UNIVERSIDAD AUTÓNOMA AGRARIA ANTONIO NARRO

SUBDIRECCIÓN DE POSTGRADO

EFECTIVIDAD DE NANOFERTILIZANTES CONTRA FERTILIZANTES CONVENCIONALES EN CULTIVOS SIN SUELO

Tesis

Que presenta CARLOS ALBERTO GARZA ALONSO

Como requisito parcial para obtener el Grado de

DOCTOR EN CIENCIAS EN AGRICULTURA PROTEGIDA

Saltillo, Coahuila Julio 2023

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Tesis

Elaborada por CARLOS ALBERTO GARZA ALONSO como requisito parcial para obtener el Grado de Doctor en Ciencias en Agricultura Protegida

Con la supervisión y aprobación del Comité de Asesoría

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A mis familiares y amigos, por todo su apoyo incondicional durante esta etapa de mi vida.

A todas las personas que de cierta forma colaboraron durante el desarrollo de este trabajo.

DEDICATORIA

A mi familia

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Dear Dr. Carlos Alberto Garza-Alonso, Yolanda González-García, Gregorio Cadenas-Pliego, Emilio Olivares-Sáenz, Libia Iris Trejo-Téllez, Adalberto Benavides-Mendoza,

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1. INTRODUCCIÓN

El constante incremento de la población mundial, así como la pérdida de superficie cultivable debido a procesos erosivos y contaminación de los suelos, son factores que obligan a buscar alternativas para incrementar la producción de alimentos de calidad, con altos rendimientos por unidad de espacio y mejores características nutricionales (Raftery y Sevcikova, 2023).

Por otro lado, es bien conocido el rol que ejercen algunos micronutrientes en las plantas, tales como zinc (Zn), hierro (Fe), cobre (Cu), entre otros, los cuales son elementos indispensables para su desarrollo. Sin embargo, en varias regiones del mundo existen problemas de deficiencia o baja disponibilidad de dichos elementos en el suelo, principalmente en zonas de clima árido-semiárido, donde predominan suelos de naturaleza calcárea (altos contenidos de $CaCO_3$ y pH>8) (Kihara *et al*., 2020).

Durante los últimos años, la nanotecnología ha cobrado importancia en la agricultura debido a sus diferentes aplicaciones como plaguicidas, herbicidas, bioestimulantes y fertilizantes (Javed *et al*., 2023). En este último grupo, los llamados nanofertilizantes han demostrado tener efectos positivos en los cultivos (Benavides-Mendoza *et al*., 2022). Existen varios reportes en la literatura que indican los efectos positivos del uso de nanofertilizantes comparados con fertilizantes convencionales (Tighe-Neira *et al*., 2022). Sin embargo, aún es necesaria más información y la constante evaluación de nuevos nanomateriales (NMs) en distintas especies vegetales. Rahman *et al*. (2021) compararon fertilizantes convencionales versus una formulación de nanofertilizantes a base de Cu, Fe y Zn en plantas de tomate, encontrando mejores características en los frutos y una eficiencia del uso de los fertilizantes (EUF) 26% superior a la encontrada en las plantas con fertilizante comercial, así como una relación beneficio/costo del 1.63 vs 1.30.

Thapa *et al*. (2023) aplicaron nanopartículas (NPs) de ZnS y ZnO en comparación con el fertilizante $ZnCl_2$ en plantas de *Vigna radiata*. Los nanofertilizantes mostraron una mejor respuesta en variables agronómicas como altura de planta, biomasa seca de hojas y raíz, así como un mayor

número de vainas y rendimiento total de grano. Por otro lado, aplicaciones de diversas nanopartículas (ZnO, FeO y MgO) comparadas con su forma convencional ($ZnSO_4$, $FeSO_4$ y $MgSO_4$) favorecieron de entre un 50-93% las características morfológicas de las plantas como altura, así como incrementos de entre 30-80% en la concentración de clorofila, donde dichas variaciones en los aumentos fueron dependientes de la concentración aplicada en cada tipo de fertilizante (Khalid *et al*., 2022).

Adicional a su aplicación como fertilizantes, los NMs tienen la capacidad de inducir bioestimulación en las células vegetales, dando como resultado una mayor actividad en el metabolismo de las plantas en procesos como la fotosíntesis y respiración celular, producción de metabolitos secundarios, mejor asimilación de nutrientes, entre otros, lo que da como resultado final un mayor rendimiento de los cultivos (Juárez-Maldonado *et al*., 2019).

A pesar de los numerosos estudios comparativos entre ambos tipos de fertilizantes, actualmente, no se ha reportado el reemplazo completo de fertilizantes convencionales con nanofertilizantes en sistemas de agricultura protegida con cultivos sin suelo, por lo que el objetivo de la presente investigación fue realizar un estudio comparativo entre fertilizantes a base de Zn, así como su capacidad para cubrir las necesidades nutrimentales de cultivos establecidos en sistemas sin suelo. La hipótesis planteada fue que los nanofertilizantes pueden reemplazar a los fertilizantes convencionales, cubriendo las necesidades nutrimentales de las plantas e induciendo bioestimulación, incrementando la productividad y la calidad de los cultivos producidos bajo condiciones sin suelo.

El reemplazo de los fertilizantes convencionales conlleva varios beneficios a corto y mediano plazo, ya que, al tener una mayor eficiencia, son aplicados en cantidades menores a los cultivos, reduciendo el impacto ambiental, además de lograr incrementar la productividad de los cultivos y la obtención de alimentos con alta calidad nutracéutica.

2. REVISIÓN DE LITERATURA

2.1. La Nanotecnología en la Agricultura

En los últimos años, la nanotecnología ha ganado importancia en la agricultura, principalmente debido a sus aplicaciones como plaguicidas, fertilizantes y bioestimulantes, aunque actualmente también se reportan aplicaciones como nanoherbicidas y nanosensores (Javed *et al*., 2023).

De forma general, la nanotecnología engloba partículas con una dimensión menor o igual a los 100 nm, sin embargo, en la literatura también se consideran materiales por encima de dichas dimensiones (Vijayakumar *et al*., 2022). Para fines prácticos, en este documento se denotarán como nanomateriales (NMs) a cualquier material con dimensiones inferiores a los 500 nm. Existe una amplia diversidad de NMs, donde los más comunes corresponden a (Ahmad *et al*., 2022):

-Nanopartículas (NPs) metálicas (ZnO, Fe_2O_3, SiO_2, MgO, TiO_2, CeO_2).

-Nanomateriales de carbono (nanotubos de carbono, grafeno, fullereno).

-Nanopartículas de zeolita y quitosán.

-Materiales cuánticos.

Los NMs con mayor estudio en la agricultura corresponden al primer grupo, principalmente las NPs de Ag, ZnO y Fe_2O_3, con los cuales se ha reportado su uso como nanofertilizantes (Liu *et al*., 2022; Khan *et al*., 2022). Por otro lado, los NMs de carbono han mostrado efectos favorables contra algunos fitopatógenos, como *Alternaria* y *Fusarium* (González-García *et al*. 2021; González-García *et al*., 2022). En el caso de los materiales cuánticos, actualmente es el grupo con menor estudio, sin embargo, representan una tecnología emergente con gran potencial de desarrollo en el corto y mediano plazo (Benavides-Mendoza *et al*., 2023). El uso de nanoformulaciones en los cultivos representa un menor impacto ambiental que los productos convencionales, debido a su mayor eficiencia y a las bajas dosis requeridas para su aplicación (Rahman *et al*., 2021).

2.2. Características de los NMs

Los efectos que producen los NMs en las plantas se explican debido a sus particulares características fisicoquímicas del material. Las principales características son la forma, tamaño, superficie de contacto, carga eléctrica, rugosidad, hidrofobicidad, entre otras (Juárez-Maldonado *et al*., 2021). Además de lo anterior, las respuestas de las plantas a la aplicación de los NMs también dependen de la dosis, tiempo de exposición, etapa fenológica y forma de aplicación (González-Morales *et al*., 2022). Con respecto a la forma, existen partículas con distintas morfologías como esférica o semiesférica, cúbicas, con forma de estrella o partículas irregulares (Benavides-Mendoza *et al*., 2021). Por otro lado, los nanotubos de carbono cuentan con formas cilíndricas, los cuales pueden tener varias capas entre sí (Safdar *et al*., 2022), mientras que el grafeno corresponde a hojas de hasta 500 nm de largo, con un grosor de apenas un átomo de carbono (Zhang *et al*., 2022). En lo que respecta al tamaño, los NMs de menores dimensiones tienen mayor facilidad de acceso a las plantas, así como una mayor superficie específica de contacto (m^2 g^{-1}), lo cual representa mayores puntos de contacto de los materiales con las células, dando como resultado mayor capacidad de bioestimulación (Méndez-López *et al*., 2022).

Por otra parte, la carga eléctrica de los NMs es relevante en su interacción con las paredes y membranas celulares. La carga neta de los NMs dependerá de la suma entre cargas positivas y negativas, formando la llamada doble capa eléctrica (EDL), lo cual varía dependiendo del material (Juárez-Maldonado *et al*., 2019). Con respecto a la rugosidad, se refiere a la textura de las capas externas de los NMs, donde materiales más rugosos, con superficie específica más grande, tienden a producir mayores interacciones con las superficies de las células (Juárez-Maldonado *et al*., 2021).

La dosis de aplicación de los NMs es uno de los factores de mayor importancia, ya que las características fisicoquímicas antes mencionadas, así como la amplia diversidad de los NMs, ocasionan que los niveles de aplicación difieran en gran medida, incluso al utilizar un mismo material. Asimismo, el tiempo en el que los NMs están en contacto con las estructuras vegetales, también influye en

la respuesta de las plantas (González-Morales *et al*., 2022). En lo que respecta a las formas de aplicación, los NMs pueden ser aplicados como pretratamiento a las semillas (seed priming), vía foliar, en drench (directo al suelo o sustrato) y como inmersión de la raíz (Méndez-López *et al*., 2022). La asimilación de los NMs por las plantas dependerá de la forma y el órgano vegetativo al que son aplicados.

2.3. Metabolismo de los NMs por las Plantas

2.3.1. Absorción de NMs por la raíz

El proceso absorción comienza desde el primer contacto de las NPs con las raíces para su posterior transporte a otros órganos de la planta. Uno de los primeros factores que influencian el contacto de las NPs con las raíces es su morfología. En este sentido, las plantas monocotiledóneas cuentan con un sistema radicular fibroso, permitiendo un mayor volumen de exploración en el suelo y favoreciendo el contacto con las NPs. Por el contrario, las dicotiledóneas tienen una raíz pivotante con menos ramificaciones (Su *et al*., 2019).

Una de las principales vías de acceso del agua y nutrientes, así como de NPs, es a través del mecanismo de intercepción de la raíz, lo cual ocurre cuando las raíces de las plantas se elongan lo suficiente para alcanzar las regiones que contienen el agua y los nutrientes (Griffiths y York, 2020). El acceso a las capas externas de la raíz puede ocurrir de forma similar que el acceso de iones, lo cual sucede mediante difusión simple o flujo de masas, aunque también ocurre directamente a través de los espacios existentes entre las uniones de las raíces secundarias con la raíz primaria o en las uniones de los pelos radiculares (Su *et al*., 2019).

Posterior al primer contacto, las raíces cuentan con varias capas de células parenquimáticas, las cuales deben ser atravesadas por las NPs hasta llegar a los haces vasculares como el xilema. Las principales vías utilizadas por las NPs son la vía apoplástica (a través de los espacios intercelulares) y la vía simplastica, la cual consiste en el movimiento a través del citoplasma de las

células, atravesando entre ellas por los plasmodesmos (uniones celulares) (Lv *et al*., 2019). A pesar de que en la vía del apoplasto el transporte se lleva a cabo mediante los espacios existentes entre las células, llega un momento en el que las NPs deben ingresar a las células para poder atravesar la banda de Caspary y posteriormente alcanzar las células del xilema (Shukla *et al*., 2016).

El acceso de las NPs a las células de la raíz también es influenciado por las características del ambiente, como lo son la presencia de materia orgánica, compuestos inorgánicos, disponibilidad de agua, salinidad del suelo, niveles de elementos minerales e incluso por factores bióticos (Ma *et al*., 2018).

2.3.2. Absorción de los NMs por las hojas

La principal vía de acceso de las NPs en las hojas es a través de las estomas. Lo anterior debido a las dimensiones en la apertura de los mismos, además de que estos cubren un área importante de la superficie foliar, facilitando el contacto y acceso de las NPs (Lv *et al*., 2019). La densidad estomática de las hojas (n° de estomas/superficie) varía entre las especies vegetales, puede ser alterada por factores ambientales como la intensidad de la luz, concentración de CO_2, entre otros (Huang *et al*., 2022). Adicionalmente, los hidátodos presentes en las hojas también funcionan como una vía de fácil acceso para las NPs, dado que se encuentran directamente conectadas con los vasos del floema (Avellan *et al*., 2021). Algo similar ocurre con los tricomas, ya que existen evidencias de su rol como vía de acceso para algunas NPs, principalmente a través de los espacios existentes entre el tricoma y la cutícula de las hojas (Hong *et al*., 2021). Las características de la cutícula de las hojas, dependiendo de la especie vegetal, también influye en la absorción de las NPs, lo anterior debido a que pueden formar reacciones de atracción o repulsión, dependiendo a su vez de la corona de las NPs (Read *et al*., 2020).

Por otro lado, además de los estomas, hidátodos y tricomas, algunos poros de las hojas pueden funcionar como vía de acceso para las NPs. Una vez que ingresan a las capas externas de las hojas, las NPs avanzan a través de la vía del apoplasto y simplasto, hasta alcanzar los vasos del floema, el cual

representa el principal medio para el transporte hacia otros órganos de la planta (White, 2012).

2.3.3. Transporte a larga distancia de las NPs a través del xilema y floema

El principal factor que influye en el transporte a través del xilema es su estructura y morfología, lo cual varía dependiendo de la especie vegetal. Algunas de las características importantes son los diámetros internos de las traqueidas, así como el diámetro de las uniones entre las células del xilema, donde se encuentra la llamada “placa de tamiz” con poros que van desde los 200 nm hasta 1.5 µm, donde en su interior también existen placas porosas con diámetros que abarcan desde los 43 hasta los 340 nm (Su *et al*., 2019). La carga de las NPs también influye en la capacidad del transporte, donde algunos materiales con cargas positivas pueden quedar adsorbidos en las paredes del xilema, mientras que NPs con carga negativa tienden a la repulsión, permitiendo el movimiento libre y fácil ascenso (Spielman-Sun *et al*., 2019). Una vez en el xilema, la principal forma de transporte de las NPs es mediante el fujo de masas, donde se mueven junto con el agua, minerales y otras sustancias absorbidas previamente por las raíces. El transporte dependerá de la velocidad de transpiración de las plantas, por lo que, a mayor transpiración, mayor será el movimiento de sustancias en el xilema y la velocidad de movimiento de las NPs también se incrementa (White, 2012).

Hu *et al*. (2020) demostraron que NPs con carga positiva mostraron mayor capacidad de acceso por medio de las células guarda de los estomas (100 % de acceso), donde fueron distribuidas hacia los espacios extracelulares (90 %) y acumuladas en los cloroplastos (55 %), lo anterior fue demostrado en plantas de *Zea mays* y *Gossypium herbaceum* con aplicaciones foliares de nanotubos de carbono (CNT), NPs de CeO_2 y NPs de SiO_2. Cuando las NPs acceden a las plantas a través de las hojas, la principal vía de transporte es el floema, tejido responsable de transportar los fotoasimilados hacia otras regiones de la planta (White, 2012). Los factores que influyen en el transporte a través del floema son las características de las células que lo conforman, las cuales son conocidas

como celdas de tamiz o tubos cribosos, los cuales se encuentran unidos a través de poros que abarcan desde los 200 nm hasta 1.5 µm, lo que permite el acceso de las NPs (Su *et al*., 2019). Las sustancias que contienen el xilema y el floema (agua, azúcares, minerales, ácidos orgánicos) también influyen en el transporte de las NPs, incluso en algunos casos, pueden promover la agregación, adsorción o incluso su biotransformación en formas iónicas y otros compuestos (Su *et al*., 2019). Esto último fue reportado por Wang *et al*. (2012), donde observaron que NPs de CuO fueron biotransformadas a Cu^{2+} en los tejidos del floema de *Zea mays*.

2.3.4. Penetración de los NMs en las células

El acceso de los NMs a las células puede llevarse a cabo de distintas maneras, donde las principales son a través de los poros celulares, mediante difusión, endocitosis y fagocitosis, lo cual depende principalmente de las dimensiones de los materiales (González-Morales *et al*., 2022). Después de ingresar a las células vegetales, las NPs tienden a acumularse entre los organelos celulares, membrana celular o transformarse en otros compuestos (Banerjee *et al*. 2019).

La compartimentalización de las NPs puede tener efectos positivos o negativos en las plantas. Por ejemplo, las NPs formadas de elementos minerales esenciales para las plantas, como Zn, Fe y Cu, tienden a producir los efectos positivos conocidos de cada elemento en el metabolismo vegetal, sin embargo, NPs de elementos considerados no esenciales como el Ti, Ce y Cd, pueden provocar toxicidad en las plantas cuando son aplicadas en altas concentraciones (Juárez-Maldonado *et al*., 2019), donde dicha concentración depende de las características del material, forma de aplicación y especie vegetal. Una vez dentro de las células, los plasmodesmos juegan un papel importante en el transporte de las NPs hacia otras células para su posterior distribución en los organelos (Banerjee *et al*. 2019).

2.3.5. Transformación de los NMs

Una vez que los NMs ingresan a las células, estos tienden a ser transformados de distintas maneras, ya sea formando agregados entre sí, uniones a otras moléculas o compuestos, así como la disolución y formación de iones libres, dependiendo del material y de las condiciones del medio en el que se encuentran (Ahmed *et al*., 2021), además de la especie vegetal a la que son aplicadas las NPs (Zhang *et al*., 2019).

Algunos reportes indican que las NPs de ZnO son ingresadas a las células vegetales mediante endocitosis, para posteriormente ser acumuladas en las vacuolas para su transformación, lo cual dependerá de la presencia de ácidos orgánicos (e.g., ácido cítrico), concentración de PO_4^{3-} y pH (Lv *et al*., 2021). En el caso del pH, se ha demostrado que las NPs de ZnO son disueltas en pH de 5.4-5.6, donde el ZnO y los iones H^+ presentes en el medio son transformados en Zn^{2+} y H_2O (Lv *et al*., 2019). También se observó que la concentración de fosfatos en el citoplasma favorece la transformación de las NPs de CeO_2 en $CePO_4$ en plantas de *Cucumis sativum* (Rui *et al*., 2015). Es importante mencionar que las NPs pueden ser biotransformadas incluso durante el proceso de transporte a larga distancia a través del xilema y floema, donde la composición de dichos tejidos favorece la liberación de los componentes de las NPs y su posterior formación de iones. Wang *et al*. (2012), demostraron que las NPs de CuO fueron biotransformadas a Cu^{2+} en los tejidos del floema de *Zea mays*. Del mismo modo, las NPs pueden ser ionizadas en los suelos, antes de ser absorbidas por las plantas, lo cual depende principalmente del pH (mayor solubilidad en medios ácidos), presencia de ácidos orgánicos y concentración de sideróforos en la rizosfera (Ahmed *et al*., 2021).

Además de las características del medio en el que se encuentran, las características fisicoquímicas de las NPs influyen en la disolución de las mismas, por ejemplo, se ha reportado que las NPs de menor tamaño (<10 nm) tienen una mayor velocidad de transformación que otros materiales de tamaño superior, lo anterior es atribuido principalmente a que las NPs cuentan con

mayor superficie de contacto entre menor sea sus dimensiones (Ahmed *et al*., 2021).

2.4. Mecanismos de bioestimulación de los NMs

Los mecanismos por los que las NPs inducen una respuesta positiva en el crecimiento y desarrollo de las plantas aún se encuentran en discusión. Sin embargo, una de las hipótesis más aceptada es la correspondiente a las interacciones interfaciales entre las NPs y las paredes y membranas celulares. Un primer contacto entre las NPs y la pared-membrana celular depende de las características de las NPs como lo son las cargas superficiales, tamaño, forma, hidrofobicidad, entre otros factores. Al entrar en contacto, el posible estímulo o daño causado o las modificaciones en las proteínas de las membranas son rápidamente detectados por las células, desencadenando cascadas de señales a través de la producción de moléculas señalizadoras (como los derivados de fosfolípidos, ROS, RNS y RSS), dando como resultado modificaciones transcripcionales y postraduccionales capaces de inducir una respuesta positiva (Juárez-Maldonado *et al*., 2019).

La dimensión de las NPs es uno de los factores de mayor importancia con relación a su capacidad de inducir bioestimulación en los tejidos vegetales. De forma general, entre menor sea el tamaño de las partículas, mayor será la superficie de contacto con las células (Juárez-Maldonado *et al*., 2021), favoreciendo así el proceso de la bioestimulación. Lo anterior permite que las plantas respondan favorablemente a dosis bajas de NPs, mientras que en altos niveles es posible observar efectos de toxicidad (Juárez-Maldonado *et al*., 2021). Por otro lado, la forma de las NPs también juega un rol importante, donde materiales irregulares (e.g., con forma de estrella) pueden producir mayores daños a las membranas y desencadenar las respuestas de las plantas, lo cual sería en menor medida para materiales de formas esféricas o semiesféricas (González-Morales *et al*., 2022).

Asimismo, la carga superficial de las NPs influye en los procesos de bioestimulación. Generalmente, la membrana celular cuenta con una carga

parcial negativa, por lo que las NPs de carga positiva serían fácilmente atraídas. Para las NPs con carga negativa, ocurre lo conocido como formación de la corona, una capa externa formada principalmente por residuos proteicos y compuestos orgánicos presentes en el medio, obteniendo al final una carga parcial positiva que permite la unión de las NPs con la membrana celular (Bing *et al*., 2021). Algo similar a lo anterior ocurre con la interacción entre las regiones hidrofóbicas e hidrofílicas de los materiales y las superficies celulares.

Una segunda fase del proceso de bioestimulación depende de la composición interna de las NPs (núcleo), donde una vez transformadas, los elementos contenidos cumplen los roles específicos en los distintos tejidos vegetales (Juárez-Maldonado *et al*., 2019). En el caso específico de NPs de ZnO, los iones liberados de Zn^{2+} cumplen las funciones conocidas para este elemento, como lo son la activación de las enzimas RNA polimerasa, superóxido dismutasa (SOD), anhidrasa carbónica, entre otras (Sturikova *et al*., 2018). Además, el mismo elemento participa en el desarrollo de los cloroplastos a través de la expresión de siete genes encargados de la estructura membranal de los tilacoides, así como en el metabolismo de carbohidratos, lípidos y ácidos nucleicos (Zhang *et al*., 2019).

2.5. NMs como nanofertilizantes

En la literatura, es posible encontrar varios reportes sobre estudios comparativos entre fertilizantes convencionales y nanofertilizantes, donde por lo general estos últimos muestran una mejor respuesta en los cultivos. La comparación de NPs de ZnS y ZnO versus una sal convencional de Zn ($ZnCl_2$) mostró que los nanofertilizantes incrementaron las variables de altura de planta, biomasa seca de hojas y raíz, además de aumentos en variables relacionadas con la productividad como número de vainas y rendimiento total de grano, en plantas de *Vigna radiata* (Thapa *et al*., 2023).

Khalid *et al*. (2022) observaron una mayor eficacia de nanofertilizantes a base de NPs de ZnO, FeO y MgO en comparación con fertilizantes convencionales de los mismos elementos ($ZnSO_4$, $FeSO_4$ y $MgSO_4$), donde estos promovieron

las características morfológicas de las plantas en un rango de 50-93%, además de aumentar la concentración de clorofila en las hojas en un 30-80%, donde todo dependió de la concentración aplicada en cada tipo de fertilizante.

Mardi *et al*. (2022) evaluaron la aplicación de NPs de ZnO contra su forma no nanométrica en plantas de *Nicotiana tabacum*, encontrando una mejor respuesta con el uso de NPs, con una mayor biomasa fresca y seca de plantas, longitud de tallo y área foliar, además de incrementar la concentración de pigmentos fotosintéticos y hormonas como el ácido indolacético (IAA) y ácido giberélico (GA). La investigación realizada por Akmal *et al*. (2022) en plantas de *Oryza sativa* sometidas a aplicaciones foliares, en drench e inmersión de la raíz en soluciones de NPs de ZnO y ZnO convencional mostró que el nanofertilizante favoreció el crecimiento general de las plantas, así como mejores características de la panícula y mayor rendimiento de grano, principalmente en las aplicaciones realizadas vía foliar.

Aplicaciones foliares de NPs de Ca mostraron una mejor respuesta que un fertilizante convencional de Ca en plantas de *Vitis vinífera*, principalmente en variables relacionadas al crecimiento vegetativo como longitud, diámetro y biomasa de los tallos, número de hojas y área foliar, así como en características de los frutos como número, diámetro, peso y contenido de sólidos solubles totales (°Brix) (Masri *et al*., 2021).

Ahmed *et al*. (2023) evaluaron la aplicación de Zn-EDTA versus NPs de ZnO en un cultivo de tomate, donde se encontró que el Zn nanométrico incrementó la tasa fotosintética, conductancia estomatal y tasa de transpiración de las plantas, además de mejorar las características morfológicas como la altura y área foliar, así como variables relacionadas con el rendimiento como el número, peso y dimensiones del fruto.

En general, numeras investigaciones han demostrado que los nanofertlizantes mejoran el crecimiento, desarrollo y rendimiento de los cultivos. Sin embargo, los efectos en las plantas dependen de numerosos factores que involucran características de los NMs y de las plantas, por lo que es necesario continuar

con investigaciones para identificar las mejores formas de aplicación y niveles que produzcan la mejor respuesta.

ARTÍCULO 1

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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 $Zn(C_6H_{10}O_6)$, 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).

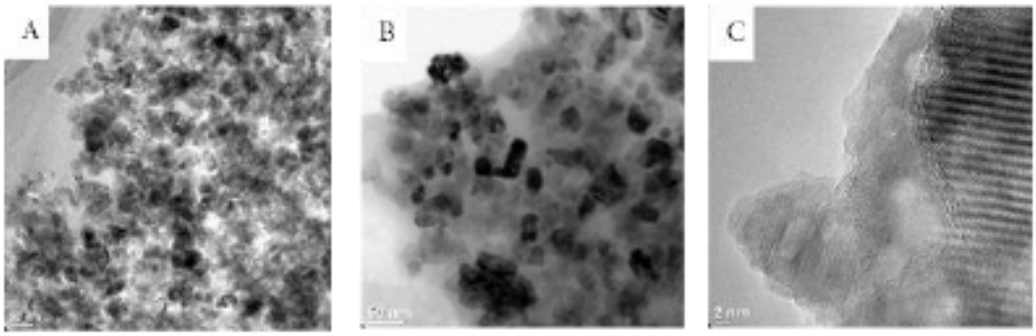

Figure 1. Characterization of ZnO nanoparticles by TEM (A-B) and HRTEM (C)

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).

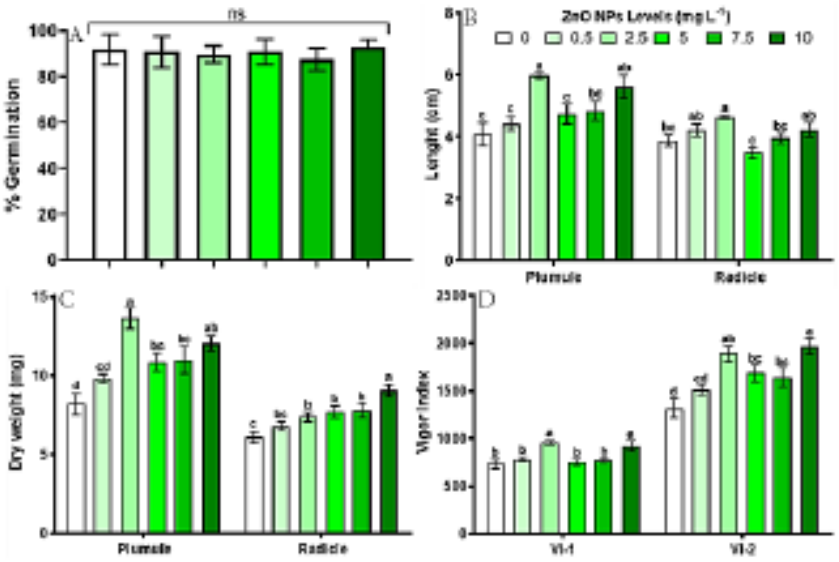

Figure 2. Germination percentage (A), plumule-radicle length (B), plumule-radicle dry weight (C), and vigor index (D) of *M. oleifera* seedlings at 15 DAS

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).

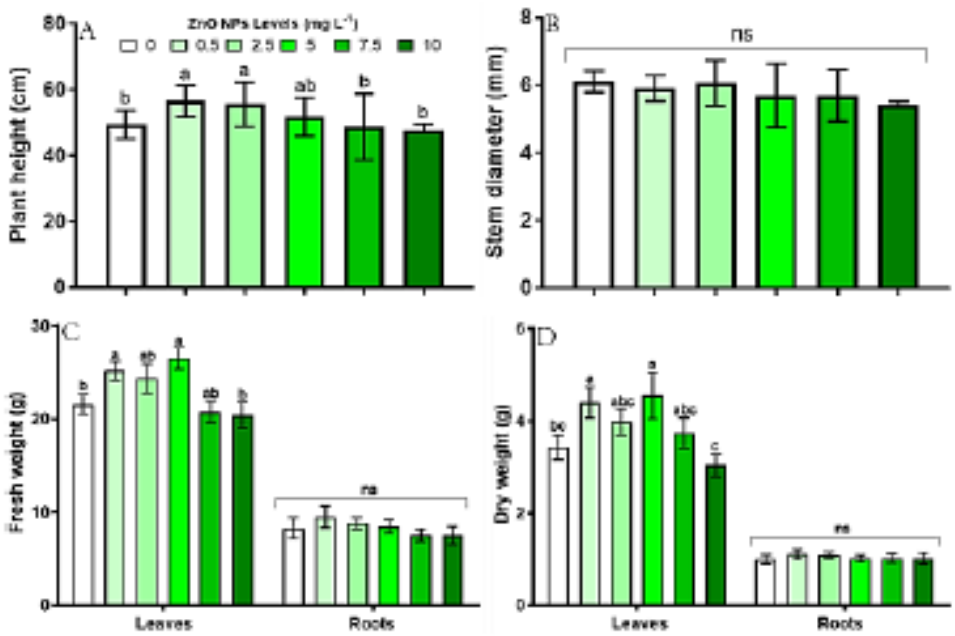

Figure 3. Plant height (A), stem diameter (B), leaf-root fresh weight (C) and leaf-root dry weight (D) of *M. oleifera* at 45 DAS

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).

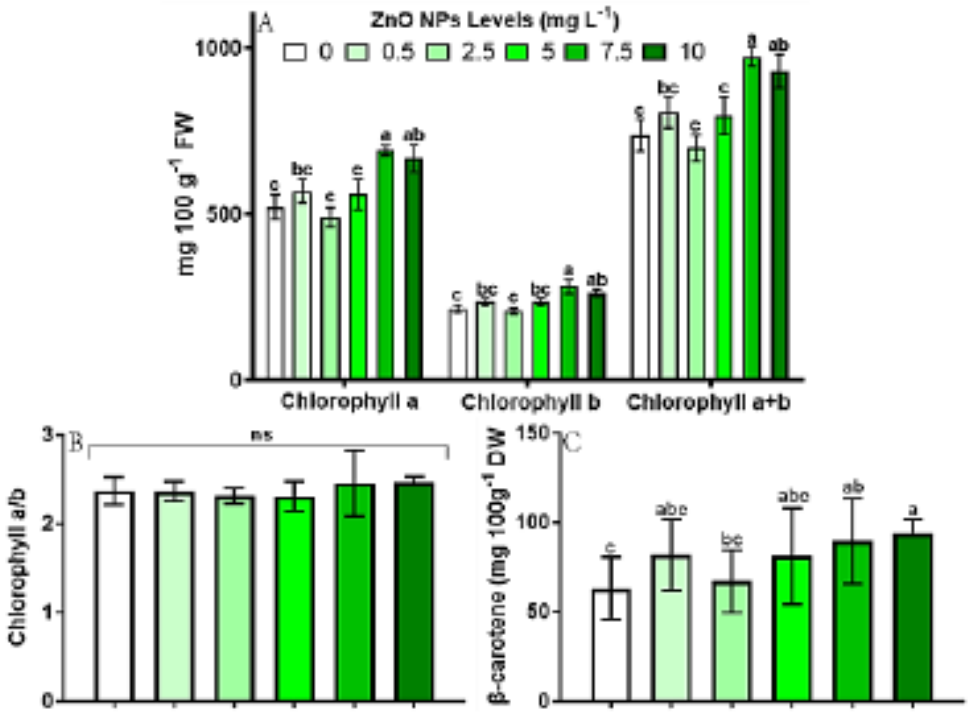

Figure 4. Chlorophyll a, chlorophyll b, total chlorophyll (A), chlorophyll a/b ratio (B), and β-carotene (C) of *M. oleifera* leaves

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)

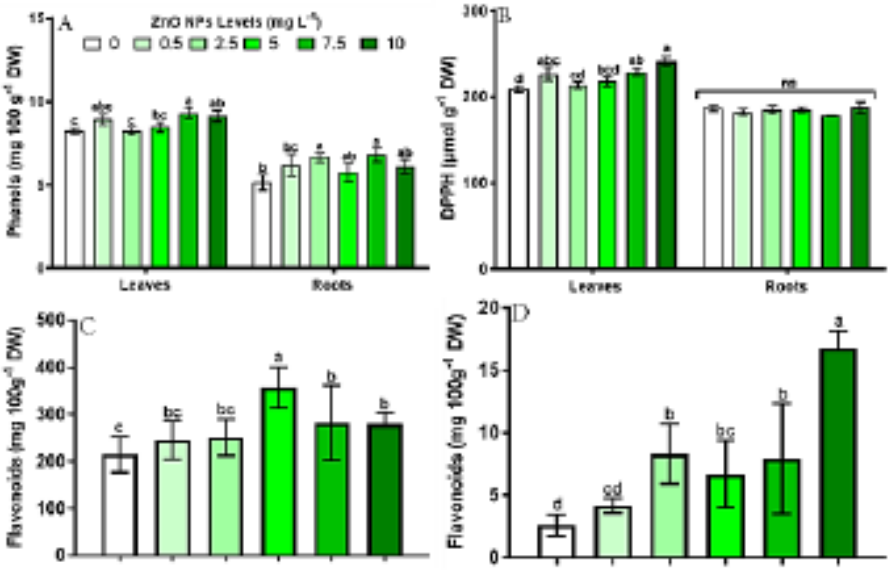

Figure 5. Phenolic compounds (A), antioxidant capacity DPPH (B), leaf and root flavonoids (C and D) of *M. oleifera*

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. $n=6$

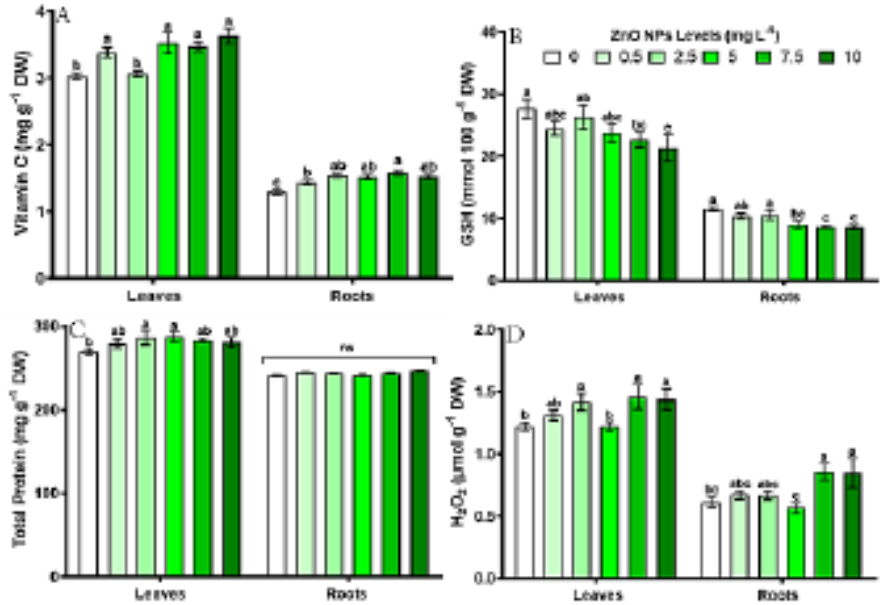

Figure 6. Vitamin C (A), GSH (B), total protein (C) and H_2O_2 (D) of *M. oleifera*

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).

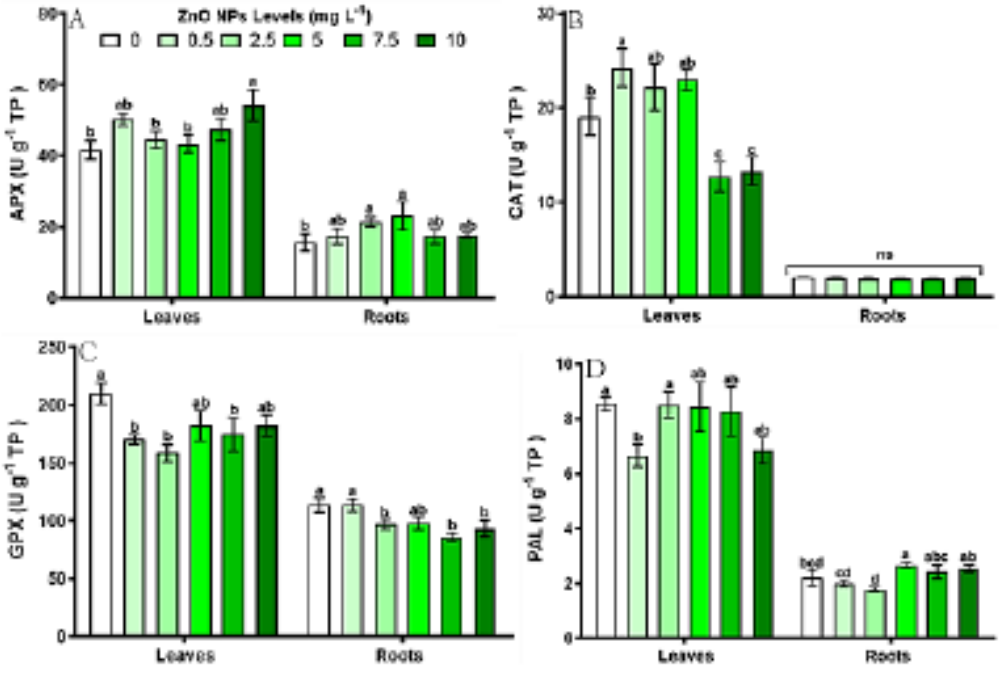

Figure 7. APX (A), CAT (B), GPX (C), and PAL (D) activity of *M. oleifera*
Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns: not significant. The lines on the bars indicate the standard error of the mean. n=6

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).

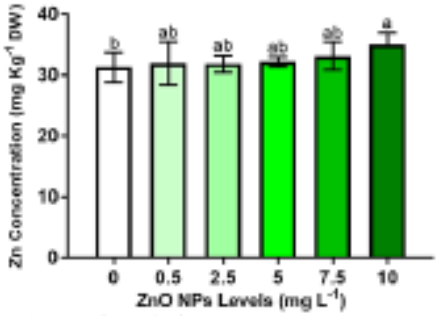

Figure 8. Zn concentration on leaves of *M. oleifera*

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. $n=6$

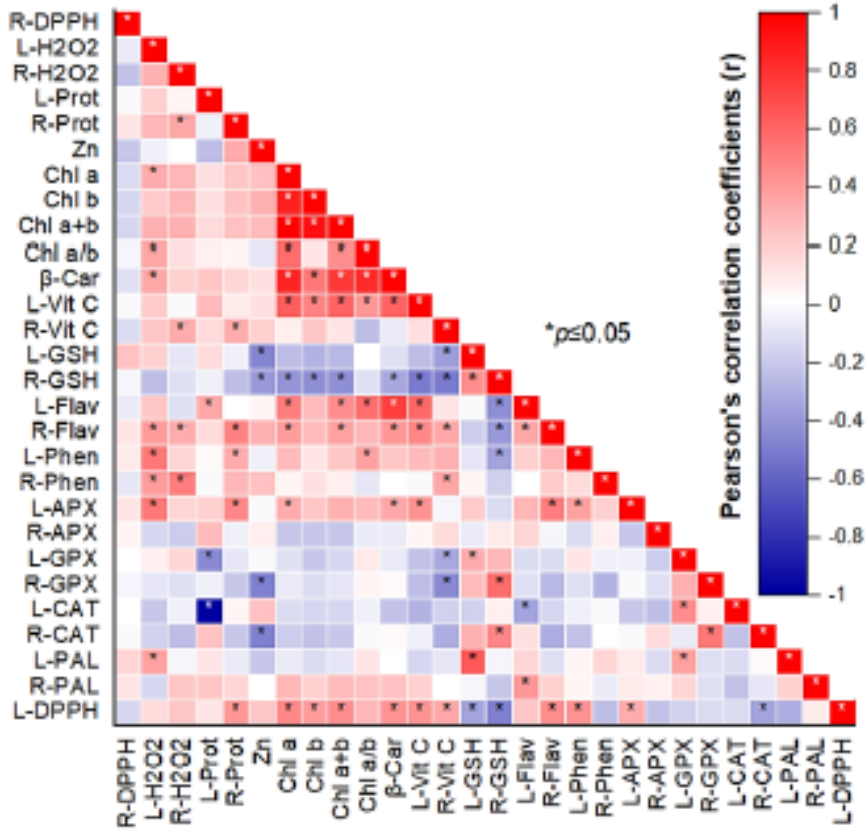

Figure 9. Matrix of correlations between variables of the antioxidant system of *M. oleifera*

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 termis* 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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Research article

ZnO nanoparticles as potential fertilizer and biostimulant for lettuce

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ABSTRACT

Zn is an indispensable nutrient for crops that usually presents low bioavailability. Different techniques have been proposed to improve the bioavailability of Zn, including the use of nano-fertilizers. The objective of the study was to evaluate the applications of drench (D) and foliar (F) ZnO nanoparticles (NZnO) compared to those of ionic Zn^{2+} ($ZnSO_4$) in lettuce. The plants cv. Great Lakes 407 was produced in pots of 4 L with perlite-peat moss (1:1) under greenhouse conditions. The treatments consisted of NZnO applications that replaced the total Zn provided with a Steiner solution, as follows: Zn^{2+} (100%D) (control); Zn^{2+} (50%D+50%F); NZnO (100% D); NZnO (50%D+50%F); NZnO (75%D); NZnO (50%D); NZnO (75%F) and NZnO (50%F). Four applications of Zn were made with a frequency of 15 days. 75 days after transplant (DAP), the fresh and dry biomass, chlorophyll a, b, and β-carotene, phenolics, flavonoids, antioxidant capacity, vitamin C, glutathione, H_2O_2, total protein, and enzymatic activity of PAL, CAT, APX, and GPX were evaluated. The mineral concentrations (N, P, K, Ca, Mg, S, Cu, Fe, Mn, Mo, Zn, Ni, and Si) in the leaves and roots of plants were also determined. The results showed that, compared to Zn^{2+}, NZnO promoted increases in biomass (14–52%), chlorophylls (32–69%), and antioxidant compounds such as phenolics, flavonoids, and vitamin C. The activity of enzymes like CAT and APX, as well as the foliar concentration of Ca, Mg, S, Fe, Mn, Zn, and Si increased with NZnO. A better response was found in the plants for most variables with foliar applications of NZnO equivalent to 50–75% of the total Zn^{2+} applied conventionally. These results demonstrate that

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total replacement of Zn^{2+} with NZnO is possible, promoting fertilizer efficiency and the nutraceutical quality of lettuce.

1. Introduction

The constant increment of the world's population leads us to be more efficient in producing food with better yields and high nutritional value. Zn is an essential element for plants; however, this element is deficient in the soils of various regions of the world. For the above, an appropriate supply of this element is considered vital to obtaining a higher yield and quality in agricultural crops [1]. Zn it is a component of some biomolecules (lipids and proteins), in addition to being a cofactor for auxins and playing an essential role in nucleic acids metabolism [2]. Additionally, this element is a component or activator of some enzymes, such as carbonic anhydrase (CA), alcohol dehydrogenase (ADH), and superoxide dismutase (SOD) [3]. Furthermore, Zn is involved in DNA transcription, RNA processing, and RNA editing in mitochondria and chloroplasts [4]. Zn is a component of cell membranes, participates in the expression and regulation of genes and biosynthesis of chlorophylls, in addition to participating in photosynthesis [5], mainly through the repair of protein D1 damaged by radiation during light harvesting in photosystem II [6].

Nanotechnology in agriculture is an alternative for increasing food production due to various applications, such as nanopesticides and nanofertilizers. The positive effects of nanomaterials (NMs) have been reported across multiple plant species, obtaining better responses in physiological processes, fruit quality, and yield [7-9]. However, NMs can also produce adverse effects, so the same material could produce biostimulation or toxicity [10] due to the size or shape of the NM, method of application, dose, exposure time, environmental conditions, and plant species [11].

Two main mechanisms have been identified in biostimulation by NMs. The first consists of the initial interactions of the NMs with the cell surface, inducing signals that trigger positive responses in plants. The second mechanism is due to the internalization of NMs, where their content becomes available for different metabolic functions of the plants [12]. The above modifies the metabolic process of plants, promoting vegetative growth and the production of antioxidant compounds, inducing greater tolerance or resistance to biotic-abiotic stresses [13].

NMs phytotoxicity (cytotoxicity, genotoxicity) is mainly due to time and levels of exposure of leaves, roots or seeds [14]. The primary mechanism by which some NMs produce genotoxicity is damage to chromosomes and interactions with DNA, causing plant mutations [15]. The interaction of NMs with cell walls and membranes induces cytotoxicity, causing oxidative stress [13], alterations in cell division, producing cells with malformations [16] and cell disorganization [17].

In addition to their biostimulant impact, NMs can be used as fertilizers, which have proven to have a higher efficiency than their conventional counterparts and a reduced environmental impact [18]. Mineral nutrition with NPs suggests greater efficiency than conventional fertilizer sources, which was demonstrated by applying nano-NPK at doses of 25 and 50% for the traditionally recommended values. As a result, higher yield, starch content, harvest index, and better efficiency in using nutrients in *Solanum tuberosum* cultivation were obtained [19]. Similarly, the application of nano NPK in chitosan formulations increased the content of N (17.04%), P (16.31%), and K (67.50%), in addition to promoting vegetative growth and chlorophyll content in *Coffea arabica* plants [20]. [21] reported the partial substitution of urea with urea-NPs in *Zea mexicana* plants, observing that the combination of both sources (50% conventional urea-50% urea NPs) showed higher levels of vegetative growth, in addition to increasing crude protein, carbohydrates, and detergent fiber.

In another study by Ref. [22]; it was found that application of hydroxyapatite-NPs to soil compared to the use of calcium superphosphate increased the leaf area and yield of *Brassica oleracea* var. Italic, while the foliar application of NPs of boron oxide vs. boric acid showed an increase in the same variables, in addition to a higher content of vitamin C. In both comparisons, the P and B contents were higher in plants with nanofertilizer applications. In another study [23], compared the application effects of Cu, Zn, Mn, and Fe in the NPs and ionic form on germination of lettuce, finding increases between 12 and 54% when nanofertilizers were used, demonstrating the potential of these agents in comparison with conventional microelement sources.

Recently, ZnO NPs (NZnO) were reported to increase antioxidant capacity and promote mineral absorption in *Cucumis sativus* [24]; however, the complete replacement of conventional fertilizers with Zn using nanoparticle formulations has not been reported. Nevertheless, this action could have significant benefits, such as the reduction of the use of conventional fertilizers, less environmental impact, and the obtaining of foods with better nutraceutical quality, in addition to facing the problems of deficiency or low availability of Zn in several regions of the world.

Based on all the above, the aim of this research was to compare different forms of application and levels of NZnO against ionic Zn ($ZnSO_4$) on vegetative growth, photosynthetic pigments, bioactive compounds, and mineral concentrations in lettuce produced in a soilless system. We hypothesized that NZnO is as effective as Zn^{2+} as a plant nutrient and shows more efficiency.

2. Materials and methods

2.1. Establishment of the experiment

The experiment was established in the Department of Horticulture - Universidad Autónoma Agraria Antonio Narro (Saltillo, México). The plant material corresponds to the genotype "Great Lakes 407" of KristenSeed, whose seeds had a germination percentage >85%. The seeds were germinated in expanded polystyrene trays with peat moss:perlite (1:1). After 28 days, the seedlings were

transplanted in 4 L pots containing the same substrate mixture (Fig. 1-A). The substrate was subjected to a physicochemical analysis [25], where the presence of Zn was not detected.

Plant nutrition for the control treatment was supplied by a Steiner nutrient solution [26]. This solution was prepared at a concentration of 50% and constant pH of 6.5, containing the following quantities of each element (in mg L^{-1}): N: 131; P: 31; K: 274; Ca: 168; Mg: 49; S: 133; B: 0.43; Fe: 3.2; Cu: 0.02; Mn: 1.94; Zn: 0.0227; Mo: 0.01, which was supplied as irrigation water, applying 1 L plant day^{-1}. Plants assigned to NZnO treatments received Steiner solution without Zn^{2+}. The water used for irrigation was chemically analyzed without detecting Zn concentrations. The plants were kept for 75 days after transplant (DAT) (Fig. 1-B and C) in a chapel-like greenhouse with homogeneous conditions of temperature (25–27 °C) and relative humidity (60–70%).

2.2. ZnO NPs and applied treatments

ZnO NPs were synthesized based on the methodology of [27]. A complete description of the synthesis method was previously reported [28]. The morphology and structure of NPs were analyzed by transmission electron microscopy (TEM) and high-resolution transmission electron microscopy (HRTEM), where most NPs resulted in a quasi-spherical shape (Fig. 2-A [28]), an average diameter of 16.49 nm (Fig. 2-B), and crystalline appearance (Fig. 2-C). Additionally, Fourier Transform Infrared Spectroscopy (FTIR) and UV–Vis tests were performed. The FTIR spectrum (Fig. 2-D) showed a strong peak between 493.1 cm^{-1}, corresponding to the stretching vibrations of ZnO bands, which indicates that the samples are well crystallized [29]. The UV–Vis spectrum illustrated in Fig. 2-E shows an adsorption peak located at 356 nm, which is attributed to the intrinsic band gap of ZnO absorption. Similar values of the absorption band that represent ZnO NPs was also reported in previous works in which the range of the absorption band was from 355 to 380 nm [30].

Treatments consisted of foliar applications (F) and drench (D) of different levels of NZnO, compared to applications of conventional Zn^{2+} ($ZnSO_4$). The application of Zn^{2+} at a concentration of 0.227 mg L^{-1} was considered the control because Zn^{2+} is the main supply for this element in soilless production systems. The total amount of Zn^{2+} calculated for a lettuce crop (17 mg Zn $plant^{-1}$ in 75 days) was taken as the basis for the applications of the treatments, which consisted of the two forms of application and percentages of Zn with respect to the control, as follows:

T1: Zn^{2+} (100%D) (control), equivalent to 17 mg Zn $plant^{-1}$.
T2: Zn^{2+} (50%D+50%F), equivalent to 17 mg Zn $plant^{-1}$.
T3: NZnO (100%D), equivalent to 17 mg Zn $plant^{-1}$.
T4: NZnO (50%D+50%F), equivalent to 17 mg Zn $plant^{-1}$.
T5: NZnO (75%D), equivalent to 12.75 mg Zn $plant^{-1}$.
T6: NZnO (50%D), equivalent to 8.5 mg Zn $plant^{-1}$.
T7: NZnO (75%F), equivalent to 12.75 mg Zn $plant^{-1}$.
T8: NZnO (50%F), equivalent to 8.5 mg Zn $plant^{-1}$.

The total number of applications of Zn was four (4.25 mg Zn^{-1} each), with an interval of 15 days between each.

2.3. Evaluated variables

2.3.1. Fresh-dry biomass of plants

After 75 DAT, two lettuce plants from each experimental unit were harvested and separated into leaves and roots to determine fresh biomass. Subsequently, the most recently mature leaves and a portion of the roots were taken and washed with distilled water and later placed in a freezer at −20 °C for future analyses. The remaining plant material was dehydrated in a drying oven at 65 °C for 72 h to determine dry biomass.

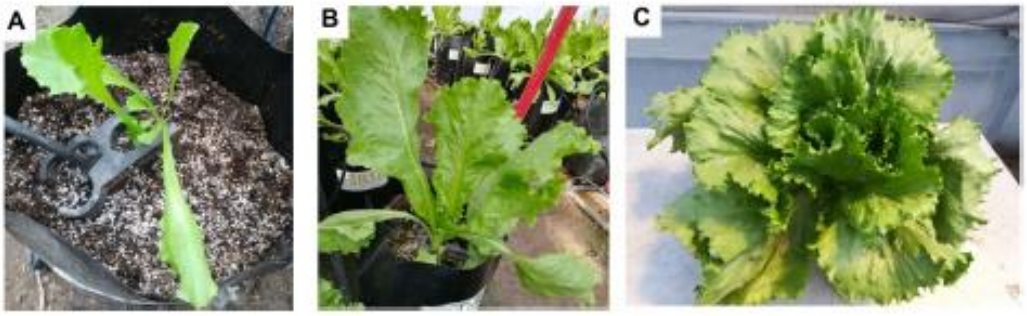

Fig. 1. Lettuce plants used in the experiment. A: Transplant; B: Lettuce growth at 30 DAT; C: Plant at 75 DAT.

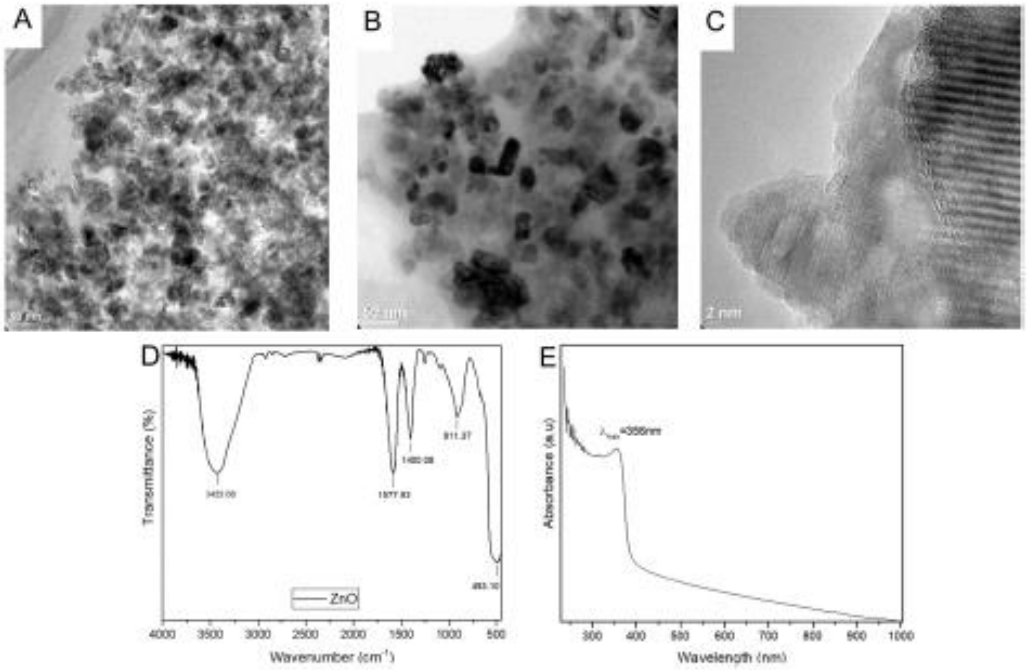

Fig. 2. TEM (A–B) and HRTEM (C) images. FTIR (D) and UV–Vis (E) spectra of ZnO nanoparticles. Subfigures A-C from Ref. [28].

2.3.2. *Sample processing for biochemical analysis*

The previously frozen leaf and root samples were lyophilized to avoid the denaturation of biochemical compounds. This process was carried out in a liophyllizer model Labconco FreeZone 4.5 (Labconco Inc., Kansas City, USA) at a temperature from −45 °C during seven days. Subsequently, the tissue was macerated for further analysis. This lyophilized tissue (LT) was used for all photosynthetic pigments, bioactive compounds, and enzymatic activity analyses.

2.3.3. *Photosynthetic pigments*

The concentrations of chlorophyll a (Chla), b (Chlb), total (Chla + b), and β-carotene (β-car) were analyzed in lyophilized leaves. A mix of 10 mg of LT + 2 mL of hexane:acetone (3:2) was centrifuged (12000 rpm, 10 min, 4 °C). The resulted extract was read in a spectrophotometer model Unico UV2150 (Unico Inc., New Jersey, USA) at different wavelengths (505, 453, 645, and 663 nm). The resulted absorbances was used for later calculation with equations proposed by Ref. [31]; expressing the results in mg 100 g^{-1} DW. Subsequently, the Chla/Chlb and Chla + b/β-car ratios were calculated.

2.3.4. *Bioactive compounds and enzymatic activity*

The concentration of total phenolics was obtained using a Folin-Ciocalteu reaction. First, 100 mg of LT+1 mL of water:acetone (1:1) was mixed and centrifuged (12500 rpm, 10 min, 4 °C). Subsequently, 50 μL of resulted supernatant +200 μL of reagent Folin Ciocalteu +0.5 mL of Na_2CO_3 (20%) + 5 mL of H_2O were homogenized and placed for 30 min in a water bath (45 °C). After this time, the sample was read at 750 nm in a spectrophotometer model Thermo Fisher G10S (Thermo Fisher Scientific, Massachusetts, USA). The same equipment was used for the readings of flavonoids, vitamin C, glutathione, H_2O_2, and antioxidant enzymes. The concentration of total phenolics was reported as mg 100 g^{-1} DW. All the above following the method described by Ref. [32].

The concentration of total flavonoids was determined mixing 100 mg of LT + 10 mL of methanol and subsequently filtered with a Whatman Filter (No 1001). Later, a mix of 2 mL of solution and 2 mL of $AlCl_3$ (2%) was incubated in dark conditions for 20 min. After this time, the sample was read at 415 nm, reporting the results as mg 100 g^{-1} DW. All the above following the techniques described by Ref. [33].

Vitamin C concentration was obtained with a mix of 10 mg of LT + 1 mL of HPO_3 (0.36 M), which later was centrifuged (5000 rpm, 10 min, 4 °C). After this, a mix of 200 μL of supernatant +1.8 mL of 2,6-diclorfenolindofenol (2,6 D-0.09 M) was read at 515 nm, expressing the results as mg g^{-1} DW. All the above according with [34].

Glutathione (GSH) was quantified with a mix of 100 mg of LT + 1.5 mL of phosphate buffer (K_2HPO_4 0.01 M + KH_2HPO_4 0.01 M) (1:1) + 10 mg of polyvinylpyrrolidone (PVP), which later was centrifuged (12500 rpm, 10 min, 4 °C) and subsequently filtered using

filters of nylon membrane (0.45 μm). This extract was used for the quantification of GSH, antioxidant capacity, protein, and activity of antioxidant enzymes. The GSH concentration was determined using a mix of 480 μL of extract +320 μL of DTNB reagent (1 mM) + 2.2 mL of Na_2HPO_4 (0.32 M), which later was read at 412 nm, reporting the results as mmol 100 g^{-1} DW. All the above following the techniques described by Ref. [35].

Antioxidant capacity was obtained using 6 μL of extract +254 μL of DPPH radical (2,2-diphenyl-1-picrylhydrazyl, 6.34 M). This mix was placed and read at 630 nm in a microplate reader model BioTek Elx808 (BioTek Inc., Vermont, USA), expressing the results as μmol g^{-1} DW, according with [36].

The concentration of H_2O_2 was quantified by the extraction of 10 mg of LT + 1 mL of trichloroacetic acid (0.1%), which latter was centrifuged (12000 rpm, 15 min, 4 °C). Subsequently, a mix of 500 μL of supernatant +750 μL of phosphate buffer (KH_2HPO_4 0.01 M + K_2HPO_4 0.01 M, 1:1) was read at 390 nm, expressing the results as μmol g^{-1} DW. All the above following the methods proposed by Ref. [37].

The concentration of total protein (TP) was quantified with a mix of 5 μL of the extract + 250 μL Bradford's reagent, which was incubated for 10 min in dark conditions. After this, the samples were read in a microplate reader at 630 nm. The results were reported as mg g^{-1} DW. All the above according with [38]. These TP values were used to calculate the enzymatic activity.

The activity of catalase (CAT) (EC 1.11.1.6) was quantified with a mix of 100 μL of the extract + 1 mL of H_2O_2 (100 mM) + 400 μL of H_2SO_4 (5%), which was directly read at 270 nm. Subsequently, a second lecture was taken after 1 min, with the objective of calculate the activity of CAT in 1 min of reaction. This activity was expressed as U g^{-1} TP, (U: mM equivalents of H_2O_2 consumed mL^{-1} min^{-1}). All the above according with [39].

Ascorbate peroxidase (APX) (EC 1.11.1.11) was determined with a mix of 100 μL of the extract + 1 mL of H_2O_2 (100 mM) + 500 μL of ascorbate + 400 μL of H_2SO_4 (5%), which was directly read at 266 nm. Subsequently, a second lecture was taken after 1 min, with the objective of calculate the activity of APX in 1 min of reaction. This activity was expressed as U g^{-1} TP, (U: μmol of oxidized ascorbate mL^{-1} min^{-1}). All the above following the methodology proposed by Ref. [40].

Phenylalanine ammonium lyase (PAL) (EC 4.3.1.5) was obtained with a mix of 100 μL of the extract + 900 μL of phenylalanine (6 mM), which was placed in water bath (40 °C, 30 min). Subsequently, were added 250 μL of HCL (5 N) + 750 μL of H_2O and directly read at 290 nm. The PAL activity was expressed as U g^{-1} TP, (U: μM of trans-cinnamic acid mL^{-1} min^{-1}). All the above according with [41].

Glutathione peroxidase (GPX) (EC 1.11.1.9) was determined through a mixture of 200 μL of the extract + 200 μL of Na_2HPO_4 (0.067 M) + 400 μL of GSH (0.01 M), which was placed in water bath (25 °C, 5 min). After this time, 200 μL of H_2O_2 (1.3 mM) was added. Ten minutes later, one milliliter of trichloroacetic acid (1%) was added and centrifuged (3000 rpm, 10 min, 4 °C). Finally, a mix of 480 μL of resulted supernatant +2.2 mL of Na_2HPO_4 (0.32 M) + 320 μL of DTNB (1 mM) was read at 412 nm. The GPX activity was reported as U g^{-1} TP, (U: mM glutathione equivalents reduced mL^{-1} min^{-1}). All the above according with [42].

2.3.5. *Leaf-root mineral concentration*

The determination of N was carried out by the micro-Kjeldahl method following the methodology of [43]. To quantify the concentrations of P, K, Ca, Mg, S, Cu, Fe, Mn, Mo, Zn, Ni, and Si, the previously dried samples were first subjected to acid digestion with HNO_3. Then, the extract obtained was read in a coupled plasma emission spectrophotometer (ICP-OES) model Optima 8300 (PerkinElmer, MA, USA) according to Ref. [44]. All results were reported as mg kg^{-1} DW.

2.4. *Statistical analysis*

The experiment was established under a completely randomized experimental design with eight treatments and five repetitions, obtaining 40 experimental units with two plants as experimental units. The Levene and Kolmogorov–Smirnov tests were previously carried out to verify the equality of variances and normal distribution of the data. The data were analyzed through an analysis of variance (ANOVA), and a Fisher's least significant difference (LSD) mean comparison test was made for variables with a $p \leq 0.05$. Additionally, a correlation analysis was made between all the variables evaluated. All the statistical analysis were performed in the Infostat v. 2020 software.

Table 1
Fresh and dried biomass in leaf and lettuce root.

TREATMENT	LFW (g)		LDW (g)		RFW (g)		RDW (g)	
Zn^{2+}(100%D)	402.88	e	18.94	c	51.14	ab	7.02	a
Zn^{2+}(50%D+ 50%F)	498.34	bc	22.26	bc	58.16	a	6.04	ab
NZnO (100%D)	461.52	cd	24.04	ab	53	ab	5.36	b
NZnO (50%D+ 50%F)	506.1	b	23.98	ab	49.9	b	6.83	a
NZnO (75%D)	407.16	e	21.62	bc	31.88	d	3.42	c
NZnO (50%D)	489.38	bc	24.54	ab	45.96	bc	5.38	b
NZnO (75%F)	612.72	a	26.34	a	50.72	ab	4.87	b
NZnO (50%F)	428.4	de	21.6	bc	41.36	c	5.88	ab

LFW: Leaf fresh weight; LDW: Leaf dry weight; RFW: Root fresh weight; RDW: Root dry weight. Values are the mean of treatments. Different letters in each column indicate a significant difference (LSD, $p \leq 0.05$). n = 5.

3. Results

3.1. Fresh-dry biomass of plants

The application of NZnO favored the gain of fresh and dry matter in lettuce plants. The most significant increase in fresh leaf biomass was found in the NZnO (75%) treatment, which was 52% higher than that in the control. Next were treatments of NZnO (50% D+50%F), Zn^{2+} (50%D+50%F), and NZnO (50%D), with increases over the control of 25, 23, and 21%, respectively. The NZnO (100% D) treatment showed an increase over the control of 14%. A similar response was found in dry weight of the leaves. The NZnO (75%) treatment favored a 39% increase over the control, followed by NZnO (50%D) treatment with 29%, and NZnO (100%D) and NZnO (50%D+50%F), both with increases of 27%.

In contrast, there was a decrease in fresh root biomass, with the NZnO (75%D) and NZnO (50%F) treatments showing reductions of 38% and 20%, respectively, in comparation of control. Similarly, the dry biomass of the roots was negatively affected by the NZnO (75%D) treatment, with a decrease of 51% compared to the control, while the NZnO (75% F), NZnO (100%D), and NZnO (50%D) treatments reduced the values of this variable by 31, 24 and 23%, respectively (Table 1).

3.2. Photosynthetic pigments

Chlorophylls in leaves increased with NZnO applications. Chla showed an increase with foliar application of NZnO, while the NZnO (75%F) and NZnO (50%F) treatments registered increases of 37% and 32%, with respect to control. In addition, Chlb resulted in a 69% increase for the NZnO (75%F) treatment and a 54% increase for the NZnO (50%F) treatment. The same trend was observed in the Chla + b concentration, with increases of 45 and 38% for the NZnO (75%F) and NZnO (50%F) treatments, respectively (Fig. 3-A). On the other hand, the treatments did not affect the concentration of β-car (Fig. 3-B).

The Chla/Chlb ratio, it resulted in a slight decrease in the NZnO (50%F) treatment, with a ratio 15% lower compared to the control, while in NZnO (50%D) and NZnO (75%F), decreases of 16% and 19% were observed. In contrast, compared with the control, the Chla + b/β-car ratio increased by 19% in plants with NZnO (50%D) and showed 28 and 33% increases with the NZnO (50%F) and NZnO (75%F) treatments, respectively (Fig. 3-C).

3.3. Bioactive compounds and enzymatic activity

The phenolic compounds in leaves resulted in increases when making NZnO applications. The highest increase was found in plants of the NZnO (100%D) treatment, which promoted the concentration of these compounds by 86%, followed by NZnO (75%F), NZnO (75%D), and NZnO (50%F), with increases of 85, 65, and 58%, respectively (Fig. 4-A). This compounds in lettuce roots were not altered by the application of Zn^{2+} or NZnO (Fig. 4-A). Similarly, the antioxidant capacity (DPPH) of the leaves resulted in increases of 15 and 9% compared to the control when applying NZnO (50%F) and NZnO (75%F), respectively. There were no differences in DPPH of the roots between treatments (Fig. 4-B). Regarding the concentration of flavonoids, no difference was found in the leaves (Fig. 4-C). However, in the case of the roots, the application of NZnO promoted an increase in these compounds, mainly in the NZnO (75%F) treatment, where a rise of 60% was observed with respect to control, followed by the NZnO (50% D+50%F), NZnO (50%D), and NZnO (50%F) treatments, with increases of 58, 42, and 33%, respectively (Fig. 4-D).

The vitamin C concentration increased with most NZnO treatments, where NZnO (50%D) showed a 145% increase over the control, followed by NZnO (75%F) and NZnO (50%F) treatments, with increases of 132% and 111%, respectively, while NZnO (75%D) and NZnO (50%D+50%F) treatments recorded 79% and 75% increases in vitamin C concentration (Fig. 5-A). In the case of the roots, the NZnO treatment (75%D) showed a rise of 178% in vitamin C concentration compared (Fig. 5-A). The concentration of GSH in leaves showed a 14% increase in the Zn^{2+} (50%D+50%F) treatment and a contrasting 15% negative effect for the NZnO (75%F) treatment.

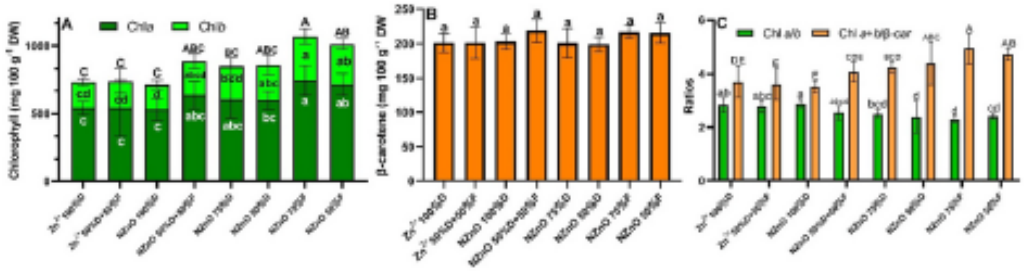

Fig. 3. Photosynthetic pigments in lettuce leaves. A: Chla, Chlb, and Chla + b. Letters inside bars are the mean comparation for each chlorophyll type. Capital letters on the bars are mean comparation of Chla + b; B: β-carotene; C: Chla/b and chla + b/β-carotene ratios. Lowercase letters are mean comparation of Chla/b, and capital letters are mean comparation of chla + b/β-carotene ratio. Different letters indicate a significant difference (LSD, $p \leq 0.05$). The lines on the bars indicate the standard error of the mean. n = 5.

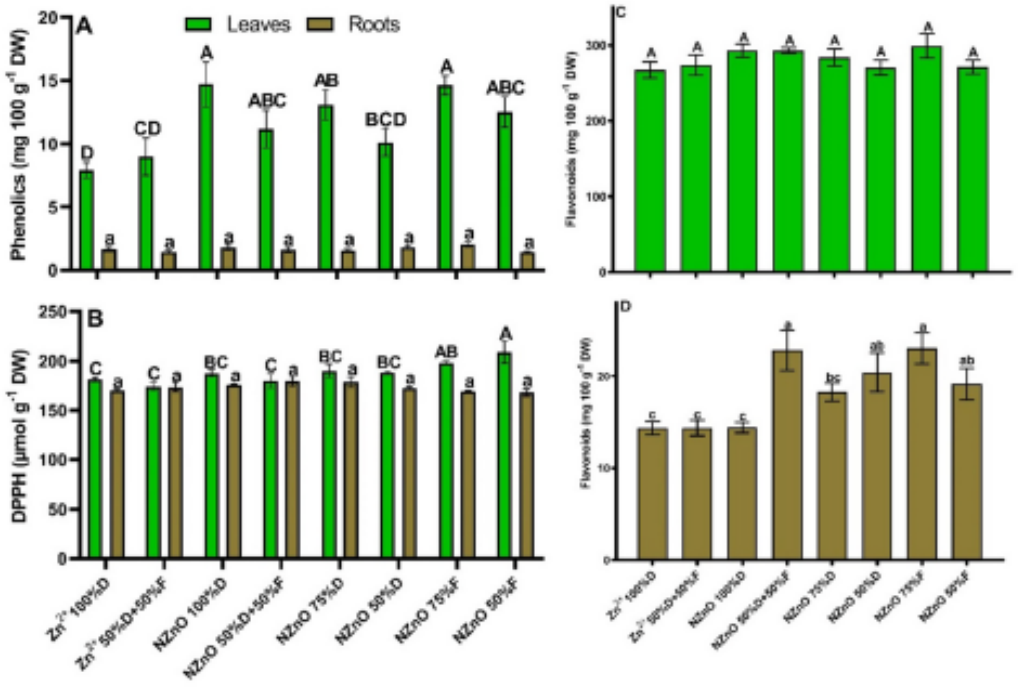

Fig. 4. Phenolics (A), antioxidant capacity DPPH (B), leaf flavonoids (C), and root flavonoids (D) in lettuce. Capital letters on the bars are the mean comparison of the leaves. Lowercase letters on the bars are the mean comparation of roots. Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). The lines on the bars indicate the standard error of the mean. n = 5.

On the other hand, the concentration of GSH in roots was not altered (Fig. 5-B). The treatments did not alter the concentration of H_2O_2 in lettuce leaves and roots (Fig. 5-C) or in the case of total protein in either vegetative organ (Fig. 5-D).

The enzymatic activity was positively or negatively affected by the applied treatments. Compared to the control, PAL activity in leaves increased 31% for the Zn^{2+} (50%D+50%F) treatment and 28% and 24% for the NZnO (75%F) and NZnO (50%F) treatments, respectively (Fig. 5-A). On the other hand, PAL activity in lettuce roots increased by 44% with the application of NZnO (75%D) but was reduced by 38% with NZnO (50%F) (Fig. 6-A). Regarding CAT activity in leaves, the Zn^{2+} (50%D+50%F) treatment promoted the activity by 170%, followed by the NZnO (50%F) treatment, which increased the activity of this enzyme by 88% compared to the control (Fig. 6-B). On the other hand, the activity of CAT in roots showed an increase of 158% with the application of the NZnO (75%F) treatment, followed by the NZnO (75%D) treatment, which promoted enzymatic activity by 78% in comparison with the control (Fig. 6-B).

On the other hand, the activity of APX in leaves increased with most treatments, being as follows: NZnO (50%F) > NZnO (50%D) > NZnO (50% D+50%F) > NZnO (100%D) > Zn^{2+} (50% D+50%F), where the increases were 43, 41, 36, 35 and 31%, respectively (Fig. 6-C). In the case of the roots, the activity of APX was only increased in plants of the NZnO (75%F) and Zn^{2+} (50% D+50%F) treatments, which showed increases of 56% and 38%, respectively, while the application of the NZnO (50%F) treatment produced a 40% reduction in the activity of this enzyme (Fig. 6-C).

Unlike the enzymes mentioned above, GPX activity in leaves was adversely affected by NPS treatments, being as follows: NZnO (50%D)> NZnO (75%D)> NZnO (50%F)> NZnO (75%F)> NZnO (50%D+50%F), with reductions of 60, 56, 53, 49 and 39%, respectively, compared to control treatment (Fig. 6-D). In contrast, the activity of this enzyme was considerably increased in the roots, where the most significant increase was found in the NZnO (50%F) treatment, with 681% higher activity compared to the control, followed by the NZnO (75%F), NZnO (50%D), and NZnO (75%D) treatments, with increases over the control of 594, 416 and 353%, respectively. Likewise, the Zn^{2+} (50%D+50%F) treatment showed a 350% of increase in activity of GPX compared to the control (Fig. 6-D).

3.4. *Leaf-root mineral concentration*

The application of NZnO significantly altered the concentration of minerals in lettuce leaves and roots. In the case of N, the ANOVA

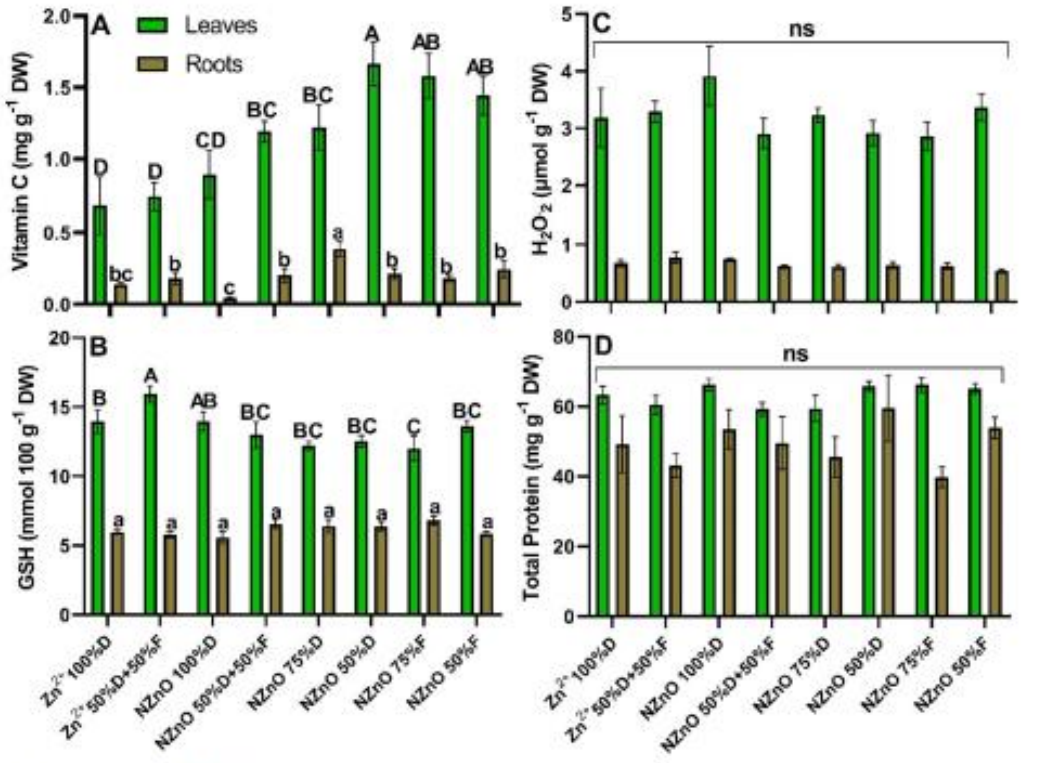

Fig. 5. Vitamin C (A), GSH (B), H_2O_2 (C), and total protein (D) in leaves and roots of lettuce. Capital letters on the bars are the mean comparison of the leaves. Lowercase letters on the bars are the mean comparation of roots. Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). ns = no significant difference. The lines on the bars indicate the standard error of the mean. n = 5.

did not show a significant difference between the treatments in either leaves or roots. However, in the concentration of P in leaves, a reduction was found for most NZnO treatments, with the most significant decrease in NZnO (75%D) treatment with a decline of 41% compared to control, followed by NZnO (50%D), NZnO (50%D+50%F), NZnO (100%D) and NZnO (75%F) treatments with decreases of 36, 35, 28, and 27%, respectively, finding no difference between the Zn^{2+} (50%D+50%F) treatment and the control.

A similar trend was found in the roots, in which the P decreased in all treatments with the application of NZnO, as follows: NZnO (50%D) > NZnO (75%D) > NZnO (50%D+50%F) > NZnO (50%F) > NZnO (100%D) > NZnO (75%F), with decreases of 65, 54, 53, 45, 35 and 29%, respectively, compared to the control; Zn^{2+} (50%D+50%F) treatment reduced the P concentration by 30%. In the case of K in leaves, only a 33% reduction was found in the NZnO (50%F) treatment compared to the control. However, the concentration of this element in the roots was negatively affected by NZnO applications, with a 62% reduction in the NZnO (50%D) treatment, followed by the NZnO (75%D), NZnO (50%D+50%F) and NZnO (50%F) treatments with reductions of 59, 58, and 36%, respectively.

On the other hand, the concentrations of Ca, Mg, and S in the leaves increased with NZnO applications. Ca increased by 71% and 66% for the NZnO (100%D) and NZnO (50%D+50%F) treatments, respectively. The Mg levels increased in the NZnO (50%F) and NZnO (50%D+50%F) treatments by 39% and 34%, respectively. For S, a similar trend was obtained, with increases of 30, 23, and 21% in the NZnO (50%F), NZnO (100%D), and NZnO (75% F) treatments, respectively. In contrast, the concentrations of Ca and Mg in the roots decreased when NZnO was applied. Ca was reduced in most treatments, remaining as follows: Zn (50%D)> NZnO(50%D+50% F)> NZnO(75%F)> NZnO(50%F)> NZnO(100%D), with decreases relative to the control of 37, 30, 28, 26, and 17%, respectively, and a decrease of 39% in plants of Zn^{2+} (50%D+50%F) treatment. A similar effect was found for Mg, for which the NZnO (75% F) and NZnO (50%D) treatments reduced the concentration of this element in the roots by 28 and 24%, respectively. For its part, the Zn^{2+} (50%D+50%F) treatment produced a Mg decrease of 39%. The concentration of S in the roots was not modified by NZnO applications (Table 2).

Concerning the leaf micronutrients, Cu was not affected by NZnO treatments; however, the Zn^{2+} (50%D+50%F) treatment increased the concentration of Cu by 55%. The remaining microelements increased their concentration in NZnO-treated plants. In the case of Fe and Mn, the treatments promoting an increment were NZnO (50%D+50%F) and NZnO (100%D), both raising the

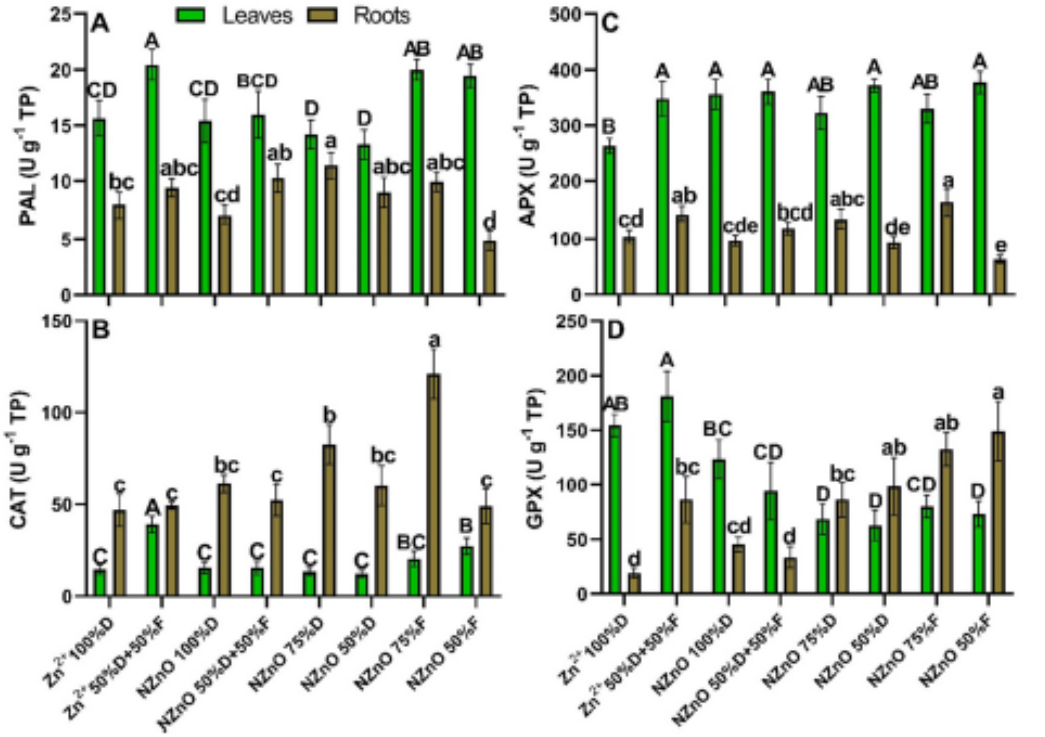

Fig. 6. PAL (A), CAT (B), APX (C), and GPX (D) activities in leaves and roots of lettuce. Capital letters on the bars are the mean comparison of the leaves. Lowercase letters on the bars are the mean comparation of roots. Different letters on the bars indicate a significant difference (LSD, $p \leq 0.05$). The lines on the bars indicate the standard error of the mean. n = 5.

Table 2
Concentration of macronutrients (mg kg^{-1} DW) in leaves and roots of lettuce.

TREATMENT	N		P		K		Ca		Mg		S	
LEAVES												
Zn^{2+}(100%D)	26310	ab	3143.13	a	35312.5	abc	14553.13	cd	2906.25	c	1935	c
Zn^{2+}(50%D+ 50%F)	27920	a	3194.06	a	29825	bcd	18253.13	cd	3924.38	ab	1948.75	c
NZnO(100%D)	26320	ab	2277.19	b	39731.25	a	24903.13	a	3034.17	c	2387.19	a
NZnO(50%D+ 50%F)	27580	a	2055	b	34709.38	abc	24237.5	ab	3904.69	ab	2023.13	bc
NZnO(75%D)	22804	b	1872.19	b	27278.13	cd	15539.69	cd	2815	c	1769.69	c
NZnO(50%D)	25900	ab	2024.38	b	23362.5	d	13335.63	d	3102.19	c	1893.44	c
NZnO(75%F)	28000	a	2317.5	b	37693.75	ab	18362.5	cd	3174.69	bc	2359.38	ab
NZnO(50%F)	28420	a	2511.25	ab	33993.75	abc	19028.13	bc	4055.31	a	2516.25	a
ROOTS												
Zn^{2+}(100%D)	15330	abc	2275.63	a	12576.56	ab	20470	a	1950.31	a	2291.88	abc
Zn^{2+}(50%D+ 50%F)	12950	c	1601.19	b	11315.63	abc	14427.5	cd	1196.91	d	1856.56	c
NZnO(100%D)	16520	ab	1468.13	bc	9358.13	bcd	16820	bc	1775.94	abc	2569.69	ab
NZnO(50%D+ 50%F)	16940	ab	1050.03	de	5270.94	de	14197.25	cd	1868.13	ab	2134.06	bc
NZnO(75%D)	15050	abc	1037.28	de	5202.84	e	17972.5	ab	1839.06	ab	2446.25	ab
NZnO(50%D)	14280	bc	798.56	e	4680.31	e	12754.75	d	1479.22	bcd	2794.69	a
NZnO(75%F)	17220	ab	1596.56	b	15113.13	a	14652.75	cd	1401.59	cd	2489.69	ab
NZnO(50%F)	17920	a	1232.03	cd	8006.56	cde	15090	bcd	1680.94	abc	2748.44	a

Values are the mean of treatments. Different letters in each column indicate a significant difference (LSD, $p \leq 0.05$). n = 5.

concentration of Fe by 29% and 26% and the concentration of Mn by 43% and 35%, with respect to control. A similar effect was observed in the concentration of Mo, where the NZnO (100% D) treatment promoted the concentration of this element by 30%. For Zn, the NZnO (50% F) and NZnO (75% F) treatments elevated the accumulation of this element by 35% and 33%, with respect to control.

No significant difference was found for the remaining treatments. On the other hand, the Ni concentration increased by 55% and 34% for the NZnO (100%D) and Zn^{2+} (50%D+50%F) treatments, respectively. The Si levels increased significantly in the NZnO (50% D+50%F), NZnO (75%D), NZnO (50%D), NZnO (75%F), and NZnO (50%F) treatments with increases of 194, 166, 145, 113, and 110%, respectively, compared to the control treatment.

For the concentration of micronutrients in roots, reductions in the levels of some elements were observed in most NZnO treatments. Compared with the control, Cu was reduced by 68% with the NZnO (50%F) treatment, followed by the NZnO (50%D), NZnO (75%D), NZnO (50%D+50%F), NZnO (100%D), and NZnO (75%F) treatments, which decreased the concentration of this element by 64, 61, 60, 59 and 53%, respectively. The Zn^{2+} (50%D+50%F) treatment produced a reduction of 41%. A similar result was found in the concentration of Fe, with the level of decrease as follows: Zn^{2+} (50%D+50%F)> NZnO (75%F)> NZnO (50%F)> NZnO (50%D)> NZnO (50%D+50%F)> NZnO (75%D), with an Fe decrease compared to the control of 50, 43, 38, 34, 27 and 13%, respectively. The same effect was found for the concentration of Mn, where the NZnO treatments produced reductions from 25 to 55%. A decrease of 23–34% for Mo was obtained compared to the control.

Decreases in Zn root concentration were also found, mainly in the NZnO (50%F), NZnO (75%F), and NZnO (50%D) treatments, with reductions of 64, 52, and 41%, respectively. However, in the NZnO (75%D) treatment, an increase of 170% was observed. The concentration of Ni increased 86% compared with the control in the plants treated with NZnO (75%D). In contrast, in the NZnO (50% D+50%F) and Zn^{2+} (50%D+50%F) treatments, Ni was reduced by 60% and 41%, respectively. The concentration of Si in the roots was not affected by the NZnO treatments (Table 3).

The correlation analysis showed positive and negative relations between some of the variables studied. For example, the fresh biomass of lettuce leaves was positively related to variables of the antioxidant compounds of plants, such as phenols, flavonoids, GSH, APX, GPX, CAT, and PAL, with coefficients ranging from 0.324 to 0.414. On the other hand, a positive relationship was found between Chla and the concentration of N in leaves, with a coefficient of 0.382. Likewise, a correlation coefficient of 0.538 was found for GSH concentration and GPX activity. On the other hand, negative relationships were observed between vitamin C concentration and GSH and between vitamin C and GPX activity, with coefficients of −0.414 and −0.563, respectively. The concentration of H_2O_2 was positively related to the concentration of phenols and the activity of the enzymes GPX and APX, with correlation coefficients of 0.451, 0.397, and 0.360, respectively. Furthermore, the antioxidant enzymes were related to each other, where the highest coefficient was found in the activity of APX and PAL (0.726), followed by CAT and PAL (0.520), APX and CAT (0.465), GPX and PAL (0.405), and the concentration of phenols with PAL, with a correlation coefficient of 0.317. Finally, some correlations were observed between the ions, for example, the concentration of K with Fe (0.724), Cu and Mo (0.689), N and S (0.579), and K and Mn (0.501) (Fig. 7).

4. Discussion

By definition, a plant biostimulant is "any substance or microorganism applied to plants to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits, regardless of its nutrient content" [45]. Due to the positive effects of NMs on plants reported in the literature [46], it is possible to consider them biostimulants.

In general, the beneficial effects of NZnO observed in lettuce plants it's explained by the two phases of bioestimulation with NMs: The first consist in the initial contact with the cell membranes, where interactions depend of several characteristics such as size, shape, surface charges, and hydrophobicity of the NMs. The above produces damage or modifications in cell membrane, producing cascades of signaling metabolites, changes on the redox balance, membrane potential, protein translation, and modifications in gene expression. These signals can travel between cells and produce a biostimulation response [47,48]. Similar response occurs when the NMs come into contact with the organelles (e.g. chloroplasts, mitochondira or nucleus), once the NMs enter the cell through membrane pores or

Table 3
Concentration of micronutrients (mg kg^{-1} DW) in leaves and roots of lettuce.

TREATMENT	Cu		Fe		Mn		Mo		Zn		Ni		Si	
LEAVES														
Zn^{2+}(100%D)	27.06	bcd	795	bc	125.78	c	5.81	bc	101.97	bc	26.53	cde	67.78	d
Zn^{2+}(50%D+50%F)	41.94	a	901.03	ab	140.94	bc	6.69	ab	105.91	abc	35.59	ab	78.66	d
NZnO(100%D)	30.88	abc	1008.47	a	170.47	ab	7.56	a	131.88	ab	41.31	a	97.94	d
NZnO(50%D+50%F)	34.94	ab	1028.06	a	180.56	a	5.72	cd	130.34	ab	21.72	e	199.84	a
NZnO(75%D)	20.84	cd	797.63	bc	134.25	bc	4.84	d	89.75	c	31.5	bc	180.72	ab
NZnO(50%D)	24.75	bcd	616.56	c	123.97	c	4.94	cd	86.69	c	24.63	de	166.69	bc
NZnO(75%F)	20.53	cd	926.44	ab	118.94	c	5.06	cd	136.25	a	29.28	bcd	144.63	c
NZnO(50%F)	18.06	d	844.47	ab	151.44	abc	5.38	cd	138.34	a	29.19	bcd	142.41	c
ROOTS														
Zn^{2+}(100%D)	39.65	a	1150.94	b	540.63	a	11.34	a	92.94	b	39.06	c	162.69	a
Zn^{2+}(50%D+50%F)	23.4	b	577.06	f	319.91	cd	10.53	a	36.28	c	16.16	e	169.28	a
NZnO(100%D)	16.23	c	1409.69	a	463.03	ab	11	a	94	b	51.88	b	185.31	a
NZnO(50%D+50%F)	15.58	c	838.94	d	295.59	d	7.66	b	49.63	c	23.41	de	157.94	a
NZnO(75%D)	15.53	c	996.34	c	383.81	bc	8.63	b	251.59	a	73.03	a	155.81	a
NZnO(50%D)	14.15	c	755.25	de	241	d	7.47	b	54.22	c	29.69	cd	145.16	a
NZnO(75%F)	18.38	bc	648.31	ef	402.84	bc	7.81	b	43.92	c	29.09	cd	167.03	a
NZnO(50%F)	12.6	c	702.44	e	389	bc	8.31	b	32.97	c	27.91	cd	157.28	a

Values are the mean of treatments. Different letters in each column indicate a significant difference (LSD, $p \leq 0.05$). n = 5.

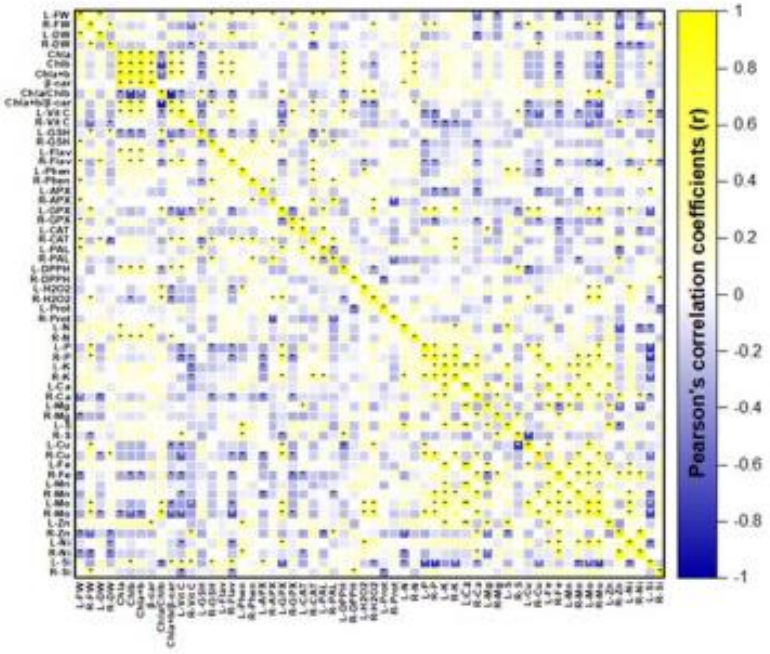

Fig. 7. Matrix of correlations between evaluated variables of lettuce plants. L: Leaves, R: Roots, FW: Fresh weight, DW: Dry weight, Chla: Chlorophyll a, Chlb: Chlorophyll b, β-car: β-carotene, Vit C: Vitamin C, GSH: Glutathione, Flav: Flavonoids, Phen: Phenols, APX: Ascorbate peroxidase, GPX: Glutathione peroxidase, CAT: Catalase, PAL: Phenylalanine ammonium lyase, DPPH: Antioxidant capacity, H_2O_2: Hydrogen peroxide, Prot: Total protein.

active mechanisms like diffusion or endocytosis, [12].

The second phase consists in the transformation (mainly ionization) of the NM core (in this case, Zn^{2+} ions) once the NMs are internalized in plant cells. The free ions in the cell cytoplasm can fulfill specific functions on several metabolic process of plants [10].

Therefore, considering the above, it is possible to explain the biostimulant impact of NZnO on growth, photosynthetic pigments, bioactive compounds, antioxidant enzyme activity, and ion concentration in lettuce plants.

4.1. Fresh-dry biomass of plants

The increase in fresh and dry biomass of lettuce can be explained principally by NMs promoting the photosynthetic activity of plants, increasing light absorption, accelerating the transport of energy between photosystems, and promoting the photolysis of water and the evolution of oxygen [49].

Dry weight gain may also be due to NPs increasing PSI and PSII activity, as well as the redox state of plastoquinol in the electron transport chain [50]. These increases in photosystem activity probably occur because NPs favor the overexpression of photosynthesis-related genes. Examples are *psaA* (Photosystem I P700 chlorophyll *a* apoprotein A1), *petA* (photosynthetic electron transfer A), *HSP90.1* (heat shock protein 1), and *pshA* (Photosystem II reaction center protein A) [51]. It is also possible that NPs form complexes with LHC proteins (light-harvesting complex) in the antennae of photosystems, improving light uptake [52]. In addition to participating in the light-dependent phase of photosynthesis, NMs also promote photosynthetic activity in light-independent reactions by increasing CO_2 assimilation by boosting the activity of beta carbonic anhydrase (BCA) and Rubisco enzymes [49].

Furthermore, NZnO can increase stomatal conductance, respiratory rate, internal CO_2 concentration, and net photosynthetic rate [53]. For example, in lettuce [54], reported that applications of 10 mg L^{-1} of NZnO increased net photosynthesis, favoring plant growth. These increases in the photosynthetic activity of plants are evidenced by the more significant accumulation of photosynthates in the leaves when applying 50–1000 mg L^{-1} of NZnO in *Brussica oleracea* [55]. On the other hand, applications of 250–500 mg L^{-1} of NZnO increase the stomatal density in the leaves, improving water balance and gas exchange, promoting the photosynthesis and respiration of plants, and resulting in greater plant growth [56].

The decrease in root biomass can be explained because NZnO causes a rearrangement of microfilaments in the epidermal cells of the elongation zones, reducing the growth of primary roots [57]. Likewise, ZnO NPs favor the generation of RNS, such as nitric oxide and peroxynitrite, causing oxidation and rearrangement of root cells and decreasing the biomass [58]. A similar result was reported by

Ref. [17]; who observed that root biomass decreased when applying 300–2000 mg L^{-1} of NZnO in *Hordeum vulgare*; the authors attributed these decreases to structural changes and disorganization of root cells.

4.2. Photosynthetic pigments

The increases in the concentration of chlorophylls are probably related to the second stage of the biostimulation process with NMs, where the core of the material is biotransformed into ions inside the cytoplasm, specifically Zn^{2+} for the case of NZnO, which was explained in previous paragraphs [10].

In this research, increases in chlorophylls were observed in plants with foliar applications of NZnO, which can be explained by the easier internalization of the material through the leaves [59]. The NMs < 2 nm can enter directly through the pores of the leaf cuticle, and NMs <20 nm can access through the stomata [60]. The NZnO used in this research has an average diameter of 16.49 nm; it follows that the access of NZnO was more efficient in the foliar application route.

Once NZnO enters cells, Zn plays a vital role in the biosynthesis of chlorophylls through the production of LHC proteins [61], increasing chlorophyll content. In addition, the same element participates in the development of chloroplasts through the expression of seven genes responsible for the membrane structure of thylakoids [62,63].

On the other hand, the decrease in the Chla/Chlb ratio can be explained because NPs promote the activity of the enzyme chlorophyll a oxygenase (EC 1.14.13.122) [64], which is responsible for synthesizing Chlb from Chla [65]. The reduction in the ratio and the increase of Chlb indicate a higher concentration of PSII against PSI, since Chlb is abundant in PSII [66]. More PSII implies a more efficient capture and use of solar radiation in the leaves. In addition to the above, Chlb fulfils essential functions, such as stabilizing LHCs and organizing thylakoidal membranes [67]. All these findings reinforce what was previously explained regarding photosynthetic efficiency and dry matter gain in plants.

The treatments did not alter the concentration of β-car in leaves, possibly due to the low production of ROS in the photosystems, since one of the functions of this pigment is the neutralization of free radicals such as O^{2-} and OH^{-} [68]. The higher Chla + b/β-car ratio shows that NPs promoted the concentration of chlorophylls compared to β-car. The latter was not altered by the treatments, possibly because the increases in β-car are more related to conditions of high solar radiation, where this compound fulfills the function of protecting chlorophylls through photoprotection and energy dissipation [69].

4.3. Bioactive compounds and enzymatic activity

The modifications of the plant antioxidant system with the application of NZnO were probably related to the first phase of biostimulation with NMs, as previously explained [47,48]. Specifically, the increase in bioactive compounds can be explained by the fact that NPs modify the activity of the electron transport chain in mitochondria, blocking the transfer of electrons from NADH to ubiquinone, inducing oxidative stress and increasing levels of O^{2-}, H_2O_2, and malondialdehyde (MDA), a compound indicating lipid peroxidation of the membrane damage [50,70,71]. On the other hand, chloroplasts are one of the main ROS production sites when plants are subjected to NMs [72].

Due to the above, when exposed to NMs, plants tend to increase the production of antioxidant compounds as a defense mechanism, for example, phenolic compounds and flavonoids [73], which was evidenced in this work by finding a positive relationship between H_2O_2 and total phenolic compounds. In addition to this response, a positive correlation was also observed between the PAL enzyme and total phenolics, where this enzyme plays an indispensable role in the biosynthesis of these compounds [74].

On the other hand, the production of antioxidant enzymes is a mechanism to counteract the production of ROS and RNS [75,76], which was proven by the positive relationships found between H_2O_2 and enzymes such as APX and GPX. The higher activity of some antioxidant enzymes in this research is also partially explained because NPs can interact with these enzymes to form protein complexes, which was demonstrated by Ref. [77]. On the other hand, the increase in CAT and APX activity can be explained by the fact that NZnO induces overexpression of the *CATa*, *CATb*, and *CATc* genes, as well as *APXa* and *APXb* [20]. The overexpression of the APX gene was also reported by Ref. [17] when applying 300–2000 mg L^{-1} of NZnO in *Hordeum vulgare*.

The increase in phenolic compounds in this research coincides with [78]. They observed that applications of 160 mg L^{-1} of NZnO promoted a rise in these compounds in *Juniperus procera*. The increase in flavonoids in lettuce roots can be explained because NZnO favors the overexpression of related genes like *Solyc08g007210.3*, *Solyc08g078030.3*, and *Solyc10g078220.2* in *Solanum lycopersicum* roots [62].

The increase in the concentration of vitamin C can be explained because the synthesis of this compound in the Smirnoff-Wheeler pathway depends on the production of photoassimilates [104], which probably increased when making NZnO applications, as explained in the previous section. In turn, vitamin C contributes to neutralizing ROS in chloroplasts [105]. As in this research, an increase in vitamin C concentration was reported by Ref. [103] in tomato fruits treated with NMs. On the other hand, the decrease in GSH levels can be explained because the transformation of DHA in vitamin C depends directly of GSH in the ascorbate-glutathione cycle [106]. A similar response was reported by Ref. [107], who observed a 20% decrease in GSH concentration when applying 5–10 mg L^{-1} of NZnO in *Hordeum vulgare*. The same trend of an increase in vitamin C and a reduction in GSH was reported by Ref. [79] when applying 50–100 mg L^{-1} NZnO to *Glycine max* plants. This effect was also observed in *Moringa oleifera* seedlings when applying NZnO 2.5–10 mg L^{-1} via seed priming [28]. The relationship between vitamin C and GSH was demonstrated in this research using correlation analyses.

The increases in CAT and APX activity found in this work are similar to those reported by Ref. [78]; where applications of 80–160 mg L^{-1} of NZnO in *Juniperus procera* increased the activity of CAT and APX enzymes. Similarly, the application of 1000 mg L^{-1} NZnO to

Oryza sativa increased the activity of CAT and other enzymes, such as superoxide dismutase (SOD) and peroxidase (POX) [53]. Similarly, PAL activity was increased in lettuce tissues with NZnO application, which has also been reported in *Capsicum annum* leaves and fruits where plants were subjected to applications of Se, Si, and Cu NPs [74].

On the other hand, the decrease in GPX activity is partially explained by the low concentration of GSH found in lettuce tissues since the above compound represents the substrate for the synthesis of GPX [42]. In addition, a reduction in GPX enzyme activity was reported in wheat and corn roots and leaves when applying NZnO 50–200 mg L^{-1} [71].

In general, NPs promote the production of antioxidant compounds to achieve cell homeostasis, keeping ROS levels in balance and mitigating damage to organelles [80]. This statement is reinforced by the positive correlations found between the compounds studied in this research.

4.4. Leaf-root mineral concentration

NMs alter the concentrations of macro-and micronutrients in plants to varying degrees [81]. This work found that most of the elements studied, mainly Ca, Mg, S, Fe, Mn, and Zn, resulted in increases in plants treated with NZnO. The increase in the concentration of these elements can be explained because NPs favor the production of organic acids exudated by the root, mainly oxalic, citric, lactic, and fumaric acids [82], which promote the absorption of nutrients by plants.

Another possible mechanism to explain the increase in minerals in lettuce is because NZnO promotes aquaporins through the overexpression of the *Tip1:1* and *Pip1:1* genes, which increases water and nutrient access to plant cells [83]. In addition to the production of water channels, NPs can create new pores in cell walls [84], promoting ion diffusion. Increases in mineral concentration in tissues may also be explained by NZnO promoting a greater abundance of root hairs [85].

On the other hand, it has been shown that applications of TiO_2 and SiO_2 NPs in plants can reduce the pH of the rhizosphere by up to 17.4% [82], while NZnO reduced the pH from 6.18 to 6.08 after just 7 days of exposure [86]. Generally, at a pH of 6–6.5, it is possible to obtain the highest availability of most of the essential nutrients by the roots, which partially can explain the increases in some elements, such as Ca, Mg, Fe, and Mn.

The increase in Ca concentration can be partially explained by the NZnO-linked overexpression of the *cation/H^+ antiporter 18-like*, *calcium-transporting ATPase 13*, and *autoinhibited Ca^{2+}-transporting ATPase 10* genes, while the higher concentration of S can be explained by overexpression of the *high affinity sulfate transporter type 1* gene [62].

Increased tissue Ca and Fe levels were reported with applications of NZnO in *Brassica chinensis* [87]. The concentration of Fe was also increased in leaves of *Spinacia oleracea* and *Coriandrum sativum* when subjected to 100 mg L^{-1} of NZnO via drench [88].

As expected, Zn was increased in plants treated with foliar application of NZnO, which is explained by the more significant absorption through the leaves [59], while in the roots, the access of NPs is carried out through other mechanisms, such as endocytosis [20].

On the other hand, some elements resulted without alterations or with specific decreases when performing NZnO applications. For example, the N concentration in the leaves and roots of lettuce plants subjected to NZnO showed no difference compared to the application of Zn^{2+}. The same effect was reported by Ref. [89]; where ZnO NPs did not alter the concentration of N in ryegrass leaves, even though N was increased in NZnO-treated soils. Currently, it is unknown how NMs applications might affect the mechanisms of absorption, transport, and accumulation of N in plants.

Similarly, there are no reports on the effect of NPs on the absorption and transport of P in plants; however, the decrease in this element in lettuce plants could be related to the inhibition of the *PHT1*, *PHT2*, *PHT3*, and *PHT4* genes [108]. In addition, it is known that NPs can affect the expression of genes linked to other elements, such as *OsLCT1* and *OsNramp5*, which are related to the absorption and transport of Cd [90], as well as a low expression of the *OsLis1* and *OsLis2* genes, which are associated with the absorption of As [91]. Another possible explanation is the P immobilization, which has been reported in soils [92] and could have occurred in the substrate.

A decrease in the concentration of P in plants has been reported when applying a wide variety of NPs (CeO_2, Fe_3O_4, SnO_2, Ag, Co, and Ni), with reductions from 7 to 38% in the levels of this element in tomato leaves [93]. A similar result was reported by Ref. [94]; where Ag NPs reduced the concentration of P in the leaves by 14%, as well as a 34% decrease in *Glycine max* roots. The same effect was recorded when applying 20–200 mg L^{-1} of NZnO in *Arabidopsis thaliana* [95].

On the other hand, the increase in S and decrease in P can be partially explained by anion transport processes, through which plants maintain the balance of ionic charges [109].

Despite reductions in some minerals in lettuce leaves and roots, element concentrations were within adequate levels for this plant species from an agronomic point of view [96,97]. There was, therefore, no decrease in the nutritional quality of the plants.

The effects of NPs on the concentration of minerals in plant tissues are still poorly understood, and a trend of increase or decrease of these elements is not easily predictable since the responses are different depending on the environment, plant species, and the characteristics of the nanomaterial studied [98].

5. Conclusions

The results indicate that it is possible to completely replace Zn^{2+} with NZnO without affecting the vegetative growth of lettuce plants, in addition to increasing aerial biomass, bioactive compounds, and the accumulation of essential minerals, obtaining a better response when making foliar applications of NZnO between 50 and 75% of the total Zn^{2+} applied conventionally. Among the limitations of this study, we can find a lack of analysis of important variables such as those related to photosynthesis and possibly the

determination of the activity of other antioxidant enzymes and concentrations of ROS, RNS and RSS, the above for a greater understanding of how lettuce plants respond to the application of NZnO.

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CONCLUSIÓN GENERAL

1. Las nanopartículas de ZnO aplicadas como seed priming a las semillas de *Moringa oleifera* favorecieron la longitud y biomasa de plúmula y radícula, además de incrementar el crecimiento inicial de las plantas y una mayor producción de compuestos antioxidantes.

2. Las nanopartículas de ZnO aplicadas vía drench y foliar en plantas de lechuga produjeron un mayor rendimiento de biomasa fresca, pigmentos fotosintéticos y compuestos antioxidantes enzimáticos y no enzimáticos.

3. Las nanopartículas de ZnO favorecieron una mayor acumulación de minerales en los tejidos de lechuga.

4. Es posible el reemplazo completo del fertilizante convencional ($ZnSO_4$) con nanofertilizante (ZnO), encontrando una mejor respuesta al realizar aplicaciones vía drench en cantidades de entre 50-25% menores a lo recomendado para el fertilizante convencional.

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