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Research Article

Seed priming with ZnO nanoparticles promotes early growth and bioactive compounds of *Moringa oleifera*

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Abstract

Nanotechnology has gained importance in agricultural production systems, with various applications such as pesticides or fertilizers. The application of nanomaterials (NMs) as a pretreatment to seeds (seed priming) has positively affected plant growth and development. On the other hand, *Moringa oleifera* is a plant appreciated for its multiple nutraceutical properties. Therefore, the objective of this study was to evaluate the effect of pretreatment of *M. oleifera* seeds with ZnO nanoparticles (NZnO) (0, 0.5, 2.5, 5, 7.5, and 10 mg L^{-1}). The study was divided into two experimental phases: the first phase consisted of evaluating germination under laboratory conditions (25 °C) at 15 DAS, while in the second phase, vegetative growth and bioactive compounds were evaluated at 45 DAS under greenhouse conditions. For phase one, the percentage of germination, length, and dry weight of the plumule and radicle were considered, and the vigor indices of seeds were determined. In phase two, we measured the plant height, stem diameter, fresh and dry biomass of aerial and root parts, and the concentration of photosynthetic pigments, phenolic compounds, flavonoids, vitamin C, glutathione (GSH), and antioxidant capacity (DPPH), such as the activity of antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and phenylalanine ammonium lyase (PAL). The results showed an increase in some variables related to seed germination, with an increase of between 30 and 25% in the vigor of the seeds subjected to 2.5 and 10 mg L^{-1} NZnO. The photosynthetic pigments resulted in increases of between 23 and 49% for the 7.5-10 mg L^{-1} NZnO treatments. Regarding bioactive compounds, the increase in phenols, flavonoids and vitamin C stands out, mainly at the levels of 7.5-10 mg L^{-1} NZnO, where increases of up to 543% were observed with respect to the control. The enzymatic activity showed different responses to the application of NZnO, where a biphasic response (hormesis) was observed on the activity of APX and CAT activities as the levels of NZnO increased. The results show that it

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is possible to promote the initial growth and bioactive compounds of *M. oleifera* by pretreatment of seeds mainly with 10 mg L^{-1} NZnO.

Keywords: antioxidants; germination; hormesis; nanomaterials

Introduction

The constant increase in population and the loss of soils due to erosion and contamination forces us to look for alternatives to increase agricultural production, with high yields, better nutritional characteristics, and less environmental impact. Success in crop production depends mainly on the stage of establishment, which is influenced by factors such as seed quality and the method of sowing or transplanting. There is a set of seed pretreatment techniques that promote the germination and initial growth of seedlings, methodologies known as "seed priming" (Waqas *et al.*, 2019). In its origins, this concept included only the action of imbibition of seeds and subsequent drying for sowing, increasing the vigor of the plants. In recent years, the concept of seed priming has included various techniques, such as extreme temperatures, UV radiation, growth regulators, minerals, and microorganisms, and recently, the use of nanomaterials (NMs) (Sher *et al.*, 2019).

Seed priming with nanomaterials is an activator of germination, promoting initial growth and biochemical characteristics in various plant species (Waqas *et al.*, 2019). NZnO at low concentrations promotes the production of ROS and phytohormones and the overexpression of new water channels (aquaporins), and the response is a more remarkable synthesis of antioxidant compounds and improvement in the absorption of water and nutrients, favoring the initial growth of seedlings (Santo *et al.*, 2021). Regarding the improvement in water and nutrient absorption, which is associated with the increase in length and biomass, it has been shown that NZnO induces overexpression of the *HvTip1:1* and *HvPip1:1* genes, both related to the generation of aquaporins in cells (Akdemir, 2021).

NZnO favors a greater capacity to mobilize the reserves contained in the seeds and increases the efficiency of using these reserves (Seyyedi *et al.,* 2015). This effect occurs because NZnO increases the activity of the enzyme α-amylase (Rai-Kalal and Jajoo, 2021), an enzyme responsible for the degradation of starch and transformation to sugars readily available for the seed embryo (Kondhare *et al.*, 2015). The above was confirmed by Itroutwar *et al.* (2020), who reported that NZnO applied to *Zea mays* seeds accumulated in the endosperm region, associated with rapid starch degradation that favored the growth of the plumule and radicle, increasing the final quality of the seedlings.

On the other hand, seed priming with NMs can increase the activity of enzymatic antioxidants and the concentration of nonenzymatic antioxidants due to two mechanisms: one is NM corona contact with the cell wall, favoring the generation of reactive chemical species, and the other is believed to occur in response to the internment and metabolism of NMs, possibly by the release of ions in plant cells (Juárez-Maldonado *et al.*, 2019). The production of reactive or oxidizing species, such as H_2O_2, activates plant defense systems, increasing the activity levels of enzymes such as CAT, APX, and GPX, as well as the concentration of nonenzymatic antioxidants such as phenols and flavonoids (Abdel-Aziz *et al.*, 2019; Ruiz-Torres *et al.*, 2021). Some studies found that the application of NZnO favored increased activity of SOD, CAT, POD, and APX in *Lupinus ternis* plants (Abdel-Latef *et al.*, 2017) and increased activity of CAT, APX, and POD in *Coriandrum sativum* (Ruiz-Torres *et al.*, 2021).

The application of NMs for seed priming has been reported in different crops. For example, in *Lactuca sativa,* the use of Cu, Zn Mn, and Fe NPs favored the germination of seeds (Liu *et al.*, 2016). On the other hand, Fe_3O_4 NPs increased vigor in *Zea mays* seeds (Neto *et al.*, 2020). In another study, López-Vargas *et al.* (2020) used carbon and graphene nanotubes in *Solanum lycopersicum* seeds, increasing the content of chlorophylls, phenols, and total flavonoids in seedlings. Zn is an indispensable element for the development of plants, and its use in ionic (Zn^{2+}) and nanometric (NZn and NZnO) forms is associated with greater vigor,

stress tolerance, and nutritional quality of crops (Hussein and Abou-Baker, 2018). Seed priming with ZnO NPs (NZnO) could be an alternative to mitigate the low availability of Zn in certain regions, with an additional biostimulant impact on germination and growth (Adhikari *et al.*, 2016; Abdel-Latef *et al.*, 2017; Itroutwar *et al.*, 2020).

Moringa oleifera is a plant highly appreciated for its high content of bioactive compounds and pharmacological properties, such as antiproliferative, antidiabetic, anti-inflammatory, and antioxidant properties (Ma *et al.*, 2020). There are few studies on the application of NMs in this plant. Juarez-Maldonado *et al.* (2018) reported that foliar applications of Cu NPs in *M. oleifera* increased the content of photosynthetic pigments and bioactive compounds such as phenols, flavonoids, vitamin C and antioxidant capacity in the leaves. To the best of our knowledge, there is no published information about the use of NZnO as a seed priming treatment for *M. oleifera.*

Based on the above, the objective of this study was to evaluate the effect of seed priming with NZnO on the germination, initial growth, and bioactive compounds of *Moringa oleifera.* The hypothesis was that NZnO interacts with the cells of the seeds, favoring the mobilization of the reserves that results in a greater initial growth of the seedlings, in addition to increasing the levels of enzymatic and nonenzymatic antioxidant compounds on plant tissues.

Materials and Methods

Location of the experiment and description of the material

The experimental work was carried out in the Department of Horticulture in the Universidad Autónoma Agraria Antonio Narro facilities in Saltillo, México. The seeds of *M. oleifera* were provided by the Center for Protected Agriculture of the Universidad Autónoma de Nuevo León. The seeds were obtained from the same tree to ensure greater homogeneity between the seeds. The plant material corresponds to the ecotype known as "Vaina corta," identified mainly by the production of pods with an average length of 24 cm (Meza-Carranco *et al.*, 2016). The germination percentage was >90%, and the weight of 100 seeds was 45 +/- 2.5 g. The wings of the seeds were carefully removed to ensure better contact of the seeds with the aqueous suspension of NMs used for priming.

Synthesis and characterization of NPs

ZnO NPs were synthesized based on the procedure reported by Patil *et al.* (2014). In a glass reactor, 100 g of $Zn(C_4H_{10}O_6)$, 25 g of citric acid (4:1, w:w) and 100 mL of distilled water were added, and the reaction mixture was stirred at 600 rpm for 60 min at 80 °C. Subsequently, a solution containing 50 g of NaOH dissolved in 50 mL of distilled water was added, and the reaction mixture was kept under constant stirring at 90-100 °C for 60 min. The ZnO NPs were separated by centrifugation in an Allegra-64R centrifuge (Beckman Coulter Inc., California, USA) and washed with distilled water and methanol. The drying of the samples was carried out at 85 °C in a vacuum oven for two hours.

The morphology and microstructure of the samples were examined by conventional and high-resolution transmission electron microscopy (TEM and HRTEM) using an FEI-TITAN 80–300 kV microscope (Fisher Scientific, Hillsboro, USA) operated at an acceleration voltage of 300 kV. Most of the NPs analyzed by TEM resulted in a quasi-spherical shape and narrow particle size distribution, with an average diameter of 16.49 nm (Figure 1-A, B), where the HRTEM image showed that they were primarily crystalline (Figure 1-C).

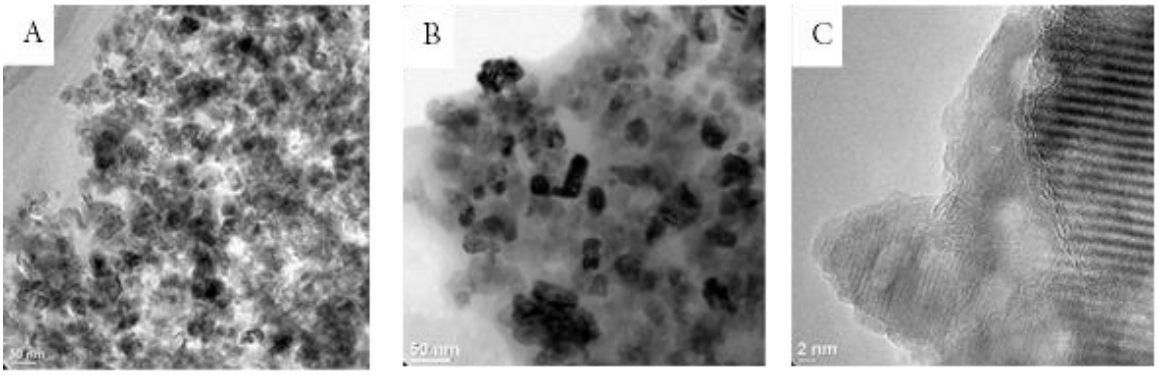

Figure 1. Characterization of ZnO nanoparticles by TEM (A-B) and HRTEM (C)

Seed priming and treatments

For the preparation of the suspensions, the corresponding amount of NZnO was placed in containers with distilled water and subsequently mixed by mechanical stirring in Science Med OS40-Pro (Science Med Inc., Helsinki, Finland) at 500 rpm for 60 min. To ensure uniform dispersion, all suspensions were sonicated with a Q500 sonicator (Qsonica Newtown, Connecticut, USA) for 25 min at 120 V and 50 GHz. The imbibition of the seeds was carried out by placing the seeds in different suspensions of NZnO with distilled water, which were kept under constant stirring for 24 h. The treatments used were T1: distilled water only (control), T2: 0.5 mg L^{-1} NZnO, T3: 2.5 mg L^{-1} NZnO, T4: 5 mg L^{-1} NZnO, T5: 7.5 mg L^{-1} NZnO and T6: 10 mg L^{-1} NZnO. After the imbibition time, the seeds were divided into two groups: one to evaluate germination characteristics at 15 days after sowing (DAS) and another to evaluate vegetative growth under greenhouse conditions at 45 DAS.

Germination stage (15 DAS)

The pretreated seeds of the first group were placed in Petri boxes with filter paper as the substrate to maintain moisture. The experimental unit was a Petri box with 15 seeds, and the experimental design was completely random, considering 6 treatments with 6 repetitions, obtaining a total of 36 experimental units. The boxes were placed in a growth chamber at a constant temperature of 25 °C, which corresponds to the optimal germination of *M. oleifera* (Carballo-Méndez *et al.*, 2019). The evaluated variables at this stage were germination percentage, plumule length, radicle length, plumule dry weight, and radicle dry weight. Additionally, the seed vigor indices (VI) were determined, which were calculated according to Carballo-Méndez *et al.* (2019), with regard to the following equations:

$$VI1 = (PL + RL) * \%G \quad (1)$$

$$VI2 = (PDW + RDW) * \%G \quad (2)$$

where PL= Plumule length, RL: Radicle length, %G: Germination percentage, PDW: Plumule dry weight, RDW: Radicle dry weight.

Greenhouse stage (45 DAS)

The pretreated seeds of the second group were placed in 2 L pots using a mixture of peat moss and perlite (1:1 v:v) as substrate. The nutrition of plants was supplied from 10 days after sowing (DAS) by a Steiner solution (Steiner, 1961) at a concentration of 50%, adjusting the pH to 6.5 with sulfuric acid. This solution was supplied as irrigation water. The plants were kept for 45 days in a chapel-type greenhouse with a constant temperature of 25-27 °C and 60-70% relative humidity. At the end of the experiment (45 days), the variables of plant height, stem diameter, fresh and dry weight of the aerial part, and radical system were determined. The experimental design was completely random with 6 treatments and 6 repetitions, obtaining 36 experimental units. Data were analyzed in Infostat v. 2020 software using an analysis of variance. In cases where a statistically

significant difference ($p \leq 0.05$) was found, a mean comparison test was performed using the minimum significant difference (DMS) method.

Sample preparation for biochemical analyses

Samples for biochemical analysis were taken at 45 DAS. For the leaf sample, the most recently matured leaves were considered, while the root was collected in its entirety. The plant material was placed in a freezer at -20 °C and then lyophilized in a Labconco FreeZone 4.5 (Labconco Corp., Kansas City, USA) at -45 °C for 7 d. Once the time was complete, the samples were macerated in a porcelain mortar until a fine powder was obtained for analysis.

Photosynthetic pigments

The photosynthetic pigments considered were chlorophyll a (CHLa), chlorophyll b (CHLb), total chlorophyll (CHLa+b), and β-carotene, which were determined according to the methodology of Nagata and Yamashita (1992). For the extraction, 10 mg of sample and 2 mL of hexane:acetone (3:2) were homogenized and centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus Corp., New Jersey, USA) at 12000 rpm for 10 min at 4 °C. The supernatant was directly read in a Unico UV2150 spectrophotometer (Unico Inc., New Jersey, USA) at wavelengths of 663, 645, 453 and 505 nm to input the absorbances into the following equations, and the results were reported as mg 100 g^{-1} of fresh weight (FW).

$$\text{Chlorophyll a} = 25.38 * A_{663} + 3.64 * A_{645} \quad (3)$$

$$\text{Chlorophyll b} = 30.38 * A_{645} - 6.58 * A_{663} \quad (4)$$

$$\text{Chlorophyll a + b} = 18.8 * A_{663} + 34.02 * A_{645} \quad (5)$$

$$\beta - \text{carotene} = 0.216 * A_{663} - 1.22 * A_{645} - 0.304 * A_{505} + 0.452 * A_{453} \quad (6)$$

Nonenzymatic antioxidant compounds and H_2O_2

Total phenols were determined according to the Singleton *et al.* (1999) method. Two hundred milligrams of sample was taken and extracted with 1 mL of water-acetone solution (1:1, v:v). The mixture was centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus Corp., New Jersey, USA) at 12500 rpm for 10 min at 4 °C. Then, 50 µL of the extract, 200 µL of Folin-Ciocalteut regent, 500 µL of Na_2CO_3 (20%), and 5 mL of distilled water were added and homogenized for 30 s. The mixture was placed in a water bath at 45 °C for 30 min to be read later in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 750 nm. The results were expressed in mg 100 g^{-1} DW.

Flavonoids were determined according to the methodology of Arvouet-Grandetal (1994). One hundred milligrams of sample and 10 mL of methanol were taken and mixed for 30 s. The mixture was filtered using Whatman paper (Cat No 1001). Then, 2 mL of the extract and 2 mL of $AlCl_3$ (2%) were mixed and placed in the dark for 20 min to be read later in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 415 nm. The results were expressed in mg 100 g^{-1} DW.

The vitamin C concentration was determined through the Klein and Perry method (1982). For the extraction, 10 mg of sample and 1 mL of HPO_3 (0.36 M) were added and centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus Corp., New Jersey, USA) at 5000 rpm for 10 min at 4 °C. Subsequently, 200 µL of the supernatant and 1.8 mL of 2,6-diclorofenolindofenol (2,6 D) (0.09 M) were homogenized to be read later in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 515 nm. The results were expressed as mg g^{-1} DW.

Glutathione (GSH) was determined with the methodology described by Xue *et al.* (2001). For the extraction, 100 mg of sample, 10 mg of polyvinylpyrrolidone (PVP), and 1.5 mL of phosphate buffer (K_2HPO_4 0.01 M:KH_2PO_4 0.01 M, 1:1) were mixed and centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus

Corp., New Jersey, USA) at 12500 rpm for 10 min at 4 °C. Then, the supernatant was collected and filtered with nylon membrane filters (0.45 µm). The same extract was used to determine GSH, total proteins, antioxidant capacity, and antioxidant enzyme activity. For GSH determination, 480 µL of the extract, 2.2 mL of Na_2HPO_4 (0.32 M) and 320 µL of 5,5-dithio-bis-2 nitrobenzoic acid (DTNB) (1 mM) were homogenized to be read later in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 412 nm. The results were expressed in mmol 100 g^{-1} DW.

The antioxidant capacity was determined by the radical DPPH (2,2-diphenyl-1-picrylhydrazyl) according to the methodology of Brand-Williams *et al.* (1995). Six microliters of the extract and 254 µL of DPPH reagent (6.34 mM) were placed in a microplate to be read later in a BioTek Elx808 microplate reader (BioTek Inc., Vermont, USA) at a wavelength of 630 nm. The results were reported as µmol g^{-1} DW.

Finally, H_2O_2 was determined according to the methodology of Patterson *et al.* (1984). For the extraction, 10 mg of sample and 1 mL of trichloroacetic acid (0.1%) were centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus Corp., New Jersey, USA) at 12000 rpm for 15 min at 4 °C. Then, 500 µL of supernatant, 750 µL of phosphate buffer (K_2HPO_4 0.01 M:KH_2PO_4 0.01 M, 1:1) and 1 mL of KI (1 M) were mixed and subsequently read in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 390 nm. The results were expressed as µmol g^{-1} DW.

Total protein and enzymatic activity

The total protein concentration (TP) was determined according to Bradford's colorimetric technique (1976). Five microliters of the extract and 250 µL of Bradford reagent were placed in a microplate and incubated for 10 min at 25 °C to be read later in a BioTek Elx808 microplate reader (BioTek Inc., Vermont, USA) at a wavelength of 630 nm. The results were expressed in mg g^{-1} DW.

Catalase (CAT) activity (EC 1.11.1.6) was determined following the methodology of Dhindsa *et al.* (1981). One hundred microliters of the extract, 400 µL of H_2SO_4 (5%), and 1 mL of H_2O_2 (100 mM) were homogenized and subsequently read in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 270 nm. After 1 min, the absorbance was read again to determine the activity of CAT at this reaction time. The results were reported as U g^{-1} TP, where U corresponds to mM equivalents of H_2O_2 consumed per milliliter per minute.

The activity of ascorbate peroxidase (APX) (EC 1.11.1.11) was quantified according to the method described by Nakano and Asada (1987). One hundred microliters of the extract, 500 µL of ascorbate (10 mg L^{-1}), 400 µL of H_2SO_4 (5%), and 1 mL of H_2O_2 (100 mM) were homogenized and subsequently read in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 266 nm. Like the determination of CAT, after 1 min, the absorbance was read again to determine the activity of APX at this reaction time. The results were reported as U g^{-1} TP, where U corresponds to µmol oxidized ascorbate per milliliter per minute.

The activity of phenylalanine ammonium lyase (PAL) (EC 4.3.1.5) was determined according to the methodology of Syklowska-Baranek *et al.* (2012). One hundred microliters of the extract and 900 µL of phenylalanine (6 mM) were taken and placed in a water bath for 30 min at 40 °C. After this time, 250 µL of HCl (5 N) was added to stop the reaction, and 750 µL of distilled water was subsequently incorporated to be read later in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 290 nm. The results were reported as U g^{-1} TP, where U corresponds to µM trans-cinnamic acid per milliliter per minute.

The activity of glutathione peroxidase (GPX) (EC 1.11.1.9) was quantified following the methodology of Flohé and Gunzler (1984). Two hundred microliters of the extract, 400 µL of GSH (0.1 mM), and 200 µL of Na_2HPO_4 (0.067 M) were homogenized and placed in a water bath at 25 °C for 5 min. Then, 200 µL of H_2O_2 (1.3 mM) was added for reaction for 10 min. The reaction was stopped by the addition of 1 mL of trichloroacetic acid (1%) and subsequently centrifuged in an Ohaus Frontier FC5515 R centrifuge (Ohaus Corp., New Jersey, USA) at 3000 rpm for 10 min at 4 °C. To determine GPX activity, 480 µL of the

supernatant, 2.2 mL of Na_2HPO_4 (0.32 M) and 320 µL of DTNB (1 mM) were homogenized to be read later in a Thermo Fisher G10S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at a wavelength of 412 nm. The results were expressed as U g^{-1} TP, where U corresponds to mM of reduced glutathione equivalents per milliliter per minute.

Concentration of Zn by ICP-AES

The total concentration of Zn in leaves was determined according to the methodology proposed by Alcántar and Sandoval (1999). Five hundred milligrams of dry samples were subjected to acid digestion in a mixture of HNO_3:$HClO_4$ (2:1 mL) and 2 mL of H_2O_2 30%. The concentration of Zn was read using an Agilent 725-ES coupled plasma induction atomic emission spectrometer (ICP-AES) (Agilent Technologies, California, USA). The results were expressed in mg kg^{-1} DW.

Results

Germination stage (15 DAS)

The germination percentage of *M. oleifera* seeds was not modified by NZnO (Figure 2-A); however, differences were found in the variables of length and dry weight of plumule and radicle. Concerning plumule length, the highest values were observed with the 2.5 mg L^{-1} treatment, increasing 46% compared to the control, followed by 10 mg L^{-1}, for which an increase of 37%. Regarding the length of the radicle, only the application of 2.5 mg L^{-1} presented a difference from the control, showing an increase of 20% in this variable (Figure 2-B).

On the other hand, the dry weight of plumule was increased when using doses between 2.5 and 10 mg L^{-1} NZnO, in the following order: 2.5>10>7.5>5 mg L^{-1}, while the level of 0.5 mg L^{-1} showed no difference from the control. A similar trend was observed in the dry weight of radicle, with this variable showing an increase as NZnO levels increased. The increase percentages to the control were 49, 28, 26, and 21%, corresponding to the levels of 10, 7.5, 5, and 2.5 mg L^{-1}, respectively. Similar to the dry weight of the plumule, the level of 0.5 mg L^{-1} was statistically equal to the control (Figure 2-C).

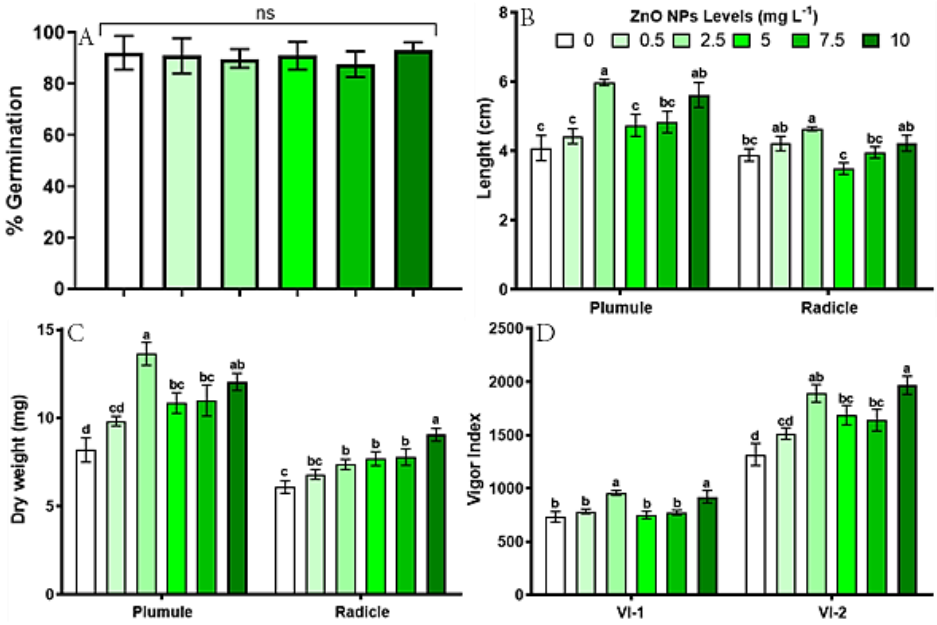

Figure 2. Germination percentage (A), plumule-radicle length (B), plumule-radicle dry weight (C), and vigor index (D) of *M. oleifera* seedlings at 15 DAS

Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns: not significant. The lines on the bars indicate the standard error of the mean. $n=6$

For VI1, the level of 2.5 mg L^{-1} of NZnO showed an increase of 30% for the control, followed by 10 mg L^{-1}, which increased the vigor of the seedlings by 25% at 15 days, without finding a difference from other treatments. On the other hand, in VI2, applications of 2.5 to 10 mg L^{-1} increased vigor in seedlings, with increments of 49, 43, 27, and 24% for treatments of 10, 2.5, 7.5, and 5 mg L^{-1}, respectively (Figure 2-D).

Greenhouse stage (45 DAS)

The vegetative growth of seedlings at 45 DAS was affected differently for most of the evaluated variables. Plant height was increased by 15% with the concentration of 0.5 mg L^{-1} NZnO, while 2.5 mg L^{-1} produced an increase of 13% compared to the control (Figure 3-A). However, the stem diameter was not modified by the different treatments with NZnO (Figure 3-B). On the other hand, the fresh weight of aerial parts was increased by 23% and 22% for the treatments of 5 and 0.5 mg L^{-1} NZnO, respectively (Figure 3-C). The same trend was found in dry weight, in which the concentration of 5 mg L^{-1} NZnO promoted a 33% increase in the dry biomass of the seedlings, while the dose of 0.5 mg L^{-1} increased the same variable by 28% over the control (Figure 3-D). For the fresh and dry weights of the roots of *M. oleifera,* no differences were found between the different levels of NZnO (Figure 3-C and D).

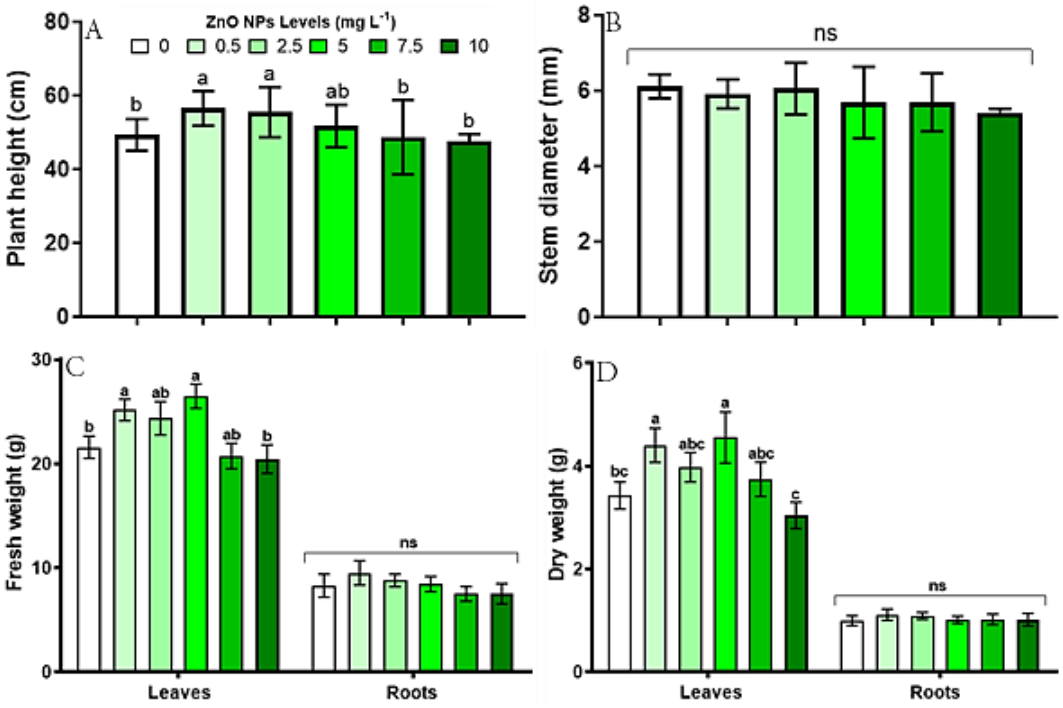

Figure 3. Plant height (A), stem diameter (B), leaf-root fresh weight (C) and leaf-root dry weight (D) of *M. oleifera* at 45 DAS

Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns: not significant. The lines on the bars indicate the standard error of the mean. $n=6$

Photosynthetic pigments

The chlorophyll and β-carotene concentration responded positively to the NZnO treatments. For chlorophyll a, an increase of 33% was found in seedlings subjected to 7.5 mg L^{-1} NZnO, while 10 mg L^{-1} increased 28% compared to the control. The same trend was found in chlorophyll b, with the concentration increasing by 30 and 23% for treatments of 7.5 and 10 mg L^{-1} NZnO, respectively. The above was confirmed with the concentration of total chlorophyll, with a total increase resulting in 32 and 26% for the same doses of NZnO (Figure 4-A). The chlorophyll a/b ratio was not different between treatments (Figure 4-B). On the other hand, in the concentration of β-carotene, the same behavior was observed for chlorophylls a and b, for

which only the doses of 10 and 7.5 mg L^{-1} NZnO favored an increase of 49 and 42%, respectively, compared to the control treatment (Figure 4-C).

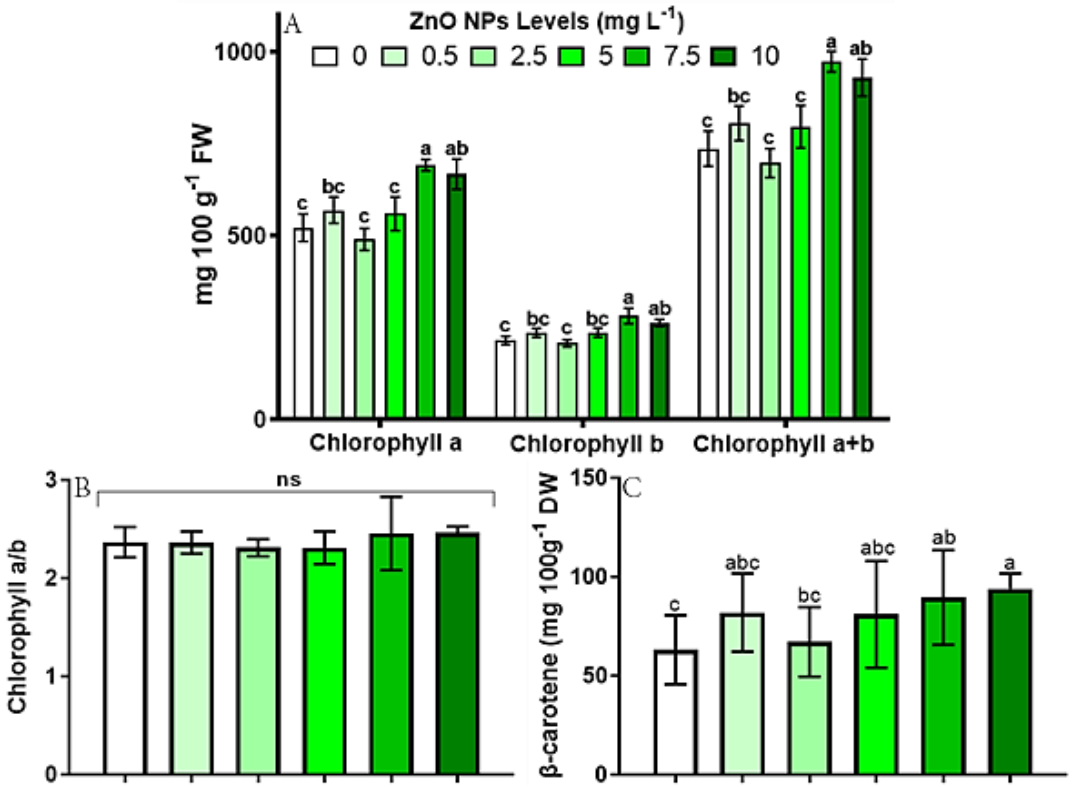

Figure 4. Chlorophyll a, chlorophyll b, total chlorophyll (A), chlorophyll a/b ratio (B), and β-carotene (C) of *M. oleifera* leaves

Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns: not significant. The lines on the bars indicate the standard error of the mean. $n=6$

Nonenzymatic antioxidant compounds

The nonenzymatic antioxidant compounds showed variation between treatments. For phenolic compounds, 7.5 and 10 mg L^{-1} NZnO promoted an increase of 13 and 11% in the leaves; in the roots, 7.5 mg L^{-1} showed an increase of 32% in phenolics, and 2.5 mg L^{-1} produced an increase of 29% compared to the control (Figure 5-A). Similarly, the DPPH antioxidant capacity of leaves showed an increase of 16% and 9% for 10 and 7.5 mg L^{-1} of NZnO, respectively, and 8% with 7.5 mg L^{-1}, over the control. However, for the roots, no statistically significant differences were found (Figure 5-B).

On the other hand, flavonoids increased 66, 31, and 30% for levels of 5, 7.5, and 10 mg L^{-1} NZnO, respectively (Figure 5-C). However, the most significant increases in flavonoids were found in roots, with the treatment of 10 mg L^{-1} showing an increase of 543% over the control, followed by levels of 7.5, 5, and 2.5 mg L^{-1}, which showed increases of 318, 304, and 257%, respectively (Figure 5-D).

Vitamin C resulted in increases between different levels of NZnO, with the treatment of 10 mg L^{-1} showing the most significant increase of 20%, followed by doses of 5, 7.5, and 0.5 mg L^{-1} with increments of 16, 14, and 11%, respectively, compared to the control. On the other hand, vitamin C in the root was increased for all treatments with NZnO, remaining as follows: 7.5>5>10>2.5>0.5 mg L^{-1}, with increases of 22, 18, 17, 16, and 11%, respectively (Figure 6-A). In contrast, the GSH concentration was negatively affected by NZnO: 7.5 and 10 mg L^{-1} produced a decrease of 23 and 19%, contrasted to the control, while at the root, the same treatments showed a reduction of 25%, as well as a decrease of 22% in the level of 5 mg L^{-1} of NZnO (Figure 6-B). Concerning protein concentration, only 2.5 and 5 mg L^{-1} NZnO increased 7%, with no difference for the

roots (Figure 6-C). On the other hand, H_2O_2 levels of the leaves increased with 7.5, 10, and 2.5 mg L^{-1} NZnO, with increases of 20, 18, and 16%, respectively. A similar trend was found in the root, with levels of 10 and 7.5 mg L^{-1} of NZnO with an increase of 37 and 32% of this compound compared to the control (Figure 6-D)

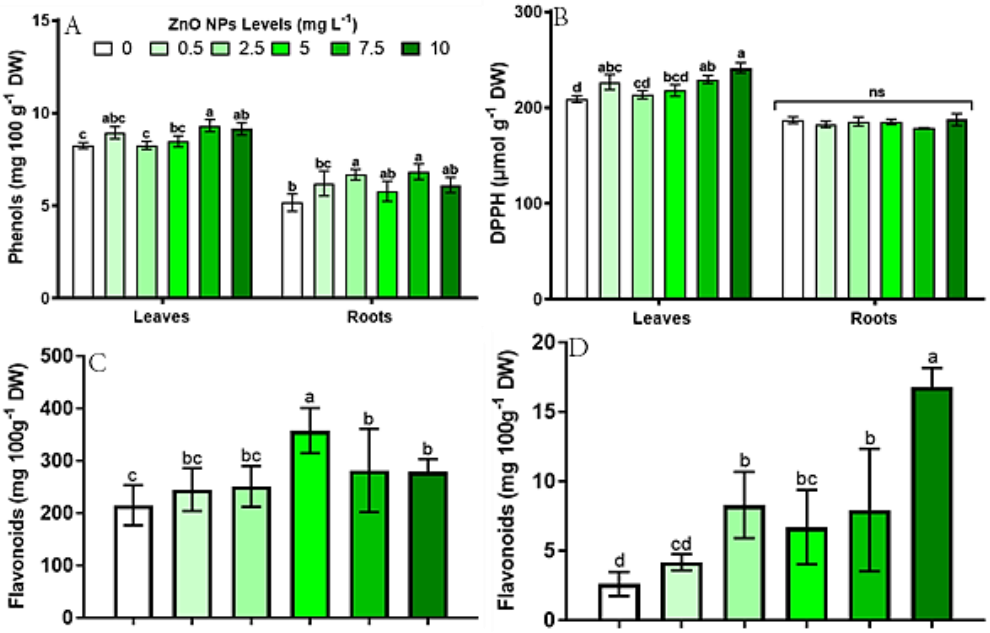

Figure 5. Phenolic compounds (A), antioxidant capacity DPPH (B), leaf and root flavonoids (C and D) of *M. oleifera*

Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns: not significant. The lines on the bars indicate the standard error of the mean. $n=6$

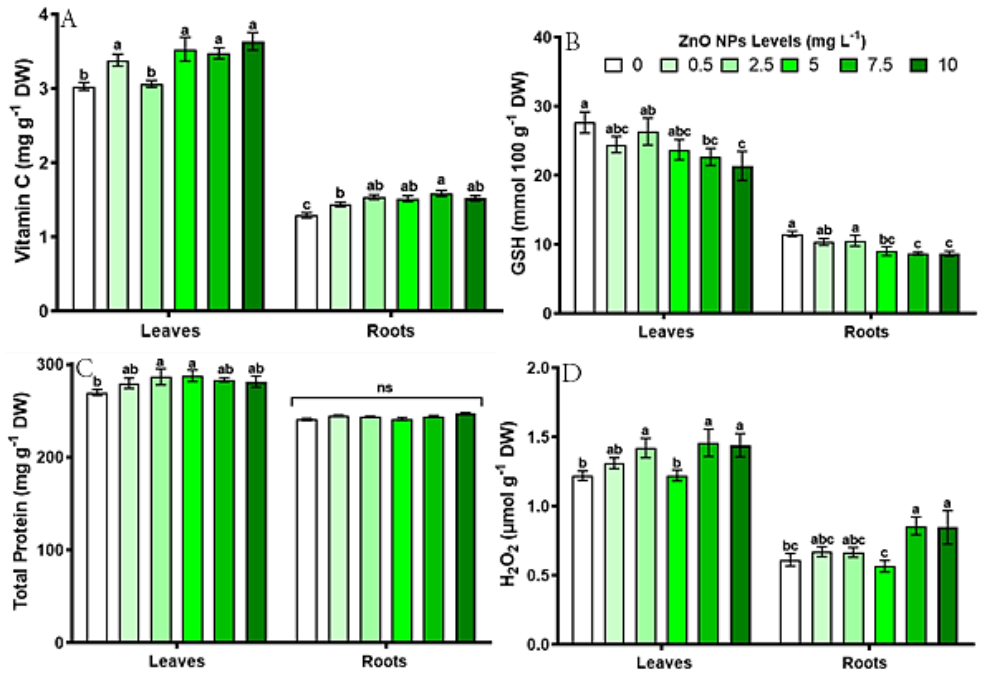

Figure 6. Vitamin C (A), GSH (B), total protein (C) and H_2O_2 (D) of *M. oleifera*

Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns: not significant. The lines on the bars indicate the standard error of the mean. $n=6$

Enzymatic antioxidant compounds

The enzymatic activity in the leaves and roots of *M. oleifera* was modified depending on the dose of NZnO. A hormetic response was observed in the activity of APX and CAT. For APX, only 10 mg L^{-1} NZnO promoted a 30% increase in the enzymatic activity in the leaves. On the other hand, the roots showed 42 and 37% more APX activity for 5 and 2.5 mg L^{-1} NZnO (Figure 7-A). Furthermore, the leaf CAT activity increased by 27% with 0.5 mg L^{-1} NZnO; however, the enzymatic activity decreased by 31 and 33% with 10 and 7.5 mg L^{-1} NZnO, respectively, without differences for the roots (Figure 7-B). Concerning GPX in leaves, decreases of 25, 19, and 17% were observed for 2.5, 0.5, and 7.5 mg L^{-1}, respectively. A similar trend was observed in roots, with decreases of 25% with 7.5 mg L^{-1}, followed by 18 and 15% with 10 and 2.5 mg L^{-1}, respectively, compared to the control (Figure 7-C). Finally, PAL activity in leaves decreased 23% with the concentration of 0.5 mg L^{-1} of NZnO; on the other hand, an increase of 30% in the level of 5 mg L^{-1} was observed for the root (Figure 7-D).

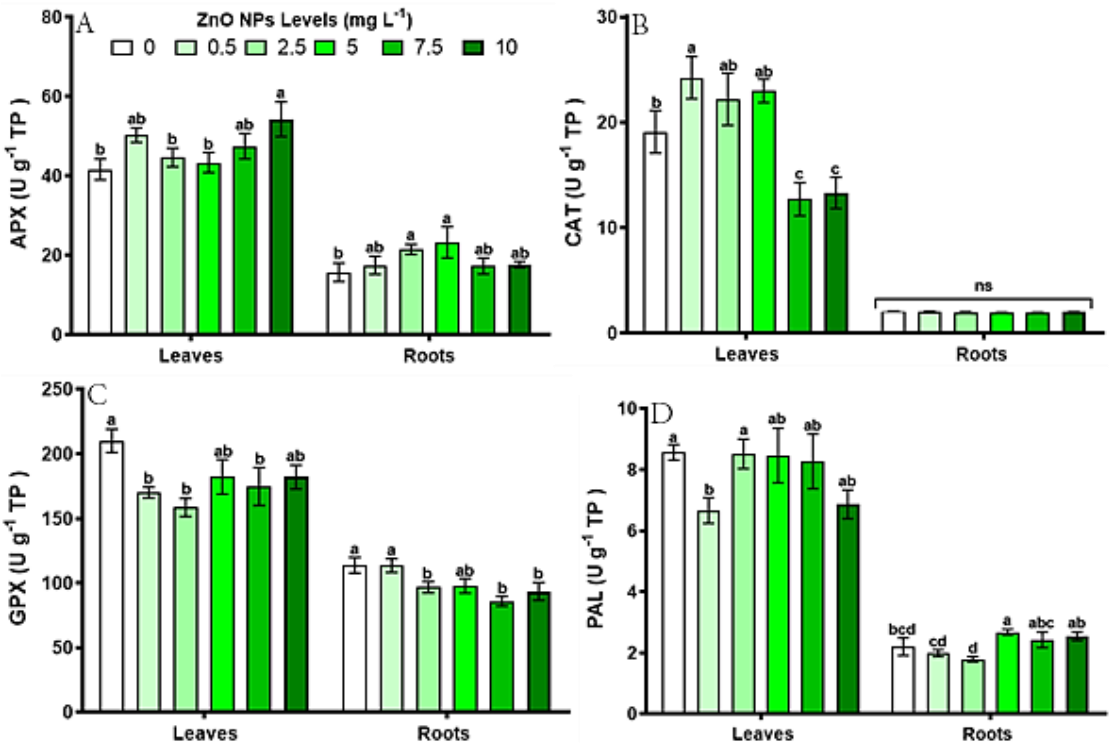

Figure 7. APX (A), CAT (B), GPX (C), and PAL (D) activity of *M. oleifera*
Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns: not significant. The lines on the bars indicate the standard error of the mean. $n=6$

Zn concentration

The concentration of Zn (Figure 8) was only increased in the 10 mg L^{-1} treatment, with an increase of 3.7 mg kg^{-1} (12%) over the control, without finding differences between the other levels of NZnO.

Correlation analysis

The results of the correlation analysis showed significant relationships between the variables related to the antioxidant system of *M. oleifera.* For vitamin C, a positive association was found with photosynthetic pigments (chlorophyll a, chlorophyll b, chlorophyll a/b, and β-carotene). The same trend was observed between flavonoids and photosynthetic pigments, such as flavonoids with vitamin C. On the other hand, the concentration of H_2O_2 in leaves and roots showed a positive correlation between most of the antioxidant compounds studied. In the case of enzymatic compounds, positive correlations were found between CAT-GPX

and PAL-GPX and APX with vitamin C, GSH, phenols, and flavonoids. The correlation analysis showed some negative relationships between the variables, highlighting the content of Zn against GSH, with the latter also negatively related to vitamin C and flavonoids. The enzymatic activity of GPX and CAT showed a negative relationship with vitamin C, and the same was true for PAL-GSH, GPX-APX, GPX, and DPPH with CAT (Figure 9).

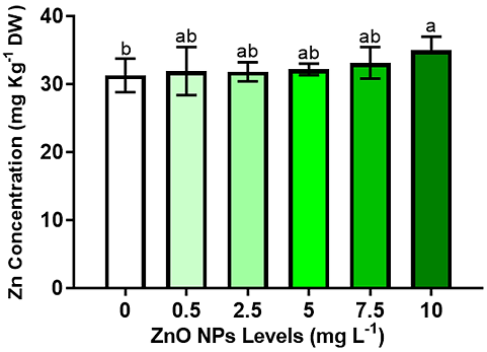

Figure 8. Zn concentration on leaves of *M. oleifera*
Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). The lines on the bars indicate the standard error of the mean. $n=6$

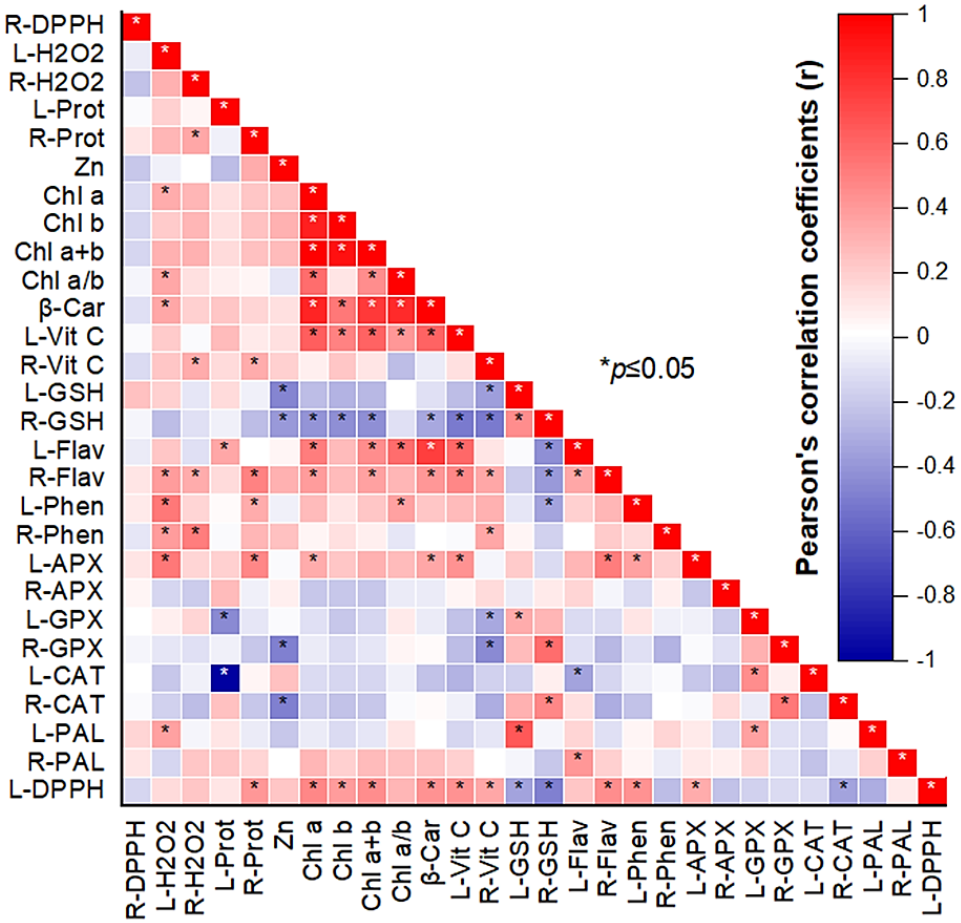

Figure 9. Matrix of correlations between variables of the antioxidant system of *M. oleifera*
L: Leaves, R: Roots, Prot: Total protein, Zn: Zn content, Chl a: Chlorophyll a, Chl b: Chlorophyll b, β-car: β-carotene, Vit C: Vitamin C, GSH: Glutathione, Flav: Flavonoids, Phen: Phenols, APX: Ascorbate peroxidase, GPX: Glutathione peroxidase, CAT: Catalase, PAL: Phenylalanine ammonium lyase, DPPH: Antioxidant capacity, H_2O_2: Hydrogen peroxide.

Discussion

Germination stage (15 DAS)

Seed priming with nanomaterials is an activator of germination, promoting seedlings' initial growth and biochemical characteristics in various plant species (Waqas *et al.*, 2019). The percentage of germination of *M. oleifera* seeds was not affected by the NZnO doses, possibly because the seeds germinated under optimal conditions, where the stimulus perceived by the nanoparticles was not sufficient to modify the dormancy of the embryo in the seeds (Rao *et al.*, 2019). However, it has been reported that NMs produce changes in the permeability of membranes and the levels of osmorregulators and can show positive effects on germination (Majeed *et al.*, 2019). In other studies, using higher concentrations of NZnO (0-500 mg L^{-1}), no changes were detected in the germination of seeds of *Capsicum annum* (García-López *et al.*, 2018) and *Cicer arietinum* with 1000 mg L^{-1} NZnO (Hajra and Kumar, 2017).

On the other hand, the length and dry weight of plumule and radicle resulted in increases in seeds treated with NZnO, possibly because NMs at low concentrations promote the production of ROS and phytohormones and the overexpression of aquaporins, and the response is a more remarkable synthesis of antioxidant compounds and improvement in the absorption of water and nutrients, favoring the initial growth of seedlings (Santo *et al.*, 2021). Regarding the improvement in water and nutrient absorption, which is associated with the increase in length and biomass, it has been shown that NZnO induces overexpression of the *HvTip1:1* and *HvPip1:1* genes, both related to the generation of new water channels (aquaporins) in cells (Akdemir, 2021).

The increases found in plumule and radicle growth can also be explained because the nanoparticles favor a greater capacity to mobilize the reserves contained in the seeds and increase the efficiency of using these reserves (Seyyedi *et al.,* 2015). This effect occurs because NZnO increases the activity of the enzyme α-amylase (Rai-Kalal and Jajoo, 2021), an enzyme responsible for the degradation of starch and transformation to sugars readily available for the seed embryo (Kondhare *et al.*, 2015). The above was confirmed by Itroutwar *et al.* (2020), who reported that NZnO applied to *Zea mays* seeds accumulated in the endosperm region, associated with rapid starch degradation that favored the growth of the plumule and radicle, increasing the final quality of the seedlings.

Regarding the vigor index, the same trend was observed as in the variables of length and dry weight of plumule and radicle, which is explained due to the mathematical construction of these indicators. As no significant difference was observed in the percentage of germination, the value of the vigor index depends mainly on the length and weight of the plumule and radicle (Carballo-Méndez *et al.*, 2019). The concept of vigor refers to the ability of seeds to germinate and develop in a wide range of environments (Rajjou *et al.*, 2012). Seed vigor is directly related to higher plant quality at other stages of development (Caverzan *et al.,* 2018). More seed vigor partially explains some of the results found in the second experimental phase of this research.

The responses of the length and dry weight of plumule and the vigor index did not show a linear trend as NZnO levels increased; instead, a biphasic response, an effect known as hormesis, was observed (Agathokleous *et al.*, 2019). This response is thought to be mainly due to aggregation phenomena, interfacial interactions, and cellular responses, resulting in a nonlinear trend (Juárez-Maldonado *et al.*, 2019). The effect of hormesis has been demonstrated in some works with the technique of seed priming, such as that carried out by Neto *et al.* (2020), where the use of NPs of Fe_3O_4 in *Zea mays* produced increased vigor of seedlings to 40 mg L^{-1}, followed by a decrease to 80 mg L^{-1} and an increase in this by increasing the dose to 160 mg L^{-1}. A similar effect was reported in seeds of *Allium cepa* with applications of 50 to 3200 mg L^{-1} NZnO (Tymoszuk and Wojnarowicz, 2020). The responses explained previously coincide with what was found in this experimental phase. The biphasic response has also been found in other species in other stages of vegetative growth; an example was presented in the study by López-Vargas *et al.* (2020), who applied seed priming with carbon NMs in tomato plants and observed nonlinear effects on vegetative growth and bioactive compounds, which coincides with the responses observed in the second experimental stage of this research.

Greenhouse stage (45 DAS)

The application of NMs as a pretreatment to seeds has been shown to favor seedling germination and initial growth, evidenced by a more significant accumulation of dry matter in the tissues of different plant species (Abbasi *et al.*, 2021). In this work, the observed increase in the vegetative growth of seedlings from seeds previously treated with NZnO could have occurred because this material can increase the number of mitotic cells in prophase, metaphase, anaphase, and telophase (Hoe *et al.*, 2018), as well as decrease the number of abnormal cells in the same phases of cellular mitosis (Reis *et al.*, 2018). The results coincide with what was reported by Tondey *et al.* (2021) in *Zea mays* plants, where NZnO increased biomass by 45% using a dose of 20 mg L^{-1} via seed priming. A similar effect was also found in plants of *Oryza sativa* (Li *et al.*, 2021).

On the other hand, the increase in the concentration of chlorophyll a and b in seedlings from seeds subjected to the highest levels of NZnO (7.5 and 10 mg $L^{-1)}$ may be associated with the essential role of Zn in chlorophyll biosynthesis (Sturikova *et al.*, 2018) through participation in LHC protein synthesis (light-harvesting complex) (Wang and Grimm, 2021), a family of proteins involved in the regulation of chlorophyll synthesis, in addition to being responsible for the repair of PSII by inserting new pigments into reaction centers (Rochaix and Bassi, 2019). On the other hand, Zn plays a vital role in developing chloroplasts (Sharma *et al.*, 2012), mainly by participating in the expression of at least seven genes related to the organization of membranes in thylakoids (Zhang *et al.*, 2019). For chlorophyll, β-carotene levels were increased in plants subjected to 7.5 and 10 mg $L^{-1.}$ This response probably depends on the functional association between chlorophyll and carotenoids that occurs in photosynthetic antennae (Rai-Kalal and Jajoo, 2021) or as a response to the production of reactive species such as H_2O_2 and O_2^- induced by NZnO (Uarrota *et al.*, 2018; Molnar et *al.*, 2020).

The results found in terms of photosynthetic pigments match other works carried out in *Lupinus termis* (Abdel-Latef *et al.*, 2017), *Cicer arietinum* (Hajra and Kumar, 2017), and *Triticum aestivum* (Solanki and Laura, 2018), all using the seed priming technique. On the other hand, NZnO increased the content of total carotenoids in seedlings of *Solanum lycopersicum* (Singh *et al.*, 2016), with the same effect observed in this research. On the other hand, the vitamin C content also increased in seedlings and showed a high positive correlation with chlorophyll a and b and β-carotene, possibly because the vitamin C synthesis pathway (Smirnoff-Wheeler) depends mainly on the photosynthates produced in the leaves (Suekawa *et al.*, 2017). One of the main functions of vitamin C is the quenching of reactive species through the ascorbate-glutathione cycle. It is also possible that this antioxidant protection partly explains the positive correlation between vitamin C and photosynthetic pigments (Smirnoff, 2018).

Increases in the activity of enzymatic antioxidants and the concentration of nonenzymatic antioxidants can be attributed to the interaction of NMs with plant cells, which occurs in two phases. The first is triggered by surface phenomena when the NM corona contacts the cell wall, favoring the generation of reactive chemical species. The second phase is believed to occur in response to the internment and metabolism of NMs, possibly by the release of Zn^{2+} ions in plant cells (Juárez-Maldonado *et al.,* 2019). The production of reactive or oxidizing species, such as H_2O_2, activates plant defense systems, increasing the activity levels of enzymes such as CAT, APX, and GPX, as well as the concentration of nonenzymatic antioxidants such as phenols and flavonoids (Abdel-Aziz *et al.*, 2019; Ruiz-Torres *et al.*, 2021).

NZnO promoted an increase in H_2O_2 levels, which could activate the response mechanisms of plants by increasing enzymatic and nonenzymatic antioxidants, which coincides with what was described above. Other studies also found that the application of NZnO favored increased activity of SOD, CAT, POD, and APX in *Lupinus ternis* plants (Abdel-Latef *et al.*, 2017) and increased activity of CAT, APX, and POD in *Coriandrum sativum* (Ruiz-Torres *et al.*, 2021).

The activity of APX coincided with the results obtained in vitamin C, evidenced by a positive correlation between these variables, since the APX enzyme uses ascorbic acid as a substrate (Nakano and Asada, 1987). The same effect was observed in GPX activity, where the results obtained agree with the content of GSH to find a positive correlation since this compound is the substrate used to promote the activity of GPX (Flohé

and Gunzler, 1984). Therefore, an increase in vitamin C and GSH levels favored a more significant activity of the enzymes APX and GPX, respectively.

The reduction in GSH levels in *M. oleifera* tissues could have occurred because, in the ascorbate-glutathione cycle, the reduction of DHA in vitamin C uses GSH as a reducing agent (Pandey *et al.*, 2015). The above seems to explain part of the results in this research, finding higher levels of vitamin C and a decrease in GSH and the consequent negative correlation coefficient between the concentrations of both compounds. The results of this research coincide with Dogaroglu and Koleli (2017), who reported that the application of NZnO at doses of 5 and 10 mg L^{-1} reduced the levels of GSH in *Hordeum vulgare* leaves by 20%.

In most of the studied antioxidant compounds, a positive trend was observed as the concentration of NZnO increased; however, some compounds showed a decrease, as was the case for CAT activity. This response probably resulted from the increase in H_2O_2 associated with the treatments of 7.5 and 10 mg L^{-1} NZnO. High levels of H_2O_2 require increased CAT activity for decomposition (Ghosh *et al.*, 2016).

The biostimulation or toxicity effects of NMs in plants depend on their physical characteristics, such as size, shape, roughness, and composition (Juárez-Maldonado *et al.*, 2021). The NPs used in this work were of a reduced size (16.5 nm), which makes us expect that the surface of contact with the cells will increase substantially (Juárez-Maldonado *et al.*, 2018), thus favoring the process of biostimulation. Furthermore, the NPs used in this research were functionalized with citric acid to decrease their aggregation and increase the biostimulant impact. Additionally, citric acid is an organic acid that induces biostimulation in plant species (Mallhi *et al.*, 2019); however, with the available information, it was not possible to verify whether citric acid was a biostimulant factor.

The stimulus or stimuli perceived in the seeds of *M. oleifera* during seed priming indeed induced changes that manifested themselves in germination and at other later stages of development, possibly as priming memory. The above occurs when the induced metabolites and hormones, their conjugated forms, and proteins subject to posttranslational modifications induce transcriptomic and metabolic signatures transmitted between successive mitotic generations (Bose *et al.*, 2018). This priming memory was presumably established during seed contact with NMs and maintained during subsequent growth and development (Chen and Arora, 2013).

Regarding the content of Zn^{2+} in plants, this element was only increased at a dose of 10 mg L^{-1}. Thus, it is likely that the lower concentrations did not allow a significant increase in the Zn content in the seedlings to manifest, presumably because, at low concentrations (e.g., 1 mg L^{-1}), NZnO interacted mainly with the seed cover, with little internalization toward the cotyledons and the embryo (Savassa *et al.*, 2018). This idea is reinforced by Munir *et al.* (2018), who reported that the content of Zn^{2+} in the tissues of *Triticum aestivum* increased linearly between ranges of 25-100 mg L^{-1} of NZnO applied by seed priming.

Conclusions

The results show that it is possible to promote the initial growth and bioactive compounds of *M. oleifera* by pretreatment of seeds with NZnO, which represents a greater possibility of success in the establishment of this plant species as well as obtaining products of higher nutraceutical quality. A limitation of this study is the evaluation of low levels of NZnO, so the response of doses higher than 10 mg L^{-1} is still unknown when using the seed priming technique in *M. oleifera*.

Authors' Contributions

Conceptualization: CAGA and ABM; Data curation: YGG, GCP and EOS; Funding acquisition: ABM; Investigation: CAGA, EOS, ABM; Methodology: CAGA, YGG, GCP and LITT; Project

administration: ABM; Supervision: All authors; Writing - original draft: CAGA; Writing - review and editing: CAGA, ABM and YGG. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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