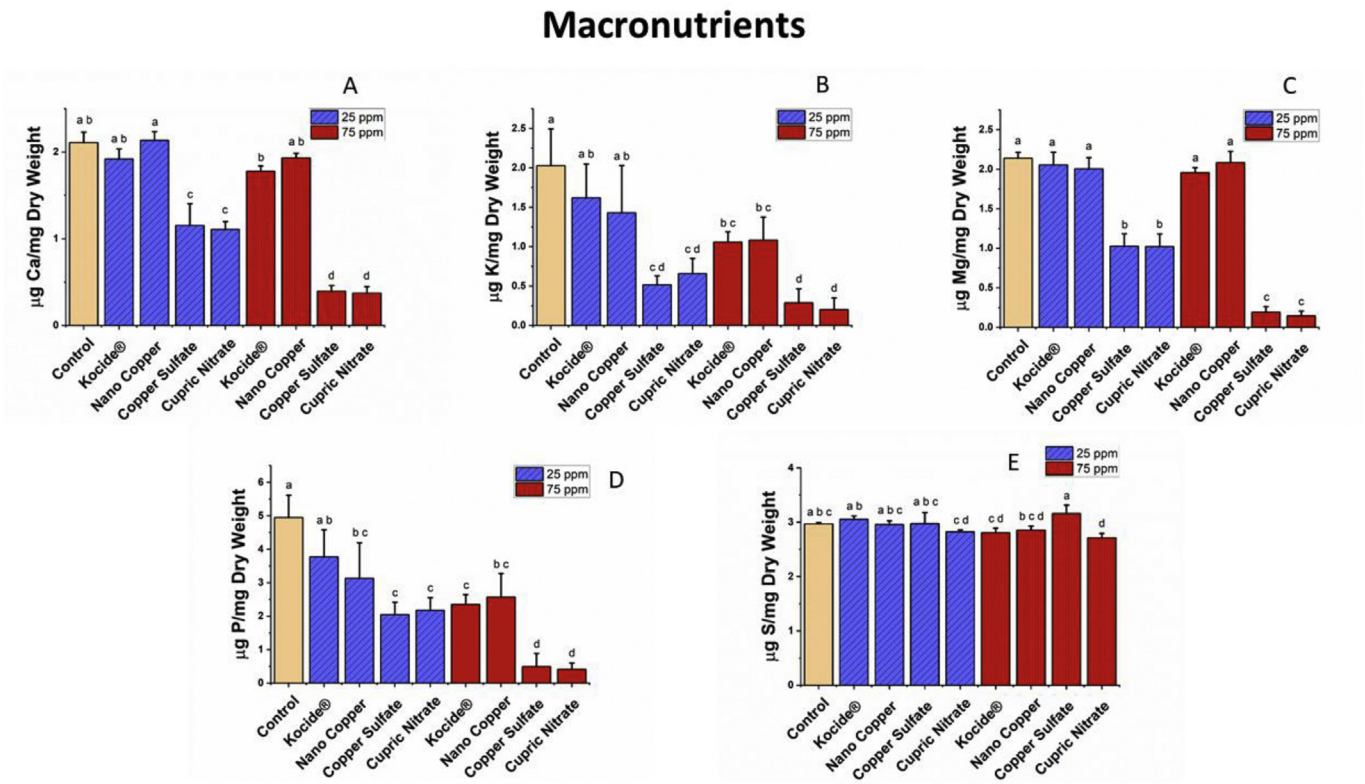

Fig. 2. Macro and micronutrient concentrations in alfalfa seedlings treated for 6 days with Kocide® ($bCu(OH)_2$), nanowire ($nCu(OH)_2$), copper sulfate ($CuSO_4$), and cupric nitrate ($Cu(NO_3)_2$) at 25 and 75 mg/L. Graphs are divided into macro: A) Ca, B) K, C) Mg, D) P, and E) S; and micronutrients: F) Zn, G) Fe, H) Mo and I) Mn. "Y" axis refers to μg of the element per mg of dry weight (DW). MW was used as a control. Error bars are the standard deviation (SD) of five independent samples per treatment. Different letters indicate significant differences at $p \leq 0.05$.

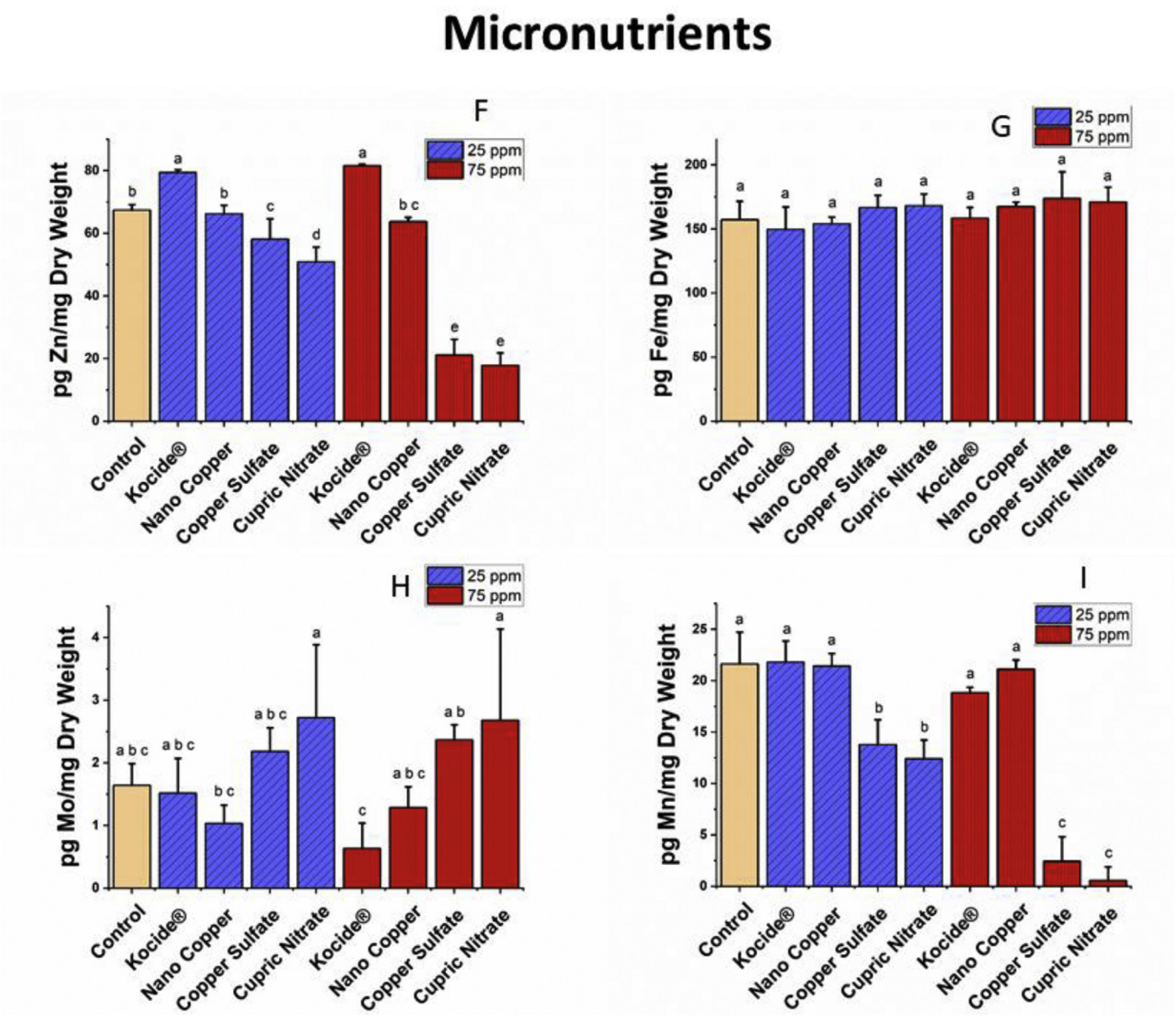

Fig. 2. (*continued*).

study, the possible accumulation of ROS caused by Cu excess could lead to cellular Ca-leakage. Thus, we believe that major impairments in Ca homeostasis are due to Cu^{2+} from ionic compounds. Österås and Greger (2006) found that Cu excess decreased Ca in root tissue of *Picea abies*.

The concentration of K in alfalfa seedlings (Fig. 2B) was significantly reduced by all ionic treatments and by $bCu(OH)_2$ and $nCu(OH)_2$ at 75 mg/L ($p < 0.001$). The highest K reduction was produced by ionic treatments at 75 mg/L, where it dropped up to 10-fold (0.2 ± 0.15 and 0.29 ± 0.18 μg K/mg DW, for $Cu(NO_3)_2$ and $CuSO_4$, respectively), compared with control (2.03 ± 0.47 μg/mg). Murphy et al. (1999) provided pioneer evidence that Cu^{2+} led to K^+ loss in *Arabidopsis* cell roots through K^+ efflux channels. In such a study, authors reported that an excess of Cu^{2+} led to citrate increase. To impede the poisonous citrate accumulation, cells exhibited citrate efflux, accompanied with K^+ efflux, which acts as counterion. At 75 mg/L, particulate Cu materials caused K^+ reduction. The reduction could be produced by a mere physical interaction, a rejection of the K^+ ions by the positive zeta potential of the nanowires, or a complexation by the negative zeta potential of the bulk compound. Previous literature has shown that Cu causes overproduction of H_2O_2, which damages the lipid bilayer, ending in K^+ leakage (Demidchik, 2015). Thus, if particulate compounds attached to cell membrane, there should be K leaking by either disturbing membrane permeability (affecting K^+ efflux channels) or by releasing Cu ions to the cell cytoplasm (Demidchik, 2014). Similar results were found in young spinach plants (20 old-days) exposed to 160 μM Cu (from $CuSO_4.5H_2O$) for a 7-day period, K was diminished 3.5 fold ($p < 0.005$) compared with control (Ouzounidou et al., 1998). In radish, K content was significantly reduced when copper ($CuSO_4 \cdot 7H_2O$) was applied at higher doses than 50 mg/kg (Vijayarengan and Uthayam, 2017). However, the effect of Cu on K reduction seems to be tissue-plant species specific since K was unaffected in roots from 20-days old of *Cucumis sativus* young plants after they were exposed to 20 mg of Cu ($CuCl_2$) per kg of sand. On the contrary, in that experiment, a significant reduction in K was found in leaves in comparison to the control (Alaoui-Sossé et al., 2004).

The concentration of Mg in alfalfa seedlings was lowered by ionic treatments at both treatment concentrations (Fig. 2C), while it remained unaffected by $bCu(OH)_2$ and $nCu(OH)_2$ treatments. So far, there are no investigations on the effect of particulate Cu and the cellular mechanism linked to Mg accumulation/releasing. However, a possible mechanism involved in Mg leakage could be similar to that of Ca, since both Ca and Mg are similar in charge. Additionally, Cu ions could be involved in Mg releasing. In this study, Cu in seedlings was basically the same at 25 mg/L of ionic treatments and at 75 mg/L of $bCu(OH)_2$ and $nCu(OH)_2$ (Fig. 1). Contrary, Mg was significantly lowered in ionic treatments, which suggest that Cu speciation is affecting Mg homeostasis. Since Cu and Mg have comparable chemical ionic forms, Cu can work as Mg competitor occupying many prosthetic groups originally available for Mg-dependent proteins (Maksymiec, 1997). Thus, depletion of Mg can be explained by the false message of no longer needed, being released by stressed cells.

The P concentration in seedlings was significantly decreased in all treatments but not by $bCu(OH)_2$ at 25 mg/L ($p \leq 0.05$) (Fig. 2D). Compared to $nCu(OH)_2$, this could occur due to the moderate release of Cu^{2+} of $bCu(OH)_2$ as demonstrated by dissolution experiments (Adeleye et al., 2014). P is an essential macronutrient involved in crucial process such as ATP production. As previously mentioned, Cu^{2+} affects cellular respiration in mitochondria, thus, it is expected that depletion in ATP would lead to a P releasing from cells. Also, damages caused by an excess of Cu could lead to the release of P as phosphate ions through compromised plasmalemma (Ait-Ali et al., 2002; Smith, 2001). Reduction of P concentration was also reported by Hong et al. (2015) in alfalfa and lettuce roots treated with nano, bulk, and ionic Cu compounds. When compared to the other macroelements (Ca, K, Mg, and S) evaluated in this study, P was the only element affected by all concentrations of the different Cu compounds tested. This result indicates that Cu compounds affect many diverse physiological processes such as DNA metabolism, signal transduction, membrane phospholipids and metabolites stabilization in which P is participating (Mourato et al., 2009). Since these processes are affected or down-regulated, P is no longer needed and thus excreted to the medium. Phosphorus is a key mineral limiting growth in terrestrial plants (Vessey, 2003). Thus, P depletion leads to root shortening (Fig. S1).

Sulfur was statistically reduced only by 75 mg/L of $Cu(NO_3)_2$ (2.71 ± 0.08 μg S/mg DW), compared to the control (2.97 ± 0.03 μg S/mg DW) (Fig. 2E). This is possibly caused by Cu ions availability. It has been demonstrated that plant cells exposed to high copper concentrations, increase their synthesis of sulfur-rich phytochelatins (Shahbaz et al., 2010), which are involved in heavy metal detoxification tasks. Additionally, availability of S promotes the production of sulfur-rich compounds (such as cysteines); these molecules participate in buffering Cu^{2+} in the cytoplasm by binding Cu^{2+} ions (Burkhead et al., 2009; Yruela, 2009). In our study, alfalfa seedlings showed S concentration stability under Cu stress, which suggest it is taking an active role in defense responses.

3.5. Micronutrient concentrations

The effect of different copper compounds on the concentration of micronutrient in alfalfa seedlings (Zn, Fe, Mo, and Mn) is depicted in Fig. 2 (2F, 2G, 2H, and 2I).

The concentration of Zn was increased at 25 mg/L and 75 mg/L of $bCu(OH)_2$ treatments (79.42 ± 0.86 and 81.58 ± 0.49 pg Zn/mg DW, respectively) compared to the control (67.39 ± 1.75 pg Zn/mg DW). As commented before, the dissolution of Cu from $bCu(OH)_2$ occurs at a slow rate (Adeleye et al., 2014), which probably reduces Cu ions uptake by seedlings. Contrary, ionic treatments caused a reduction in Zn content at 25 and 75 mg/L (Fig. 2F). A comparison between treatments showed no significant differences in Cu content between $bCu(OH)_2$ at 75 mg/L and at $CuSO_4$ at 25 mg/L (Fig. 1), while Zn content was markedly increased by the former treatment (Fig. 2F). The same behavior was observed in seedlings exposed to $bCu(OH)_2$ and $nCu(OH)_2$ at 25 mg/L; Cu concentration did not change (Fig. 1), but Zn concentration rises in the bulk treatment (Fig. 2F). Thus, it seems that the form of Cu inside seedling cells is biasing Zn response, since Zn was significantly augmented by $bCu(OH)_2$ in comparison to the others treatments. The negativity of the $bCu(OH)_2$ (as per the zeta potential value) could be involved in Zn increasing. The positive charge of Zn can be attracted by $bCu(OH)_2$ compounds and retained in cells. An increase in Zn concentration may be used by antioxidant proteins to cope with important physiological functions in stressed cells. For instance, when cells are subjected to heavy metal exposure, reactive oxygen species (ROS) are promptly produced causing oxidative damage. Under these circumstances, superoxide dismutases (SODs) represent an immediate “line of protection” against ROS. Some particular class of SODs uses Zn/Cu as their cofactors (Alscher et al., 2002), which could explain the accumulation of zinc in order to respond to this affectation. Finally, drastic fall in Zn concentration caused by ionic compounds at 25 and 75 mg/L evidence detrimental impacts of these compounds on alfalfa seedlings physiology.

The concentration of manganese was only affected by ionic compounds which correlate with the observed pattern for Ca, K, P, Mg, and Zn. Since Mn plays active role as protein cofactor in the glucose metabolism and in the fatty acids synthesis (Ma et al., 2018), the possible downregulation of these processes, as consequence of Cu ions overloaded, could lead to Mn release. On the other hand, some antioxidant enzymes including a form of SOD, use Mn as a cofactor (Alscher et al., 2002); thus, under $bCu(OH)_2$ and $nCu(OH)_2$ stress conditions, Mn concentration probably remains unaffected to stabilize protein function.

3.6. Nitric oxide production

Nitric oxide was significantly accumulated in higher amounts in control group than the rest of the treatments (Fig. 3A). The ROIs analysis showed a significant reduction in signal intensity in all treatments, compared with control ($p \leq 0.05$) (Fig. 3F). NO production/accumulation tended to fall from $nCu(OH)_2$, 3.74 fold-reduction (Fig. 3C), to $bCu(OH)_2$, 7.26 fold-reduction (Fig. 3B), and to ionic treatments, 10.56 fold-reduction in average (Fig. 3D and E). Currently, there are no reports that explain the differences associated to these compounds. Nevertheless, possibly Cu ions are involved. For instance, the major decrease in NO concentration was caused by Cu ionic treatments, which correlate with shortenings of root lengths registered under same comparable treatments (Fig. S1). This latter suggest that NO has an active role in root elongation and it is affected by Cu. Moreover, since an excess of Cu causes imbalances in the respiratory chain, possibly, in this study Cu is affecting NO synthesis by interfering in this process (Xie et al., 2013). Also, within the reductive pathway, NO can be enzymatically produced by the nitrate reductase (NR), that uses nitrate as substrate (Kolbert et al., 2008). This enzyme is regulated by post-translational phosphorylation and de-phosphorylation mechanisms (Sharma and Shanker Dubey, 2005). In this study, it was shown before that Cu treatments reduced P availability, which suggests that low P content affects NR activity with a concomitant reduction in NO. On the other hand, NOS activity is highly stimulated by Ca^{2+} (Crawford, 2006). Since ionic Cu severely reduced Ca in alfalfa seedlings, the NO reduction observed in this study, could be affected by NOS down-regulated activity. However, further measurement of NR and NOS enzymatic activities under these

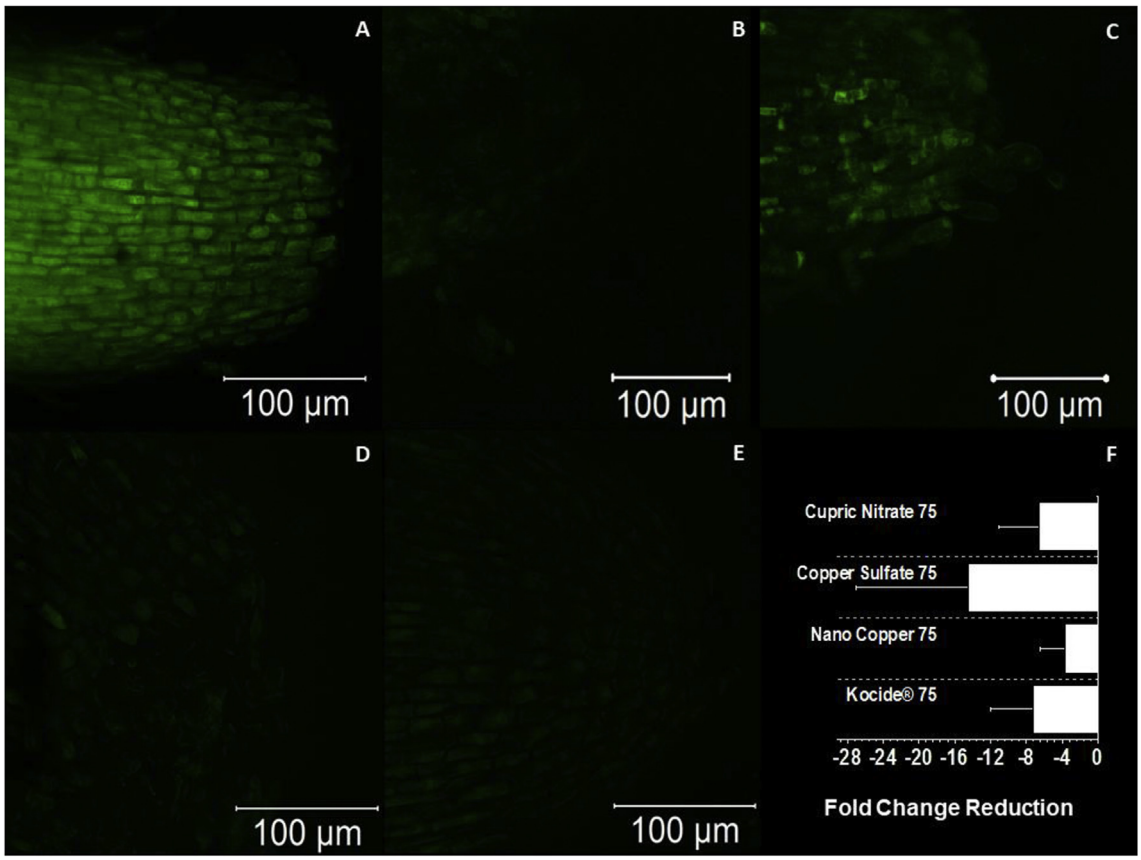

Fig. 3. Confocal microscopic images showing nitric oxide (NO) production/accumulation in 6-day old roots of alfalfa seedling germinated and grown under exposure to A) Millipore water (Control), B) Kocide® ($bCu(OH)_2$), C) nanowire ($nCu(OH)_2$), D) copper sulfate ($CuSO_4$), and E) cupric nitrate ($Cu(NO_3)_2$), at 75 mg/L. F) Denotes regions of interest (ROIs) analysis, expressed as fold change reduction in mean ± SD (error bars) fluorescence intensity regarding the control (all treatments were different against control but not among them at $p \leq 0.05$). Nitric oxide detection was revealed by the dye DAF-FMDA. Images are representatives of at least three independent studies.

circumstances need to be performed for conclusive evidence. In agreement with these observations, 6-day old radish seedlings subjected to 500 mg/L of nano Cu (nCu), accumulated Cu, as demonstrated by scanning transmission electron microscopy-energy dispersive spectroscopy (STEM-EDS), which was correlated with a concomitant radish root reduction (Atha et al., 2012). That study also showed that ionic treatments caused major impairments in root development. Additionally, a reduction in NO accumulation was reported in 14-days old pea roots that were treated with 50 µM of $CdCl_2$ (Rodríguez-Serrano et al., 2006). Interestingly, a recent investigation showed that impairments caused by NMs, its released ions, and/or heavy metals, can be mitigated when exogenous NO is supplied, improving also the antioxidant response (Tripathi et al., 2017).

3.7. Cu/Zn SOD and MT expression

Significant up-regulation of Cu/Zn SOD (1.92 fold) was found in alfalfa seedlings exposed to 25 mg/L of $nCu(OH)_2$ ($p \leq 0.013$) in comparison with control group (Fig. 4A). Since no significant Cu uptake was observed by 25 mg/L of either $nCu(OH)_2$ or $bCu(OH)_2$, the increase in mRNA levels observed for the former treatment, was possibly driven by their particular zeta potential values. The positive value of the $nCu(OH)_2$ (24.1 ± 0.32 mV), compared with the negative $bCu(OH)_2$ (−40.9 ± 2.7 mV), may reject positive ions such as Zn, making them available to proteins that participate in the overall gene expression process. For instance, Zn is used by the Zn finger proteins that play crucial roles in the regulation of the transcription (Ciftci-Yilmaz and Mittler, 2008). The rest of the treatments did not affect the expression of Cu/Zn SOD gene (Fig. 4A). As Cu in excess interferes with many metabolic functions and leads to oxidative stress (Mourato et al., 2009), in our study, it was demonstrated that alfalfa seedlings respond to this physiological threatening by increasing and/or keeping their expression of SOD in a Cu compound/doses dependent manner. The SOD differential expression observed in this investigation can occur by the presence of different SODs, which use different microelements as cofactors: Cu/Zn-dependent SOD found in the cytosol and also in chloroplasts, Fe-dependent SOD found in plastids, and Mn-dependent SOD found in mitochondria (Gill et al., 2012). In this study, we focused on the study of Cu/Zn SOD. Taking into consideration that the Fe content was not affected by any of the Cu treatments (Fig. 2G), a possible scenario where Fe-dependent SOD are up-regulated to help cells to combat ROS might occur. However, it needs to be experimentally demonstrated. Additionally, there is a lack of information about the presence of Cu/Zn SOD isoforms in *M. sativa*, which can also be differentially expressed in response to different forms of compounds, or different compound concentrations. For instance, the presence of three Cu/Zn SOD isoforms that are expressed in different organelles or the occurrence of up to four different Cu/Zn SOD isoenzymes, have been reported in *A. thaliana* and in *Zea mays*, respectively (Yruela, 2009). Therefore, the use of transcriptomics to analyze broad expression of genes and its isoforms involved in Cu stress responses will be very helpful to gain a deeper knowledge on the plant cell responses to heavy metal stress.

In a recent report, Nair and Chung (2014) reported that the

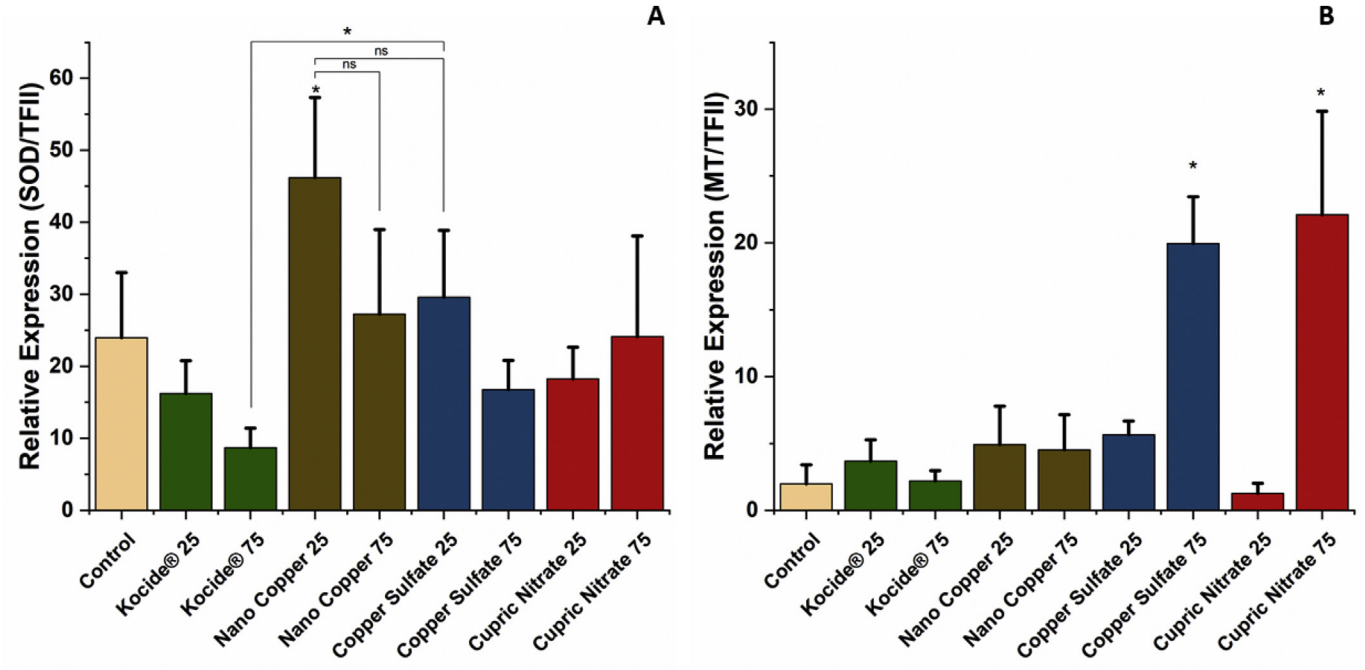

Fig. 4. Expression of antioxidant genes in alfalfa seedlings exposed for 6 days with 25 and 75 mg/L of Cu NPs/compounds. The relative expression of (A) Cu/Zn superoxide dismutase (Cu/Zn SOD) and (B) metallothionein (MT) have been normalized to translational factor protein (TFIIA). There were five cDNAs per treatment and each cDNA was amplified by triplicates. Bars show the mean + SD of five samples (organisms) per treatment. The * stands for a significant difference, compared with control ($p \leq 0.05$).

expression of CuSOD gene was up-regulated in young *A. thaliana* plants exposed at 2–5 mg/L of nCuO. Subsequently, a reduction in transcripts content was observed as nCuO increased (10–20 mg/L). In our study, the same pattern was observed since $nCu(OH)_2$ induced the expression of CuSOD gene at 25 mg/L, followed by transcripts reduction when $nCu(OH)_2$ concentration was increased. Additionally, the activity of SOD has been reported to be augmented in tomato seedlings at 100 mg/L of CuO NPs (Singh et al., 2017), and in wheat seedlings treated with 200 mg/L of Al_2O_3 NPs (Riahi-Madvar et al., 2012). Interestingly, higher doses of the corresponding NPs caused no effect or a decrease in the enzyme activity. Although in our experiments we did not include enzymatic activity, a possible negative feedback caused by a fall in enzymatic activity could control the amount of the corresponding SOD transcripts. Thus, futures studies considering enzymatic activities will be important to describe the role of the enzymatic antioxidant machinery under Cu stress conditions.

Metallothioneins are proteins capable of binding heavy metals including Cu, Zn, and Cd. Thus, they act as metal "regulators". They also show the ability to counteract ROS due to their high cysteine content (Mustafa and Komatsu, 2016). Here, we detected an up-regulation in mRNAs levels in $CuSO_4$ (10.05 Fold-change) and $Cu(NO_3)_2$ (11.12 Fold-change) treatments at 75 mg/L, compared to the control (Fig. 4B). Remarkably, we observed that ionic treatments caused detrimental impacts to alfalfa root physiology (Fig. 3D and E), macroelement content (Fig. 2) and root development (Fig. S1), which correlates with the higher copper concentrations found inside seedling cells (Fig. 1). At higher doses of ionic treatments (75 mg/L), we detected a significant induction of MTs transcripts. Thus, MT molecules are over-expressed to counterattack damages caused by copper excess. These results are in accordance with a previous study where Zn^{2+} from ZnO NPs triggered the increment in MT transcripts in *A. thaliana* roots (Landa et al., 2012). In 14-day old young wheat plants, roots expressed MT in higher amounts when seedlings were exposed to high Ag ions concentration (2.5 mg Ag/kg) or Ag NPs (2.5 mg/kg) (Dimkpa et al., 2013).

4. Conclusions

This study gives information about the effect of Cu stress on alfalfa seedlings at physiological and molecular levels. Major impairments were caused when alfalfa seedlings were exposed to ionic treatments. It was demonstrated that 25 mg/L doses of all treatments reduced root elongation. Excluding Zn and P, the macro and micronutrient content were not affected when seedlings were exposed to bulk or nano Cu compounds at 25 mg/L. At 75 mg/L, all Cu compounds affected root elongation and NO production. Ionic compounds caused major reduction in both root growth and NO concentration. The concentration of K and P were reduced at all 75 mg/L treatments. Also, alfalfa seedlings activated the gene antioxidant machinery by up-regulating Cu/Zn SOD and MT expression depending on the Cu stress to deal with deleterious impairments such as ROS production. Further studies, which include Cu speciation inside cells, enzyme activities, as well as the use of "omics" to explore the broad expression of genes, will be helpful to understand and potentially improve alfalfa responses to Cu stress.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.09.028.

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