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ORIGINAL RESEARCH

Nanomaterials modify the growth of crops and some characteristics of organisms from agricultural or forest soils: An experimental study at laboratory, greenhouse and land level

Los nanomateriales modifican el crecimiento de los cultivos y algunas características de organismos de suelos agrícolas o forestales: Un estudio experimental a nivel laboratorio, invernadero y campo

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ABSTRACT

Currently, some concerns regarding the potential toxicity of nanoparticles (NP) on the environment have emerged. The effect of ZnO, TiO_2, and Fe_2O_3 NP on corn (*Zea mays* L.), common beans (*Phaseolus vulgaris* L.), nanobioremediation of polycyclic aromatic

hydrocarbons (PAH), and soil organisms from agricultural or forest soils was studied at laboratory, greenhouse, and land level. The samples were analyzed by X-ray diffraction (XRD), field emission scanning electron microscopy with X-ray energy dispersion spectrometry (FESEM-EDS), scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDS) gas chromatography (GC), ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) and Fourier transform infrared spectrometry with attenuated total reflectance (FTIR-ATR). ZnO-NP did not harm the mycorrhizal root colonization but, the presence of ZnO-NP decreased the degradation of PAH. The synthesis of metabolites from corn was more affected by the PAH than by ZnO-NP. FTIR spectra showed that NP affected the synthesis of compounds from specific functional groups in common bean plants. Fe_2O_3-NP were attached to the body of forest-soil organisms and significantly increased the concentration of Fe in their body, while TiO_2-NP changed the morphological tissue of roots and stems of common bean as witnessed by micrographs of longitudinal and cross-sections. The NP used in this research significantly changed some response variables on the experiments carried-out at laboratory, greenhouse, and land level.

Keywords: crop production, environmental pollution, phytoremediation, nanotoxicity, PAH degradation, soil organisms.

RESUMEN

Actualmente han surgido algunas preocupaciones con respecto a la toxicidad potencial de las nanopartículas (NP) en el medio ambiente. Se estudió el efecto de las NP de ZnO, TiO_2 y Fe_2O_3 en el maíz (*Zea mays* L.), frijoles comunes (*Phaseolus vulgaris* L.), nanobiorremediación de hidrocarburos aromáticos policíclicos (PAH) y organismos de suelos agrícolas o forestales a nivel de laboratorio, invernadero y campo. Las muestras se analizaron por difracción de rayos X (XRD), microscopía electrónica de barrido de emisión de campo con espectrometría de dispersión de energía de rayos X (FESEM-EDS), microscopía electrónica de barrido con espectroscopia de rayos X de dispersión de energía (SEM-EDS), cromatografía de gases (GC), cromatografía líquida de ultra alto desempeño acoplada a espectrometría de masas (UPLC-MS) y espectroscopia infrarroja por transformada de Fourier con reflectancia total atenuada (FTIR-ATR). Las ZnO-NP no tuvieron un efecto negativo en la colonización de la raíz micorrízica, pero la presencia de ZnO-NP disminuyó la degradación de los PAH. La síntesis de metabolitos del maíz fue más afectada por los PAH que por las ZnO-NP. Los espectros de FTIR mostraron que las NP afectaron la síntesis de compuestos de grupos funcionales específicos en plantas de frijol común. Las Fe_2O_3-NP se unieron al cuerpo de los organismos del suelo forestal y aumentaron significativamente la concentración de Fe en su cuerpo, mientras que las TiO_2-NP cambiaron la morfología del tejido de raíces y los tallos del frijol común, como lo demuestran las micrografías de las secciones longitudinales y transversales. Las NP utilizadas en esta investigación cambiaron significativamente algunas variables de respuesta en los experimentos llevados a cabo a nivel de laboratorio, invernadero y campo.

Palabras clave: Contaminación ambiental, degradación de PAH, fitorremediación, nanotoxicidad, organismos del suelo, producción de cultivos.

1. INTRODUCTION

Hundreds of different kinds of nanoparticles (NP) have been synthesized during the last years, and these have specific properties never seen before. TiO_2-, Fe_2O_3-, and ZnO-NP have been widely applied in industry in several products, such as paints, colorants, plastic cosmetics, cleaning, and personal care products (Fan *et al*., 2014; Jacobs *et al*., 2010). TiO_2-NP is an allowed, and registered additive by the Food and Drug Administration of the United States of America (FDA; E171) classified as save and "biologically inert" (human and animals) (Rodriguez-Escamilla *et al*., 2019). However, there is no available information regarding the regulatory status of Fe_2O_3-, and ZnO-NP, though the FDA stated that there remains a need to learn more about the potential role and importance of dimensions in the physical and chemical characteristics and biological effects exhibited by FDA-regulated products that involve the application of nanotechnology (FDA, 2014). In the USA, the production of TiO_2-NP could reach 2.5 million tons by 2025 (Robichaud *et al*., 2009). TiO_2-, Fe_2O_3-, and ZnO-NP could affect plants and soil organisms, and it has become a big concern because of their poorly documented toxicity effects (Kibbey & Strevett, 2019; Medina-Pérez *et al*., 2018; Medina-Perez & Fernández-Luqueño, 2018).

The latest studies about the behavior of some NP showed that they cause effects via activation of oxidative stress by producing cell damage, and also the activation of several defense mechanisms, like immune response or inflammation in mammalian cells (Iavicoli *et al*., 2011). Positive and negative effects of titanium compounds have been reported in different crops including the increase of other elements in plant tissue, effects on enzyme activity, and increase in other metabolites such as chlorophyll (Feizi *et al*., 2012). At certain concentrations, the NP of TiO_2, Fe_2O_3, and ZnO improve the physiological responses such as germination, antioxidant and enzymatic activity, chlorophyll synthesis and growth of different plants (Feizi *et al*., 2012, Suriyaprabha *et al*., 2012). The biological activity and biokinetics of nanoparticles depend on parameters such as size, shape, chemistry, crystallinity, surface properties (area, porosity, charge, surface modifications, coating), agglomeration state, biopersistence, and dose (Casals *et al*., 2008). However, other authors reported negative or absent effects. Asli and Neumann (2009) found that TiO_2-NP inhibits foliar growth and respiration, as well as root development affecting the transport system in corn seedlings. Contradictorily, Boonyanitipong *et al.* (2011) applied TiO_2-NP to germinated rice seeds (*Oryza sativa* L.) and did not find effect on root length.

The objective of this manuscript was determinate the effect of ZnO-, TiO_2-, and Fe_2O_3-NP on corn (*Zea mays* L.), common beans (*Phaseolus vulgaris* L.), nanobioremediation of a PAH-polluted soil, and soil organisms from agricultural or forest soils at the laboratory, greenhouse, and land level. These studies were carried out through the use of cutting-edge technologies such as X-ray diffraction (XRD), field emission scanning electron microscopy with X-ray energy dispersion spectrometry (FESEM-EDS), scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDS) gas chromatography (GC), ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) and Fourier transform infrared spectrometry with attenuated total reflectance (FTIR-ATR).

2. MATERIALS AND METHODS

This manuscript briefly describes four experiments in lab, greenhouse, and land, but in each experiment NP with the same characteristics were used; actually, the NP were synthesized in the same production batch.

2.1 Vegetable materials and arbuscular mycorrhizal fungi

Corn (*Zea mays* L.) seeds, variety Jaguan, were provided by the Department of Phytotechnics of the 'Universidad Autónoma Agraria Antonio Narro.' Besides, basic seeds of common beans (*Phaseolus vulgaris* L.) pinto Saltillo variety were also used, which were acquired in the 'Campo Experimental Saltillo' from the 'Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP).' The seeds were kept refrigerated until their use but, two days before their use the seeds were washed with 70% ethanol [v/v] and 2.5% sodium hypochlorite [v/v].

Arbuscular mycorrhizal fungi (AMF) inoculum was obtained from a native fungal strain sampled from the agricultural soil, which was the same to experiment. The inoculum was propagated in common wheat roots (*Triticum aestivum* L.). The harvested inoculum consisted of a mixture of rhizospheric soil, fine root fragments, hyphae, and spore.

2.2 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) such as anthracene and phenanthrene were purchased to Sigma Aldrich, with 97 and 98% of purity, respectively. The experimental design and procedures regarding nanobioremediation technology in a PAH-polluted soil are described in the section 2.5.

2.3 Soil sampling and physicochemical characteristics of soils

Agricultural soil was sampled from an experimental field (0-25 cm depth) in the 'Universidad Autónoma Agraria Antonio Narro.' The sampling site is located at 25° 21' 30.51" North latitude and 101° 02' 22.20" West longitude.

2.4 Physicochemical characterization of NP and the preparation of the suspensions

Metal oxide nanoparticles such as ZnO, TiO_2, and Fe_2O_3 were purchased to 'Investigación y Desarrollo de Nanomateriales (ID-nano).' XRD technique was used to identify crystalline phase of nanoparticles, with CuKα radiation (1.54 Å) in the range of 20 a 75 (2θ). The morphological characterization was carried out in a FESEM trademark JEOL (JSM-7800F) with 10.0 kV acceleration voltage, in the same equipment, determined chemical compositions by EDS. The chemical suspensions were prepared in 10 mL of deionized water using an ultrasonic bath.

2.5 Experimental design and procedures regarding phytonanoremediation technology in a PAH-polluted soil

The experiment was carried out under greenhouse conditions. Twelve treatments were established (Table 1), with a factorial design and a completely random distribution. The levels of three factors were: 1) Arbuscular mycorrhizal fungus (AMF) (with or without); 2) PAH (with or without a mixture of anthracene and phenanthrene), and 3) ZnO-NP (0, 150 or 300 mg kg^{-1} dry soil). The concentrations of PAH and NP where defined according to

previous studies carried out in our facilities (Fernández-Luqueño *et al*., 2016; Sánchez-López *et al*., 2019).

Table 1. Treatments design to evaluate the effect of ZnO-NP in the degradation of anthracene and phenanthrene (PAH) from a polluted agricultural soil inoculated with arbuscular mycorrhizal fungus (AMF).

Treatment	Description
T1	Soil^{Ø} + Plant
T2	Soil + Plant + PAH$^{€}$
T3	Soil + Plant + AMF
T4	Soil + Plant + ZnO-NP↓
T5	Soil + Plant + ZnO-NP↑
T6	Soil + Plant + PAH + ZnO-NP↓
T7	Soil + Plant + PAH + ZnO-NP↑
T8	Soil + Plant + AMF + ZnO-NP↓
T9	Soil + Plant + AMF + ZnO-NP↑
T10	Soil + Plant + PAH + AMF
T11	Soil + Plant + PAH + AMF + ZnO-NP↓
T12	Soil + Plant + PAH + AMF + ZnO-NP↑

^{Ø} 3.5 kg dry soil; $^{€}$ 200 mg anthracene plus 400 mg phenanthrene kg^{-1} dry soil; ↓= 150 mg ZnO-NP kg^{-1} of soil dried; ↑= 300 mg ZnO-NP kg^{-1} of soil dried.

The treatments were established for triplicate in polypropylene bags. Five days before the onset of the experiment, the experimental units were watered to saturation and allowed to drain by three days. Later phenanthrene and anthracene were added but, previously dissolved in acetone, mixed into the soil and left to evaporate for 48 hours. Three corn seeds were sown for each experimental unit. The mycorrhizal inoculum was added at the time of planting, and the ZnO-NP suspension was added three days after the emergence of the seedlings.

2.5.1 *Roots analysis*

For evaluation of the AMF colonization, AMF structures in roots were stained using the method from Phillips & Hayman (1970). The roots were cut in 1 cm segments and soaked in 1 M KOH solution at 90 °C for 15 minutes after acidified in 1 % [v/v] HCl and then stained with trypan blue 0.01% [v/v]. For each treatment ten root segments were placed on slides for triplicate, the colonization percent and frequency percentage of association indicators were determined according to the method described by Sánchez de Prager *et al*. (2010).

2.5.2 *Preparation for metabolomic profiling*

The root was completely separated from the soil and washed at the time of destructive sampling, subsequently treated with liquid nitrogen. Extracts plants were prepared according to the method of Rendón-Anaya *et al*. (2017). Mixing 50 mg vegetal material in 1000 µL methanol and formic acid solution (75% [v/v] and 0.15% [v/v]). The mixture was sonicated for 15 min in a water bath at maximum frequency and centrifuged at 10,000 g for 10 min at 4 °C. The extraction evaporated. Before the analysis the extracts were

reconstituted whit one mixture of water and methanol (70% [v/v] and 30% [v/v]).The supernatant was filtered through a 0.22 µm filter for the analysis.

2.5.3 *LC/MS analysis*

Liquid chromatography (LC) was performed on an Acquity UPLC system trademark Waters, coupled to mass spectrometry (UPLC-MS) equipped with an electrospray ionization source and a single quadrupole analyzer. The extracts samples was analyzed with BHC C18 analytical column (130Å, 1.7 µm, 2.1 mm × 150 mm), the injection flow rate of 10 µL, while the elution of the compounds was carried out at a flow rate of 500 µL min^{-1}, where the mobile phase (A) corresponded to a 0.1% water solution of formic acid, as mobile phase (B) acetonitrile with 0.1% formic acid was used, the latter was supplied in a linear gradient of 2 to 100%. The retention time was 16 min, using 4 min for washing and rebalancing the column between sample and sample (Rendón-Anaya *et al*., 2017).

2.5.4 *Statistical Analyses*

The data were subjected to an analysis of variance (ANOVA), and means were compared with the Tukey test, using Statistic Software. Statistical significance was determined at $P<0.05$. The metabolomic data analysis the spectrum were transformed to standard mass and processed using a Progenesis QI, for comparison with the control using means compared to t-student. We employed a MetaboAnalyst for Partial least square (PLS) with cross-validation.

2.6 Effect of nanoparticles on common bean (*Phaseolus vulgaris* L.)

The source, disinfection, storage and use of seeds of the common bean are described in subsection 2.1. Three different nanoparticles were used in this test *i.e.*, TiO_2, ZnO, and Fe_2O_3 which were described in subsection 2.4.

2.6.1 *Greenhouse experiment*

An experiment was carried out under greenhouse conditions, for 60 days. Seven treatments were used with 18 replicates each, obtaining a total of 126 experimental units (EU), which were distributed in a completely randomized design. Each EU consisted of a black plastic nursery bag (30 × 24.5 cm). Each bag was filled with three kg of dry soil. Three days before planting, each EU was irrigated with drinking water to saturate the soil and allowed to stand for three days, after this time, the doses of TiO_2, ZnO, and Fe_2O_3, NP were applied in concentration of 0, 150 and 300 mg kg^{-1} of dry soil. The NP were added using the methodology described by Kibbey & Strevett (2019), which consists in using the NP as they were received from the supplier, adding to the soil in a suspension in 10 mL of distilled water without sonicating. After adding the NP to each EU, the soil was vigorously mixed, and three disinfected seeds of *Phaseolus vulgaris* L. were planted. During sowing the seeds were distributed homogeneously in each EU at a depth of 1.5 cm and tap water was added (250 mL).

During their stay in the greenhouse, irrigation was carried out with tap water according to the needs of the crop, as well as with additional irrigation with 250 mL of the nutrient solution once a week, as well as the complete rotation of the EU to ensure the same growth conditions. At 15 days after sowing (DAS) a thinning was done to adjust the number of plants leaving a single plant for each EU.

2.6.2 *Destructive sampling and data collection*

Two samples were taken during the permanence of the greenhouse experiment, at 30 and 60 DAS, which consisted of selecting nine EU at random of each treatment. To each unit, the soil column was removed, taking care not to damage the root system, which was washed with common water and then with distilled water. Subsequently, the stems and root will be separated, and the length of the stem and root measured, in addition to counting root nodules.

Once this has been done, the root system and stems of three randomly selected plants will be placed in paper bags and dehydrated in a forced air oven at 70 °C for 72 h, then a record of the dry weight of each sample was done. Of the remaining plants, samples of leaf, stem, and root were taken for later analysis by the techniques of FTIR-ATR and SEM-EDS.

2.7 Effect of NP on macroinvertebrates from forest soil

2.7.1 *Experimental description*

This experiment was conducted in forest soil in Santa Rita, municipality of Arteaga, Coahuila. The type of soil corresponds to a sandy loam soil (92% sand, 6.0% silt and 2.0% clay) with an electrical conductivity (EC) of 446.13 μS cm^{-1}, pH 7.5 and 3.06% organic matter (MO). The NP of Fe_2O_3 were described in section 2.4. In the forest, an area of 48 × 50 m was allocated, within which five points of 4 m^2 were assigned. At each point, the treatments were randomly assigned with three replicates each. The treatments were control (0), 1, 10, 100 and 1000 mg of Fe_2O_3-NP per kg^{-1} of dry soil. For the application of the NP, a metal frame (20 × 10 × 10 cm; length, width, height) was used. The metal structure was used to ensure that the corresponding concentration infiltrates the specific area (experimental unit).

Macroinvertebrate sampling was performed at 0 (sampling two hours after the application of NP), 30 and 60 days after the start of the experiment (DDE). Arthropod organisms and worms collected were concentrated in plastic bottles with 70% alcohol and 4% formaldehyde, respectively. At this time, the organisms were sampled while soil samples were obtained. The acid digestion method (HNO_3, H_2O_2 and HF, at a temperature of 180 °C for two hours) was used to determine the amount of Fe in the soil, while, for the analysis of organisms, only HNO_3 and H_2O_2 were used. All samples were digested in triplicate and analyzed by plasma atomic emission spectrometry (ICP-MS). The methodology used (with some variants) was based on Mariyadas *et al*. (2018).

2.7.2 *Statistical analysis*

An analysis was performed using the general linear model procedure to evaluate the effect of Fe_2O_3-NP concentrations over time, on the presence of Fe in organisms and soil ($p < 0.05$). When the analysis showed significant differences, we compared the means with the Fisher test ($p < 0.05$). The data were analyzed using the Minitab software (version 18).

2.8 Micromorphological changes in the common been tissue by TiO_2-NP

2.8.1 *Common bean, soil, and nanoparticles*

The source of seeds and NP is described in sections 2.1 and 2.4, respectively. The soil was sampled at the Institute of Agricultural Sciences of the Autonomous University of Hidalgo

State (Hidalgo State, Mexico). The layer was sampled from 0 to 20 cm deep, and then soil samples were 2 mm mesh sieved and characterized. According to FAO/UNESCO soil classification system, the soil was a haplic phaeozem with pH 7.54 and electrolytic conductivity 5.3 dS m^{-1}, a water holding capacity (WHC) of 625.01g kg^{-1}, the organic carbon content of 3.6 g C kg^{-1} soil, and a total inorganic N content of 0.21 g N kg^{-1} soil.

2.8.2 *Experimental design and greenhouse experiment*

Plants of common bean (*Phaseolus vulgaris* L.) were produced in a plant-growth chamber and after that in a greenhouse at Cinvestav-Zacatenco in one hundred sixty-two PVC columns (17 cm in diameter × 60 cm in height). The PVC columns were closed on the bottom with perforated PVC lid and containing 0.5 kg of tezontle (a porous, highly oxidized volcanic rock). After that, each PVC column was filled with seven kg of soil with sampled agricultural soil described in section 2.8.1. TiO_2-NP addition and the common bean sowing were done according to section 2.6.1 but, NP were suspended in 200 mL deionized water and sonicated for 30 min while each soil column was amended with 0, 0.15 or 0.30 g TiO_2-NP kg^{-1} at the time of sowing. The response of the plants cultivated during 90 days was measured. In completely randomized block design six treatments were applied to the soil combining the cultivation of bean plants and the application of TiO_2-NP at two concentrations. Irrigation was well controlled so that no leaching was observed. The temperature and moisture content inside the greenhouse during the experiment was 25 °C and 35-48%, respectively.

2.8.3 *Plant sampling*

When plants reached 90 days, they were random selected. Six plants of the treatment amended with 300 mg kg^{-1} were aleatory choose. For preparing cuttings for SEM, 20 cm segments of plants (stem, root, and nodules) were first collected in the greenhouse and preserved in phosphate-buffered saline (PBS) until the preparation of cuttings.

2.8.4 *SEM and EDS*

For SEM analyses tissues from nodules, steam, and roots were washed with sterile water and PBS, fixed for one h with 2.5% glutaraldehyde in PBS and adhered to poly-L-lysine-coated coverslips. The fixed tissues were washed three times with PBS and post-fixed for two h in 1% OsO4 in distilled H_2O. Next, tissues were washed with PBS, dehydrated in alcohol, subjected to critical-point drying with CO_2, coated with gold and analyzed by SEM (JOEL-JSM6510LV). Secondary electron images of the plants' cuttings were obtained by the EDS technique coupled to an SEM manufactured by AURIGA-39-16 in a 25-kV setting.

3. RESULTS AND DISCUSSION

3.1 Soils characteristics

The agricultural soil used in the experiment with corn was a silty-clay, with a pH (1:2.5 soil/water) of 8.2, 2.7% organic matter, 1.78 g kg^{-1} total N, 1.31 g kg^{-1} total P, 1.60 g kg^{-1} total K and 0.048 g kg^{-1} total Zn.

3.2 Physicochemical characterization of nanoparticles

Nanoparticle morphologies and XRD patterns of ZnO, TiO_2, and Fe_2O_3 nanoparticles are shown in Figure 1. The low-intensity signals correspond to pure phase in the case of ZnO, while TiO_2 and Fe_2O_3 have a biphasic state, the crystallography system is hexagonal for all materials. The chemical composition of nanoparticles can be seen in Table 2.

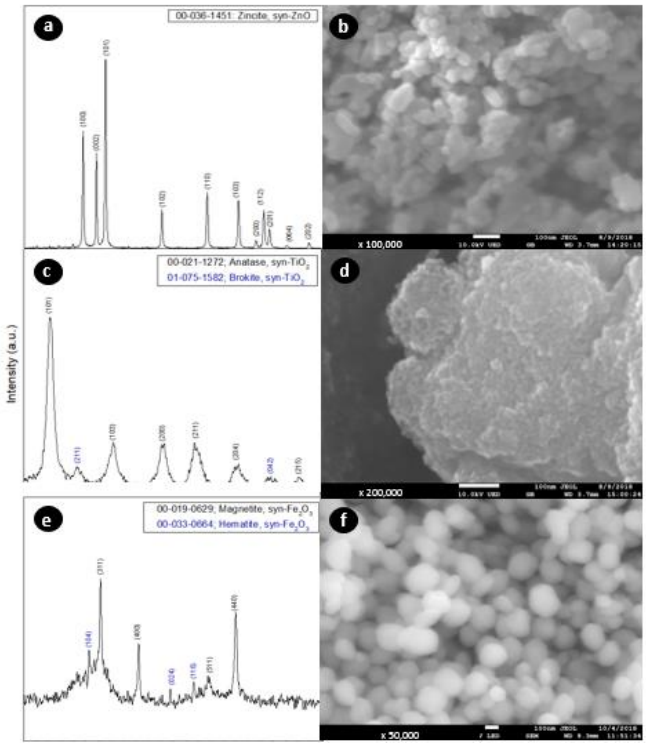

Fig. 1. Characterization of nanoparticles by XRD and FESEM. XRD patterns and FESEM micrographs of ZnO (a,b), TiO_2 (c,d), and Fe_2O_3 (e,f) nanoparticles.

Table 2. Chemical composition of ZnO, TiO_2, and Fe_2O_3 nanoparticles by X-ray energy dispersion spectrometry (EDS) characterization.

Nanoparticle	**Chemical elements** (wt. %)			
	O	Zn	Ti	Fe
ZnO	25.76	74.24	—	—
TiO_2	24.63	—	47.68	—
Fe_2O_3	21.77	—	—	78.24

—, Unidentified item

3.3 Phytonanoremediation technology

AMF root colonization was detected in all established treatments because the soil has not undergone initial sterilization and there were significant differences in colonization by AMF ($p < 0.05$) of the ANOVA. ZnO-NP did not harm the mycorrhizal root colonization as witnessed by treatments T8 and T9 compared to T3. ZnO-NP increased the AMF colonization in roots from treatments T8 and T9, 63.0 and 59.3%, respectively, in

comparison to the control treatment (T1) and the T3. In contrast, Wang et al., (2018) reported that the addition of 500 mg kg^{-1} ZnO-NP were decreased by 23% to 52% root colonization, during an experiment in which evaluated the phytotoxicity of ZnO-NP in *Zea mays* L. However, in treatments T6 and T11 the AMF infection decreased to 44.0 and 37.8%, compared to T1, which could be attributed to the presence of PAH (Figures 2 & 3). It is well known that effective AMF-mediated phytoremediation exists (Rajtor & Piotrowska-Seget, 2016) but, there is no more information regarding AMF-mediated phytoremediation in presence of engineered nanomaterials.

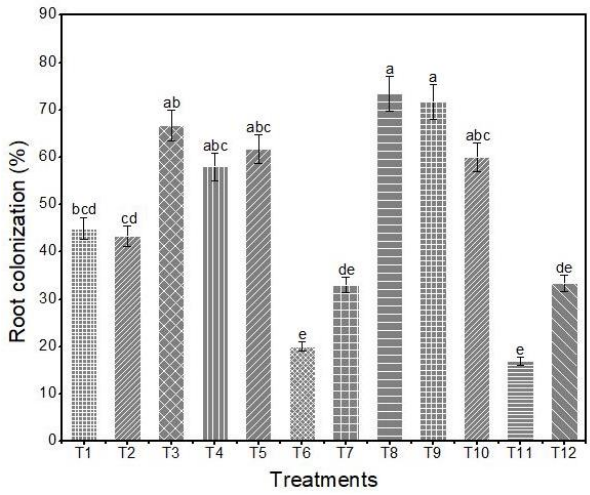

Fig. 2. Root colonization of AMF (%). Different letters denote significant differences ($P < 0.05$) between treatments.

On the other hand, factorial ANOVA test showed the significant effect ($p < 0.05$) of association indicators, PAH contamination ($F = 281.0303$) concerning the presence of arbuscules, followed by PAH × HMA interactions ($F = 42.1921$) and PAH × ZnO-NP ($F = 8.4297$). The coexistence of factors induced a degree of oxidative stress (data not shown) that affected the biochemistry of plants, increasing communication between symbionts.

The dissipation of PAH was significantly higher in the Treatment 10 (Soil + Plant + PAH + AMF) for both hydrocarbons, i.e., phenanthrene and anthracene with 42.2 y 10.8%, respectively. However, the presence of ZnO-NP decreased the PAH degradation in treatments T6, T7, T11, and T12, compared with treatment T10, i.e. the AMF increased the degradation of PAH, but the ZnO-NP reduced their degradation. Medina-Pérez *et al*. (2018) stated that NP affect the growth of plants, the health of earthworms and abundance and diversity of soil microorganisms. However, NP could also increase the dissipation of pollutants through specific remediation technologies such as nanofiltration, catalytic nanomotors, heterogeneous catalysts, among others (Medina-Pérez *et al.*, 2019).

Around 500 different mass/charge (m/z) signals were recovered. When performing the statistical filtering and identification of the m / z signals with the Progenesis QI software libraries, we maintained 101 positively charged and 20 negative signals. Partial least square (PLS) method allowed modeling the relationship between the metabolite matrices identified in the twelve treatments along with grouping relationships between treatments (Figure 4). The synthesis of metabolites from corn was more affected by the PAH than by ZnO-NP as witnessed in Figure 4. Marchiol *et al*. (2019) stated that hydroxyapatite nanoparticles did

not have phytotoxic effects on tomato plants grown in hydroponics while the synthesis of plant metabolites neither was affected.

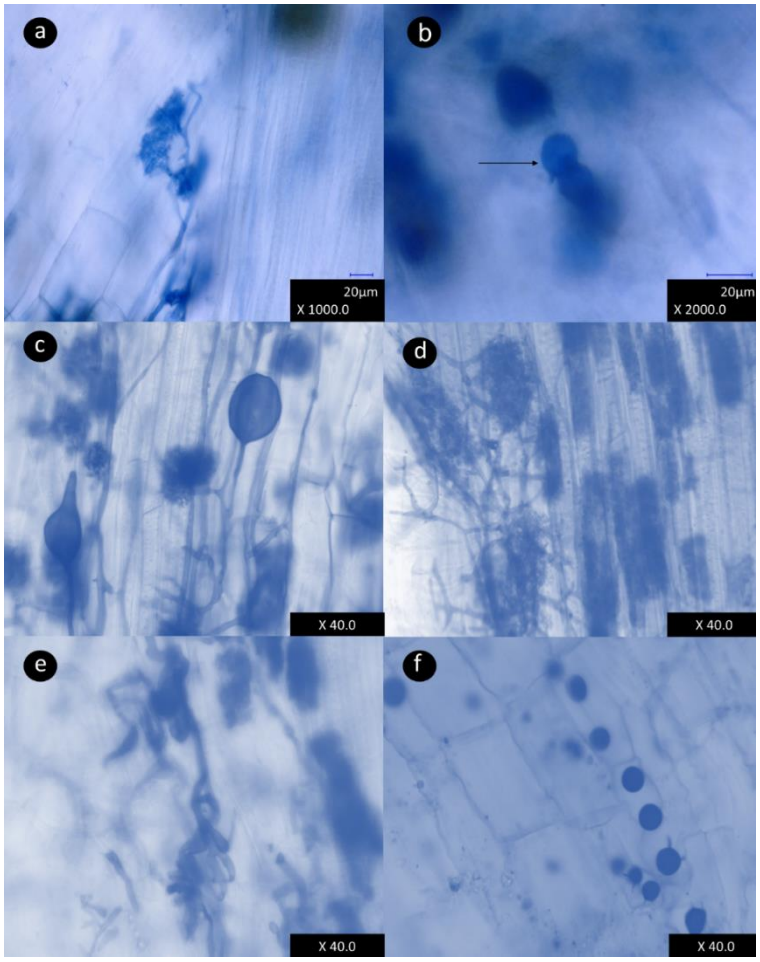

Fig. 3. Arbuscular mycorrhizal fungal colonization in root from *Zea mays* L. Arbuscules in treatment with interaction HMA + ZnO-NP (150 mg kg^{-1}) (a). Latent spore in treatment with interaction PAH + AMF + ZnO-NP (300 mg kg^{-1}) (b). Advanced colonization in treatment with only inoculation AMF (c). Mature arbuscules in treatment with only ZnO-NP (150 mg kg^{-1}) (d). Collapsed arbuscules in interaction PAH + ZnO-NP↑ (e) Abundant latency spores in interaction PAH + HMA (f).

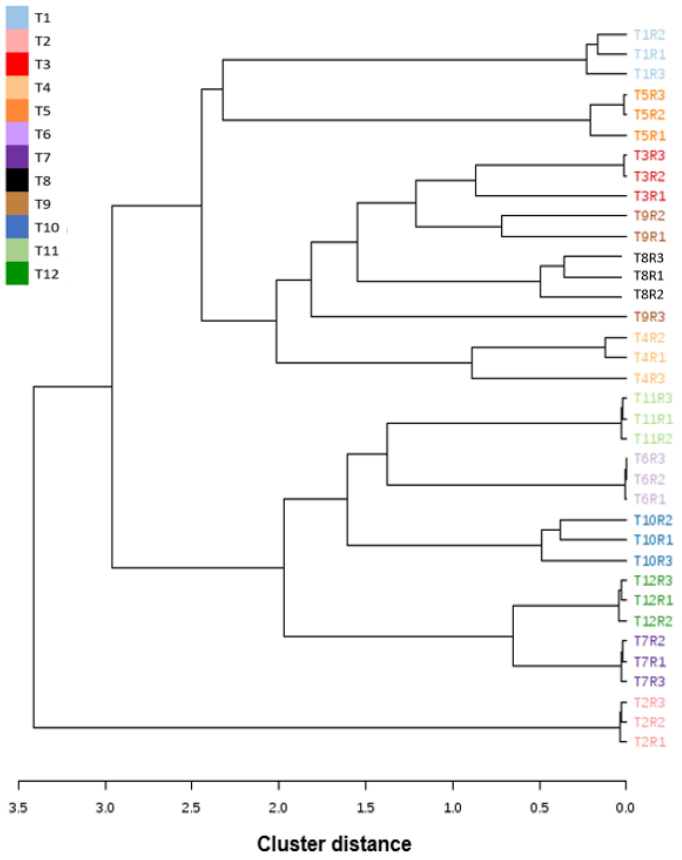

Fig. 4. Partial least square (PLS)-dendrogram for the recorded LC/MS metabolite profiles of Zea mays L. 56 days after sowing (DAS), treatment with Arbuscular mycorrhizal fungus (AMF), polycyclic aromatic hydrocarbons (PAH) or ZnO nanoparticles. Cluster distances were calculated using Sperman's method.

3.4 Effect of nanoparticles on common bean (*Phaseolus vulgaris* L.)

3.4.1 Biomass production

Both root length and stem height were affected by at least one type of NP. In the case of root length, this was significantly reduced by the presence of TiO_2-NP at a concentration of 300 mg kg^{-1} when exposed for a period of 60 days after sowing (DAS), result that coincides with that reported by Kibbey & Strevett (2019), who found that the dose of 100 mg L^{-1} of TiO_2-NP reduced the length of lettuce seedling roots at 21 days of exposure. On the other hand, the height of the stem was significantly reduced by being exposed to TiO_2-NP at 300 mg kg^{-1} and Fe_2O_3-NP at 150 mg kg^{-1} both at 30 and 60 DAS, compared to the control treatment (Figure 5).

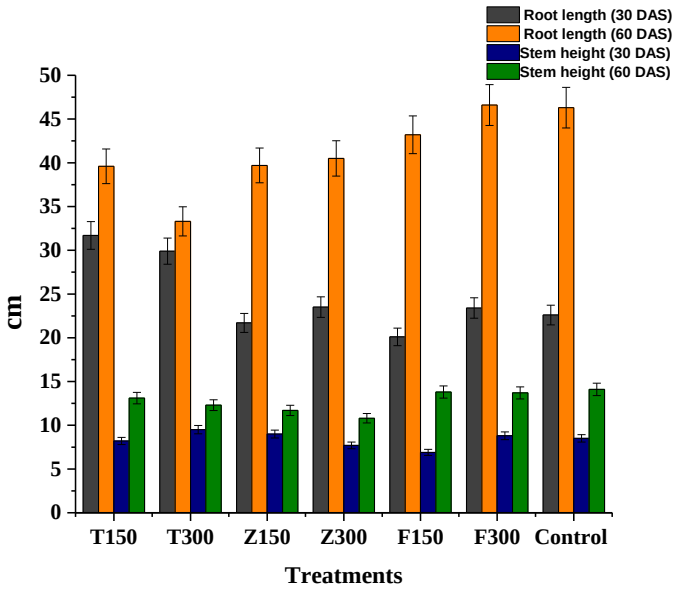

Fig. 5. Morphological characteristics of common bean plants, grown under greenhouse conditions and in a soil conditioned with TiO_2, ZnO and Fe_2O_3 nanoparticles at concentrations of 150 and 300 mg kg^{-1}. n=9, (Tukey $\alpha = 0.05$).

Other characteristics, such as dry biomass production and the number of nodules were also evaluated. In the case of dry root weight, it was recorded that the presence of TiO_2-NP in both concentrations increased the dry weight of this section of the plant, on the other hand, none of the nanoparticles significantly affected the dry weight of the plant stem. However, only Fe_2O_3-NP positively influenced root nodule formation at 60 DAS, compared to the control. Medina-Pérez & Fernández-Luqueño (2019), reported and discussed several reviews regarding the advantages and drawbacks of engineering nanomaterials, among these NP, could increase or decrease the biomass production in crops and the possible collateral damage of NP to the environment.

3.4.2 FTIR-ATR analysis

For the present study, the FTIR-ATR spectra were taken into account from the sampling at 30 DAS of three sections of common bean plants exposed to NP. In the leaf spectra (Figure 6), all treatments have the same signals corresponding to the functional groups related to the lipids of the waxy layer of the leaves (In 2920 and 2850 cm^{-1} the asymmetric stretch of the bond C-H, from 1750 to 1720 cm^{-1} the bond C=O and from 1380 to 1440 cm^{-1} the symmetrical flexion of the CH_3 group) consistent with that reported by Warren *et al*. (2015) and Barraza-Garza *et al*. (2013). Likewise, there was a band at 1150 cm^{-1} corresponding to the flexion of C-O bond associated with the carbohydrates produced in the leaf, however, it was observed that in the signal of the region between 1540 and 1520 cm^{-1} corresponding to symmetric flexion of the N-H bond associated with proteins (Barraza-Garza *et al*., 2013), it occurs with greater intensity in treatment with Fe_2O_3-NP at a concentration of 150 mg kg^{-1} of dry soil, but this signal is not distinguished in treatments with TiO_2- or Fe_2O_3-NP at a dose of 300 mg kg^{-1}.

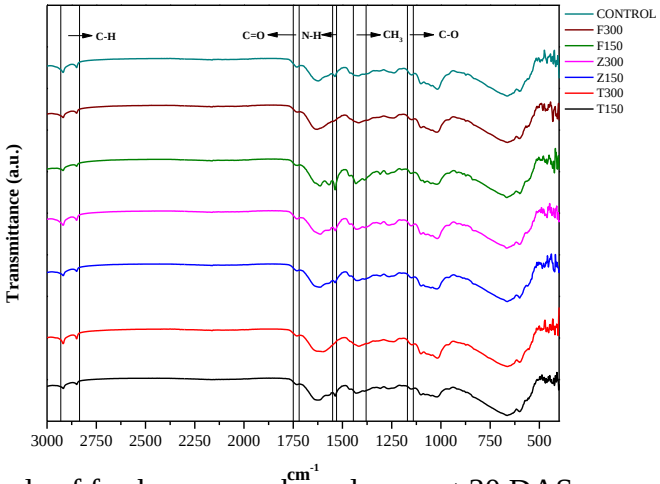

Fig. 6. Infrared spectra made of fresh common bean leaves at 30 DAS

In the spectra of the stem (Figure 7) a band in the region of 1150 cm^{-1} corresponding to the flexion of the C-O bond of carbohydrates can be observed. On the contrary, in the region of 2920 to 2850 cm^{-1} where two bands specific to the asymmetric stretching of the C-H bond must be identified, but it was only detected in the treatment with 300 mg kg^{-1} of ZnO-NP. On the other hand, there were detected C=O bonds around 1650 cm^{-1} present in the amide groups of some protein, just like the P=O group in the 1250 cm^{-1} position belonging to nucleic acids (Barraza-Garza *et al*., 2013), it composition coincides with the EDS analysis shown in the SEM micrograph in Figure 9.

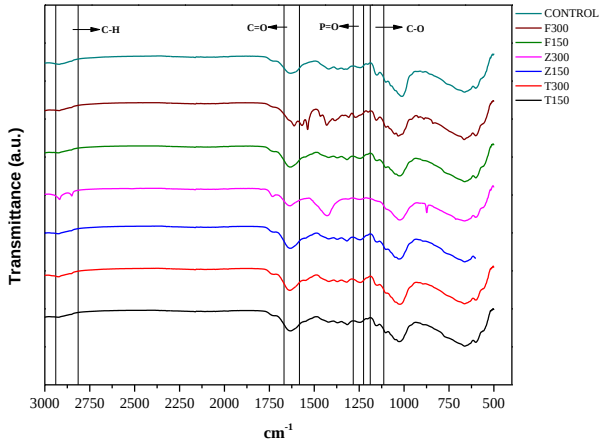

Fig. 7. Infrared spectra made of fresh common bean stem at 30 DAS

Like the stem spectra, the spectra obtained from the root analysis (Figure 8) of the plants have the same signals in the same regions, indicating that they are similar in their composition. However, in the region of 2920 at 2850 cm^{-1} only signals corresponding to the C-H group were detected in the treatment with 150 mg kg^{-1} of TiO_2-NP. However, it should be noted that because various organic compounds are in plant tissues, they produce complex spectra, making it difficult to identify specific compounds due to the over position of signals (Ribeiro-da Luz, 2006).

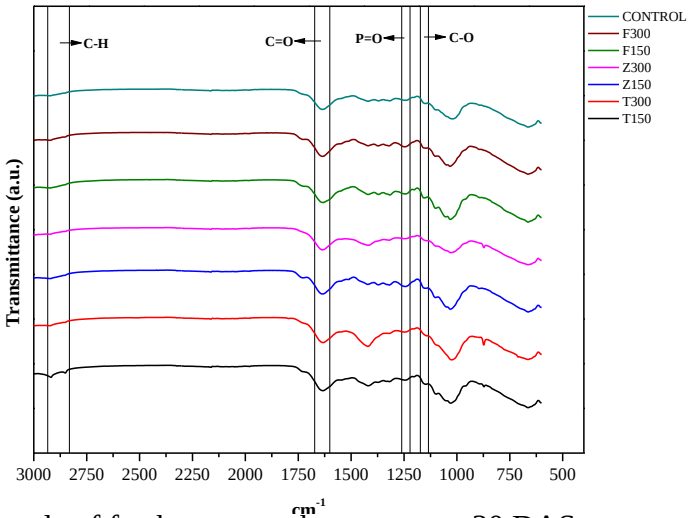

Fig. 8. Infrared spectra made of fresh common bean roots at 30 DAS

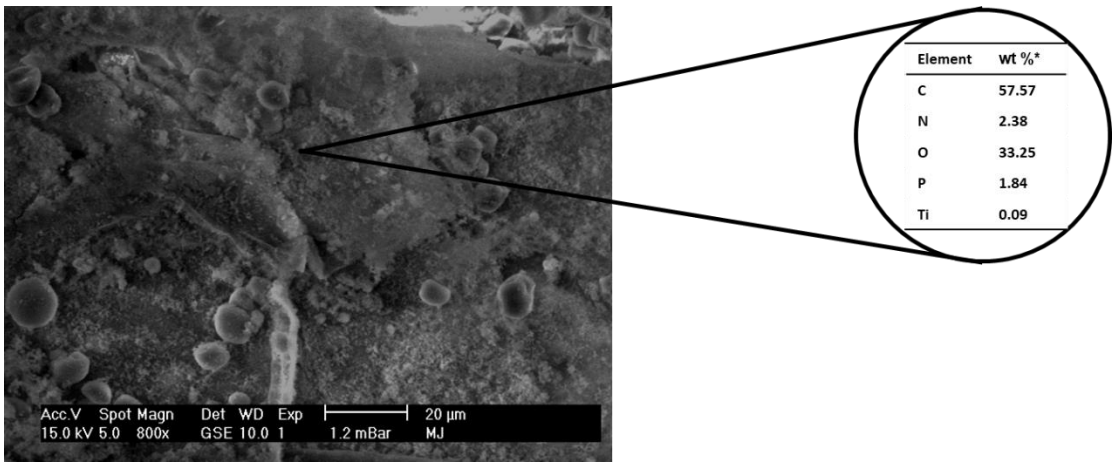

Fig. 9. SEM micrograph and EDS analysis of common bean stem exposed to 150 mg kg^{-1} of TiO_2-NP. * The rest of the percentage corresponds to elements in a smaller proportion in the tissue (Na, Mg, S, K, Mn, and Ca).

3.5 Effect of NP on macroinvertebrates from forest soil

Currently, there are no protocols for the evaluation of NP on soil organisms under natural conditions. However, our research team has initiated experiments on forest soil to evaluate the concentrations of iron NP in different classes of macroinvertebrates. The results revealed that earthworms are those that indicated the presence or avoidance of Fe_2O_3-NP in the soil, compared to the rest of the organisms. When the effect of Fe_2O_3-NP at time 0 was evaluated, earthworms showed high Fe concentrations (7870 mg Fe kg^{-1} dry weight) in the treatment of 100 mg of Fe_2O_3-NP kg^{-1} dry soil, while at 1000 mg kg^{-1} dry soil, presence of iron in their bodies was lower (200 mg Fe kg^{-1} dry weight). Particularly, at 60 days after the addition of the NP, the concentration of Fe in earthworms was significantly higher in treatments with 1 and 10 mg kg^{-1}, compared with the control and with the treatment of 1000 mg Fe_2O_3-NP kg^{-1} dry soil. It was observed that always the concentrations of Fe in macroinvertebrates decreased in the concentration of 1000 mg Fe_2O_3-NP kg^{-1}, while at 1

and 10 mg Fe_2O_3-NP kg^{-1} dry soils macroinvertebrates remained constant over time, with higher concentrations of Fe in macroinvertebrates (Table 3).

Table 3. Concentrations of Fe in macroinvertebrates (mg Fe kg^{-1} dry weight [D.W]) at 0 and 60 days after the onset of the experiment. The treatments evaluated were control (0), 1, 10, 100, and 1000 mg kg^{-1} of Fe_2O_3-NP. Different lowercase letters in each column indicate statistical difference between treatments for each time ($p < 0.05$).

Treatments	**Concentrations of Fe in organisms** (mg $kg^{-1}$of D.W)	
	Time 0	Time 60
1	1555.00 ab	9265.00 a
10	100.00 b	8720.00 a
100	7870.00 a	4533.33 b
1000	200.00 b	2100.00 bc
Control	173.33 b	850.00 c

Although these results correspond to a first part of the field studies, we can argue that endogenic earthworms could be bioindicators of the presence at low concentrations of Fe_2O_3-NP (10 mg Fe_2O_3-NP kg^{-1} dry soil), because earthworms limit soil consumption when high concentrations of Fe ions are detected. Figures 10 and 11 are an example of the micrographs taken and the values obtained from the earthworm samples (treatment of 10 mg Fe_2O_3-NP kg^{-1} dry soil) that were in contact with the Fe_2O_3-NP at 0 and 60 days after the addition of the NP. This type of analysis helps to elucidate whether there is a directly proportional relationship between the adsorption of NP in their bodies and high concentrations of Fe inside their bodies. In this case, when we analyzed the presence of Fe adhered to the body of the organisms, a low amount of Fe_2O_3-NP was observed, even, the values obtained were similar to the start and end of the experiment. However, Fe concentrations inside their bodies were higher after 60 days (Table 3, see treatment at 10 mg Fe_2O_3-NP kg^{-1} dry soil).

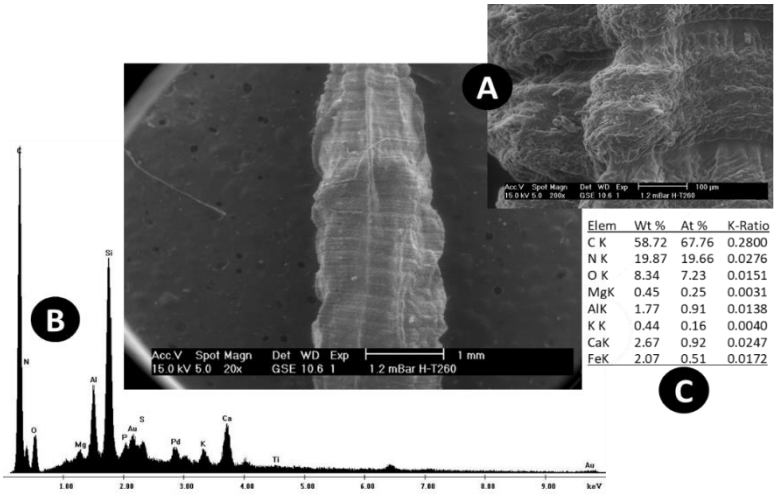

Fig. 10. Analysis of Fe adhered to the body of the earthworms when organisms were exposed to 10 mg Fe_2O_3 nanoparticles per kg^{-1} dry soil (at 0 days after the addition of the NP). (A) SEM micrograph of Fe_2O_3 dispersed over body earthworms (20× and 200×), (B) results qualitative and (C) quantitative of the EDS analysis.

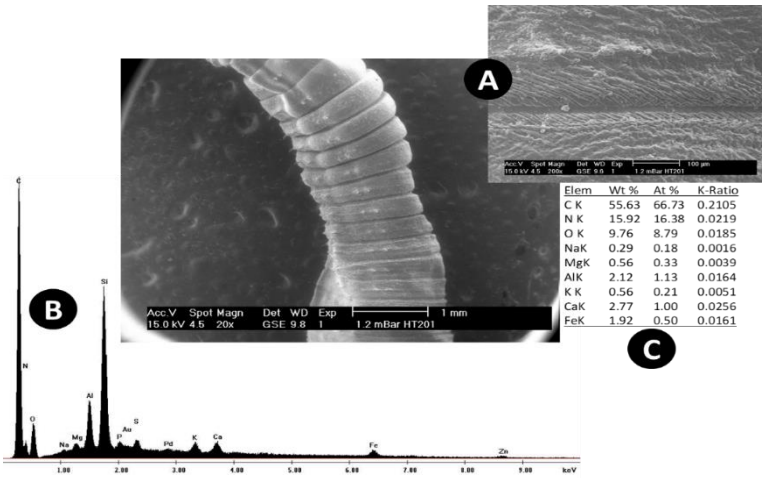

Fig. 11. Analysis of Fe adhered to the body of the earthworms when organisms were exposed to 10 mg Fe_2O_3 nanoparticles kg^{-1} dry soil (at 60 days after the addition of the NP). (A) SEM micrograph of Fe_2O_3 nanoparticles dispersed over body earthworms (20× and 200×), (B) results qualitative and (C) quantitative of the EDS analysis.

3.6 Micromorphological changes in the common been tissue by TiO_2-NP

The results of the phenological characteristics of the plants showed that the treatments applied in this experiment had no significant effects (Data not shown). It was the main reason why the plants' growth in the treatment with 300 mg TiO_2-NP kg^{-1} dry soil were selected to the SEM study. The elongation of the shoot, the root, and the seedlings was not significantly affected by the concentrations of TiO_2-NP. It is possible that the

physicochemical characteristics of the soil alleviated the adverse effects on the growth and weight of the plants (Frenk *et al*., 2013).

Longitudinal and cross-sections of the roots and stems showed an increment in the diameter and number of vascular bundles. In the observed cuttings of roots exposed to TiO_2-NP, rhizodermis was more thickened and pigmented than the cuttings of control plants. In the case of sections of stem, size, and shape of the xylem ring, notable variations were observed in the treatments with TiO_2-NP and the central extensions of the xylem elements were more pronounced in them, in the same way, the intercellular spaces were not were altered, but crystals were not found in cortical cells (Figure 12). In fact, TiO_2-NP could pass through the epidermis and the bark of the plant via apoplast. Du *et al*. (2011) reported that small TiO_2-NP had penetrated through the cell wall of wheat plants, which they identified by TEM. But in this study the adsorption phenomenon of TiO_2-NP was identified in rhizodermis tissues, which was observed on the surface of the nodules and roots (Figure 13). The TiO_2-NP was mainly adhered to the cell wall of the periderm cells, indicating that most of the TiO_2-NP applied could not enter the internal bean root cells which coincides in those reported by Du *et al*. (2011) for wheat plants.

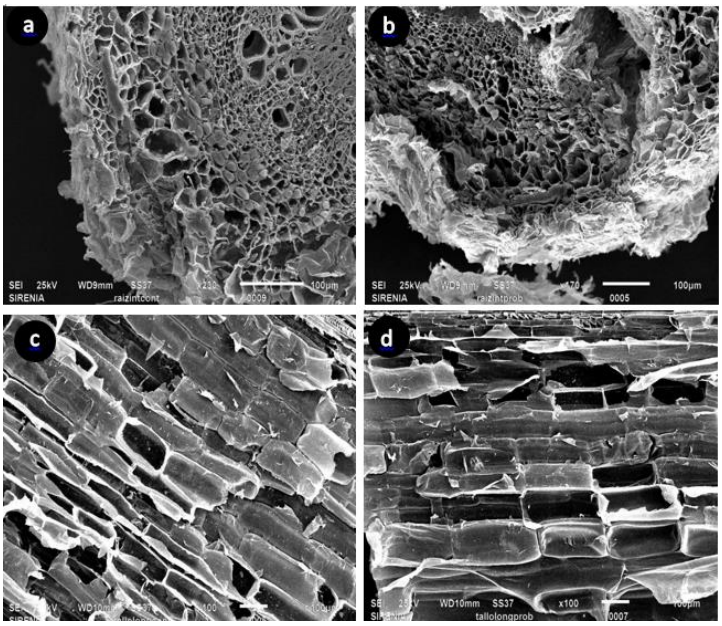

Fig. 12. a) SEM micrograph (230×) of a cross-section of bean plant root treated with TiO_2-NP (0 mg kg^{-1}); b) SEM micrograph (170×) of a cross-section of bean plant root treated with TiO_2-NP (300 mg kg^{-1}); c) SEM micrograph (100×) of a longitudinal-section of bean plant stem treated with TiO_2-NP (0 mg kg^{-1}) and d) TiO_2-NP (300 mg kg^{-1}).

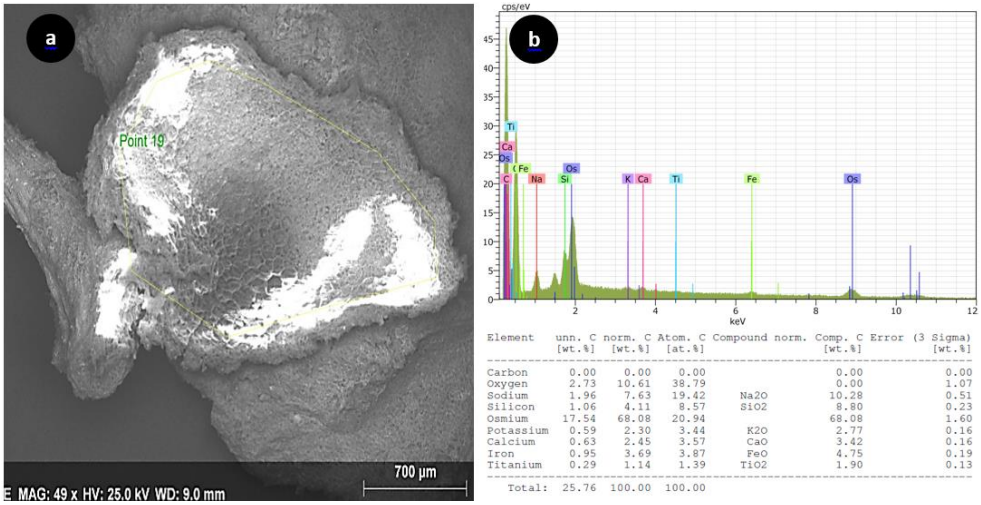

Fig. 13. a) SEM (300×) and elemental analysis by EDS spectra of roots from exposed common bean (*Phaseolus vulgaris* L.) plants exposed to 300 mg TiO_2-NP kg^{-1} dry soil. b) Elemental analysis confirms the presence of TiO_2 on the surface of a segment of a secondary root.

The NP used in this research significantly changed some response variables on the experiments carried-out at laboratory, greenhouse and land level. ZnO-NP did not jeopardize the mycorrhizal root colonization but, the presence of ZnO-NP decreased the degradation of PAH. The synthesis of metabolites from corn was more affected by the PAH than by ZnO-NP. In addition, NP affected the synthesis of compounds from specific functional groups in common bean plants. Fe_2O_3-NP were attached to the body of forest-soil organisms and significantly increased the concentration of Fe in their body, while TiO_2-NP changed the morphological tissue of roots and stems of common bean. Additional studies during long-time periods and under real conditions should be established, focusing on the ecological toxicology; otherwise, the ecosystems could be jeopardized.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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