Centro de Investigación en Alimentación y Desarrollo, A.C.

BIOFORTIFICACIÓN CON NANOPARTÍCULAS DE ZINC MÁS QUITOSANO EN EL DESARROLLO Y LA CALIDAD NUTRIMENTAL DEL FRIJOL

Por:

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"*Una buena madre vale por cien maestros*"
George Herbert

Te lo prometí hace 10 años y para cuando leas esto lo habré cumplido, quizás las cosas no salieron como lo había pensado, pero hoy después de mucho esfuerzo y sacrificios principalmente tuyos, soy doctor. No podría escribir lo suficiente para describir el mundo de emociones que me hace sentir esto, tú que estuviste ahí desde el inicio, que dejaste muchas cosas de lado por verme feliz, por verme alcanzar mis sueños, te mereces este título y todo lo que venga después porque gracias a ti soy lo que soy y por tú apoyo pude hacer lo que me apasiona. Sencillamente, gracias.

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"Como ya es usual, detrás de cada idiota siempre hay una gran mujer"
John Lennon

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"*La familia no es algo importante. Lo es todo*."
Michael J. Fox

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CONTENIDO

CONTENIDO (continuación)

RESUMEN

La deficiencia de micronutrientes supone una amenaza para la salud de la población mundial. En el caso del zinc, se ha reportado que niveles por debajo de lo recomendado ocasionan problemas en el crecimiento y el sistema inmunológico. Una estrategia innovadora es la biofortificación, definida como el proceso de incrementar las concentraciones de elementos esenciales en porciones comestibles de plantas a través de la fertilización. El uso de nanofertilizantes surge como una forma de llevar a cabo la biofortificación de forma sustentable y precisa. Por lo que, el objetivo del presente trabajo de investigación fue biofortificar, promover el desarrollo y aumentar la calidad nutrimental del frijol mediante la adición de nanopartículas de zinc más quitosano. Se utilizaron plantas de frijol cv. Strike y se les aplicaron vía foliar, nanopartículas de zinc y nitrato de zinc a dosis de 25, 50 y 100 ppm con y sin quitosano. Se evaluó el contenido de zinc en los distintos órganos de la planta, la acumulación de biomasa, rendimiento, actividad fotosintética, actividad de la enzima nitrato reductasa, aminoácidos, proteínas solubles, pigmentos fotosintéticos y contenido mineral, además del grado de biofortificación alcanzado en fruto y el patrón de distribución del Zn dentro de la planta. Los resultados obtenidos indican que la aplicación de zinc favorece el proceso de biofortificación, encontrando incrementos para todos los tratamientos utilizados. Los tratamientos que se destacaron fueron nitrato de zinc más quitosano a 50 y 100 ppm, que incrementaron el contenido de zinc en frutos en más del 110%. El tratamiento de nanopartículas de ZnO a 25 ppm, obtuvo resultados sobresalientes para las variables de biomasa y producción, sus resultados fueron similares estadísticamente al tratamiento de 50 ppm del NZN. Esto sugiere que las nanopartículas de ZnO pueden reducir la cantidad de fertilizante a emplearse sin afectar los rendimientos en los cultivos, por lo que podrían ser utilizadas para solucionar la problemática ambiental ocasionada por el uso excesivo de fertilizantes. El patrón de distribución del Zn dentro de la planta sugiere una mayor translocación de Zn a los diferentes órganos de la planta cuando se utilizó quitosano. La aplicación de quitosano favoreció la biomasa, producción y los parámetros relacionados con la fotosíntesis. Finalmente, indicar que se requiere un mayor número de estudios respecto al uso de nanopartículas y quitosano en horticultura para determinar con certeza su efecto en la fisiología y nutrición de las plantas.

Palabras clave: nanotecnología, micronutrientes, sustentabilidad, seguridad alimentaria.

ABSTRACT

Micronutrient deficiency poses a threat to the health of the world's population. In the case of zinc, it has been reported that levels below the recommended level cause problems in growth and the impermeable system. An innovative strategy is biofortification, defined as the process of increasing the concentrations of essential elements in edible portions of plants through fertilization. The use of nanofertilizers emerges as a way to carry out biofortification in a sustainable and precise way. Therefore, the objective of this research work was to biofortify, promote development and increase the nutritional quality of beans by reducing zinc nanoparticles plus chitosan. Bean plants cv. Strike and applied via foliar, zinc nanoparticles and zinc nitrate at doses of 25, 50 and 100 ppm with and without chitosan. The content of zinc in the different organs of the plant, the accumulation of biomass, yield, photosynthetic activity, activity of the nitrate reductase enzyme, amino acids, soluble proteins, photosynthetic pigments and mineral content, in addition to the degree of biofortification reached in the fruit and the distribution pattern of Zn within the plant, were evaluated. The results obtained indicate that the application of zinc favors the biofortification process, finding increases for all the treatments used. The treatments that stood out were zinc nitrate plus chitosan at 50 and 100 ppm, which increased the zinc content in fruits by more than 110%. The ZnO nanoparticle treatment at 25 ppm obtained outstanding results for the biomass and production variables, its results were statistically similar to the 50 ppm NZN treatment. This suggests that ZnO nanoparticles can reduce the amount of fertilizer to be used without affecting crop yields, so they could be used to solve the environmental problem caused by the excessive use of fertilizers. The distribution pattern of Zn within the plant suggests a greater translocation of Zn to the different organs of the plant when chitosan is applied. The application of chitosan favors biomass, production and parameters related to photosynthesis. Finally, indicate that a greater number of studies are required regarding the use of nanoparticles and chitosan in horticulture to determine with certainty their effect on the physiology and nutrition of plants.

Keywords: nanotechnology, micronutrients, sustainability, food safety.

1. SINOPSIS

1.1. Justificación

Actualmente existen alrededor de 2 mil millones de personas que padecen hambre (FAO, 2020). En conjunto, el problema de la malnutrición afecta a una de cada tres personas, de las cuales los principales problemas que padecen son: emaciación, retraso del crecimiento, insuficiencia ponderal, y deficiencias de micronutrientes (OMS, 2018).

Los micronutrientes, son elementos esenciales para la formación de huesos, cerebro y cuerpos saludables, que se requieren en pequeñas cantidades. Dichos nutrientes deben obtenerse de los alimentos, idealmente de una dieta balanceada. Deficiencias en yodo, vitamina A, hierro y zinc son los problemas más importantes y suponen una amenaza para la salud y el desarrollo de las poblaciones de todo el mundo, en particular para los niños y las mujeres embarazadas de los países subdesarrollados (OMS, 2018; Dahdouh *et al.*, 2019).

En el caso del zinc, se estima que un 33 % de la población mundial se encuentra afectada o en riesgo de padecer dicha deficiencia. Se ha reportado que niveles por debajo de lo recomendado pueden provocar problemas en el embarazo, crecimiento en niños, problemas con el sistema inmunológico, además de un bajo desarrollo neuro conductual. A su vez, una deficiencia severa de este microelemento ocasiona dermatitis pustulosa, alopecia, diarrea crónica, trastorno emocional, pérdida de peso, infecciones recurrentes, hipogonadismo en hombres, trastornos neuro-sensoriales y problemas de cicatrización de úlceras, problemas que pudieran llegar a ser fatales si no se reconocen y se tratan a tiempo (Prasad, 2012; Wessells *et al.*, 2012; Sida-Arreola *et al.*, 2017; Dahdouh *et al.*, 2019).

Una estrategia innovadora a la problemática de la desnutrición de micronutrientes en la dieta alimentaria es la "biofortificación". La cual ha sido definida como el proceso de incrementar las concentraciones biodisponibles de elementos esenciales en porciones comestibles de plantas cultivadas a través del manejo agronómico (fertilización), el cual se enfoca en mejorar el contenido nutricional de arroz, camote, frijol y maíz (White y Broadley, 2005; Pachón, 2010). El frijol común (*Phaseolus vulgaris* L.) es la leguminosa de mayor consumo directo en el mundo ya que es la principal comida para más de 300 millones de personas en regiones de África y América Latina.

En términos nutricionales, es la principal fuente de proteínas de origen vegetal, además de su alto contenido de minerales, especialmente hierro y zinc, y su gran aporte de vitaminas; en base a esto, la biofortificación del frijol es clave con nutrientes como el hierro o zinc (Blair, 2013; Petry *et al.*, 2014).

Para llevar a cabo el proceso de incrementar la concentración de minerales en los tejidos vegetales, es necesaria la aplicación exógena de dichos nutrientes a través de la fertilización (Blasco, 2015); diversos autores indican las consecuencias negativas que podría tener dicho proceso para el medio ambiente, por lo tanto, es necesario desarrollar nuevas estrategias que den como resultado una producción de cultivos de mayor calidad, además de minimizar el uso de recursos y fertilizantes (Blasco, 2015; Raliya *et al.*, 2017).

En base a lo anterior, el uso de la nanotecnología surge como una forma posible para lograr el desarrollo óptimo de las plantas de forma sustentable y precisa; dado su tamaño, las plantas pueden absorberlas con una dinámica diferente, lo que presenta una ventaja adicional. Sin embargo, el estudio de las nanopartículas y su interacción con las plantas está en desarrollo, por lo que se requieren más estudios para determinar el alcance de sus beneficios (Raliya *et al.*, 2017; Sturikova *et al.,* 2018).

Otra alternativa viable para enfrentar esta situación es el uso de biorreguladores de crecimiento, el cual se presenta como una opción viable en la mejora del desarrollo vegetal y la disminución en la aplicación de fertilizantes; especialmente destaca el quitosano, el cual es la forma desacetilada de la quitina, que a su vez es el segundo material de desecho más abundante y proviene principalmente del exoesqueleto de crustáceos e insectos favorece el crecimiento en las raíces y la absorción de nutrientes, además de que ayuda a la planta a enfrentar situaciones de estrés y contra ataques de patógenos (Abdel-Mawgoud *et al.,* 2010; Guzmán-Antonio *et al.,* 2012).

1.2. Antecedentes

1.2.1. El Problema de la Malnutrición

La mayor problemática a nivel mundial se centra en cómo garantizar que la creciente población mundial tenga suficientes alimentos y que estos sean de una calidad necesaria para satisfacer las necesidades nutricionales que exige un estilo de vida sano (FAO, 2020). En adición a lo anterior, los recursos como el agua y el suelo cada vez son más escasos, además de los cambios climáticos cada vez más severos y frecuentes que agravan la situación. Desafortunadamente, los sistemas de producción agrícola tienen como principal objetivo el aumentar la rentabilidad para los agricultores y las industrias agrícolas, dejando a segundo término lo relacionado con la calidad de los productos y los problemas de salud humana; dificultando el trabajo para los científicos agrícolas (FAO, 2020; Singh y Prasad, 2014).

En los últimos años el término malnutrición ha tomado relevancia internacional debido a que es la principal causa de muertes con más de 20 millones de decesos al año. La malnutrición se puede definir como un problema causado por la falta o el exceso de ingesta y absorción de alimentos y nutrientes, generando un desorden en la composición corporal y masa celular ocasionando una actividad física y mental disminuida que a largo plazo deteriora el estado clínico del individuo. El concepto de malnutrición engloba tanto la desnutrición como los trastornos de sobrepeso y obesidad, a pesar de manifestarse como problemas físicos distintos, diversos estudios indican que comparten síntomas como el metabolismo alterado, la inflamación crónica y la disfunción intestinal (enteropatía); también el consumo excesivo de energía y macronutrientes a menudo coincide con las deficiencias de micronutrientes en personas con sobrepeso (Cederholm *et al.,* 2015; Bourke *et al.,* 2016; OMS, 2018).

A nivel mundial, la mala alimentación afecta a más de 2 mil millones de personas alrededor del mundo, acarreando problemas como enfermedades cardíacas, cáncer, derrame cerebral y diabetes. Más recientemente, la malnutrición se ha asociado con un mayor riesgo de infección por SARS-CoV-2 y a su vez se presentan casos más graves en personas contagiadas con un mayor número de hospitalizaciones que requieran ventilación mecánica. El brote de la pandemia COVID-19 ha causado una emergencia alimentaria transcontinental, con amenazas inminentes en la nutrición (Huizar *et al.,* 2020).

La estimación más reciente muestra que casi 690 millones de personas, o el 8.9 % de la población mundial, estaban desnutridos, y tomando en cuenta las proyecciones preliminares, en base a las últimas perspectivas económicas mundiales, sugieren que para el término de la pandemia de COVID-19 se podrían contabilizar entre 83 y 132 millones de personas más que se encuentren en problemas de desnutrición (FAO, IFAD, UNICEF, WFP and WHO, 2020).

En base a lo anterior, la desnutrición se ha convertido en un grave problema de salud, especialmente pediátrico, siendo la causa de muerte de cerca de 3.1 millones a nivel mundial de niños menores de 5 años, lo cual representa el 45 % del total de muertes de niños anualmente. También contribuye al aumento de la mortalidad infecciosa, la discapacidad neurológica, el retraso del crecimiento físico y mental; además la desnutrición temprana aumenta el riesgo de obesidad en la edad adulta lo que ocasiona problemas clínicos como alto porcentaje de tejido adiposo, altos niveles circulantes de triglicéridos, presión arterial alta, provocando las enfermedades anteriormente mencionadas. Una de las causas de este problema es la deficiencia de nutrientes como lo son Fe, Zn, así como por diversas vitaminas (Bouis y Welch, 2010; Kane *et al.*, 2015; Bourke *et al.*, 2016; OMS, 2018).

1.2.1.1. Deficiencia de Micronutrientes en Humanos. Las deficiencias de micronutrientes, conocida mundialmente como "hambre oculta" debido a que su desarrollo es progresivo a través del tiempo y sus efectos no son apreciables hasta que han causado un daño irreversible, son de gran importancia para la salud y tienen un alto impacto socioeconómico en todo el mundo. El problema afecta principalmente a los países de bajos ingresos, siendo principalmente las mujeres, los niños, las personas de mediana edad y los ancianos, los grupos con mayor riesgo de presentar este padecimiento o que ya experimentan los efectos de este problema (UNICEF, 2019; OMS, 2018).

En el mundo, cerca de 2 billones de personas sufren una deficiencia crónica de micronutrientes y alrededor de 462 millones de adultos tienen insuficiencia ponderal, además se calcula que 159 millones de niños menores de 5 años tienen retraso del crecimiento, y 50 millones más presentan emaciación. También existen 528 millones de mujeres en edad reproductiva que sufren anemia, la mitad de las cuales podría tratase con suplementos de hierro (OMS, 2018; UNICEF, 2019).

Entre los síntomas característicos de la deficiencia de micronutrientes se encuentran el retraso en el crecimiento, retraso cognitivo, debilidad inmunológica y enfermedades infecciosas constantes, las cuales son las principales causas de morbilidad y mortalidad en países de bajo desarrollo (Tulchinsky, 2010). A su vez, Song et al. (2015), relacionan la deficiencia de micronutrientes con un mayor índice de padecer depresión, encontrando que las personas con este problema sufren de ansiedad y tristeza crónica.

Deficiencias en nutrientes como hierro y vitamina B_{12} generan anemia, el cual es uno de los padecimientos más importantes debido a la carencia de estos nutrientes, esta enfermedad afecta a un número significativo de mujeres en edad fértil, se estima que reduce en 20 % la capacidad mental y laboral de las personas. La deficiencia de vitamina A es la principal causa de ceguera prevenible en niños, pero que progresa a ceguera permanente en casos más fuertes, también en mujeres en lactancia e infantes, dicha deficiencia potencializa las enfermedades graves aumentando las tasas de mortalidad. Mientras que el iodo es la principal causa de daño cerebral en niños, además de que puede causar una pérdida de cerca de 13 puntos de coeficiente intelectual, generando retraso mental y está relacionado con problemas de tiroides. También existen otros nutrientes esenciales, cuya deficiencia provoca varios problemas en la salud humana como lo son el ácido fólico y el zinc (Ritchie y Roser, 2017).

1.2.1.2. Deficiencias de Zinc en Humanos. El zinc (Zn) es un nutriente esencial para casi todos los organismos, y tiene un papel fundamental como cofactor metálico en más de 300 proteínas del cuerpo humano. Además, juega un papel esencial como estabilizador y receptor de hormonas esteroides, tiroideas, retinoides y de las membranas celulares; también actúa como regulador siendo fundamental para la síntesis de biomoléculas como el ADN y se une a proteínas nucleares y forma complejos llamados "zinc fingers". Estas funciones del Zn están relacionadas con su capacidad de unirse a la histidina y la cisteína, estabilizar los sitios activos e intervenir en el mantenimiento de la integridad del sistema inmune (Caro *et al*., 2016; Taboada-Lugo, 2017).

El requerimiento del Zn en adultos es de 11 mg. día^{-1} y se encuentra presente en una cantidad de 2 a 3 g y es superado solo por el hierro como el micronutriente más abundante. La deficiencia de zinc se asocia con respuestas inmunitarias deterioradas y conduce a un mayor riesgo de infecciones virales respiratorias, particularmente en personas de edad avanzada. Además, genera una disminución en la producción de anticuerpos a través de la alteración de la función y el número de varias células inmunes (Gorji y Ghadiri, 2020).

Este problema es muy común en países donde la dieta es desbalanceada, principalmente en lugares con dietas basadas en cereales y baja ingesta proteica. La deficiencia de zinc, a diferencia de la anemia, puede presentarse indistintamente en hombres, mujeres y niños. Aproximadamente el 33% de la población mundial se ve afectada por la deficiencia de Zn, siendo la causante del 5% de las muertes en niños menores de 5 años; en los países en desarrollo, la deficiencia de Zn es la quinta causa principal de enfermedades y mortalidad humana (Bilski *et al.,* 2012; Singh y Prasad, 2014; Reed *et al.,* 2015).

Esta deficiencia puede tener una serie de consecuencias negativas para la salud, que afectan el sistema nervioso central, gastrointestinal, inmune, epidérmico, reproductivo y esquelético. Se ha reportado que niveles por debajo de lo recomendado pueden acarrear problemas en el desarrollo del embarazo, crecimiento en niños, problemas con el sistema inmunológico, además de un bajo desarrollo neuro conductual. A su vez, una deficiencia severa de este microelemento ocasiona dermatitis pustulosa, alopecia, diarrea crónica, trastorno emocional, pérdida de peso, infecciones recurrentes, hipogonadismo en hombres, trastornos neuro-sensoriales y problemas de cicatrización de úlceras, padecimientos que pudieran llegar a ser fatales si no se reconocen y se tratan a tiempo (Prasad, 2012; Wessells *et al.*, 2012; Sida-Arreola *et al.*, 2017; Dahdouh *et al.*, 2019).

El zinc se considera esencial en la recuperación después de alguna enfermedad o lesión, por lo que las deficiencias de este elemento pueden aumentar la susceptibilidad a enfermedades e infecciones e incrementar el tiempo de recuperación, o en algunos casos, perjudicar el avance de esta última. Además, dicha deficiencia puede reducir la capacidad mental y aumentar el predominio de complicaciones maternas, neonatales e infantiles (Ritchie y Roser, 2017).

1.2.2. Biofortificación

Actualmente, una estrategia innovadora a la problemática de la desnutrición de micronutrientes en la dieta alimentaria es la "Biofortificación". Aunque aún no existe un consenso sobre el término, la biofortificación ha sido definida como el proceso de incrementar las concentraciones biodisponibles de elementos esenciales en porciones comestibles de plantas cultivadas a través del manejo agronómico (fertilización) o mejoramiento genético (White y Broadley, 2005).

La biofortificación por fitomejoramiento convencional mejora una característica nutricional o agronómica deseable y ya existente en el cultivo convencional. Para ello, se hacen cruzamientos entre materiales con cualidades de interés que permiten obtener variedades con las características deseadas por los fitomejoradores. Por su parte, la biotecnología es una aplicación tecnológica que es utilizada para crear o modificar productos o procesos para usos específicos a través de procesos como marcadores moleculares y transgénesis. La biofortificación de cultivos básicos es una estrategia reciente, que se suma a otras como la fortificación de alimentos mediante procesos industriales, la adición de suplementos al momento de consumir un alimento o consumir una dieta balanceada (White y Broadley, 2005; Pachón, 2010; FAO, 2014).

A través de sus diferentes alternativas, la biofortificación intenta ser una herramienta que busca mejorar el contenido nutricional de los alimentos básicos que consumen las personas de bajos recursos, proporcionando una solución a mediano y largo plazo para el problema grave de desnutrición que presentan dichas poblaciones, a su vez se presenta como una solución relativamente económica, rentable y sostenible ya que para la mayoría de los cultivos solo se requiere una inversión única para la propagación de variedades con los niveles elevados de nutrientes para que esta se convierta en autosuficiente (Pachón, 2010; Bouis *et al.,* 2011).

Las investigaciones han demostrado que es posible cultivar alimentos básicos para producir cultivares con niveles elevados de elementos esenciales. La premisa de la biofortificación es que las dietas de las personas con desnutrición se basan principalmente en alimentos básicos, ya que carecen del poder adquisitivo para una dieta más balanceada que contenga cantidades suficientes de alimentos ricos en los nutrientes esenciales. Los alimentos básicos biofortificados no pueden ofrecer un nivel elevado de minerales y vitaminas por día como los suplementos comerciales o los alimentos fortificados industrialmente, pero pueden ayudar a que los millones de personas que se encuentran en desnutrición superen dicho problema (Meenakshi *et al.,* 2010; Pachón, 2010; Bouis *et al.,* 2011).

Desde un punto de vista estratégico, y dado que la biofortificación es una técnica basada en la producción de cultivos, la elección de las zonas de intervención debe realizarse teniendo en cuenta variables clave tales como zonas de producción, rangos de adaptación, infraestructura para el fitomejoramiento, distribución de semillas y adopción de variedades. Desde el año 2004, el proyecto HarvestPlus, coordinado por el International Food Policy Research Institute (IFPRI) y el Centro Internacional de Agricultura Tropical (CIAT), conducen programas con el objetivo de aumentar el contenido de beta-caroteno, lisina, triptófano, hierro y/o zinc en los cultivos básicos de mayor importancia en el mundo en vía de desarrollo: arroz (*Oryza sativa*), camote (*Ipomoea batatas*), frijol (*Phaseolus vulgaris*), maíz (*Zea mays* L), trigo (*Triticum spp*.) y yuca (*Manihot esculenta*) (Monserrate-Rojas *et al.,* 2009; Pachón, 2010).

Diversos logros han sido alcanzados a través de los tiempos, en 2008, expertos economistas clasificaron a la biofortificación entre las soluciones más rentables y sustentables para abordar la problemática mundial de la mala alimentación y los problemas que ocasiona. También en 2016, el

Premio Mundial de la Alimentación se otorgó a Howarth Bouis de la organización Harvest Plus y María Andrade, Robert Mwanga y Jan Low del CIP (International Potato Center), por sus trabajos relacionados a la biofortificación de alimentos. En trabajos concretos la organización HarvestPlus produjo maíz, papa y yuca con un nivel de vitamina A arriba del 50 % de la ingesta diaria, también trabajos con hierro en frijol y mijo alcanzaron el 60 % de la ingesta de hierro recomendada, por último, trabajos en trigo y arroz produjeron variedades con niveles de zinc tan altos que proporcionarían hasta el 80 % de la recomendación diaria (Andersson *et al*., 2017; Bouis, 2018).

Sin embargo, a pesar de la efectividad y los grandes avances que hasta el momento han demostrado los programas de biofortificación para aumentar el consumo de algún elemento en particular a través de la dieta de los seres humanos, ninguno de estos trabajos ha estudiado el efecto de la biofortificación sobre otros aspectos de la calidad nutricional, particularmente la capacidad antioxidante y su relación con los compuestos bioactivos (FAO, 2014).

Existe un consenso generalizado en torno a la importancia de proteger parámetros de calidad deseables en los alimentos biofortificados, esta preocupación nace debido a que existen evidencias de que una aplicación excesiva de nutrientes puede dar lugar a diversas complicaciones, como lo son la toxicidad para las plantas que a su vez ocasiona una mala calidad nutricional, además de cambios en la coloración de los alimentos (como el caso del maíz que se tornó naranja debido a las altas concentraciones de carotenoides), lo que complica su comercialización (Blasco, 2015).

1.2.2.1. Biofortificación del Frijol. El frijol común (*Phaseolus vulgaris* L.) es una de las cinco principales especies que se han tomado en cuenta para los programas de biofortificación debido a que es la leguminosa de mayor consumo directo en el mundo y la tercera más importante después de la soya y el cacahuate, aunado a esto es la comida principal para más de 300 millones de personas en regiones de África y América Latina. En términos nutricionales, el frijol es comúnmente conocido como “la carne de los pobres" debido a su precio económico y a que es la principal fuente de proteína de origen vegetal, además de su alto contenido de minerales, especialmente hierro y zinc, y su gran aporte de vitaminas, en base a esto la biofortificación del frijol es clave con nutrientes como el hierro o zinc, principalmente el primero, debido a que se estima que la deficiencia del mismo afecta a casi 2 mil millones de personas en todo el mundo, principalmente mujeres y niños en países en desarrollo (Blair, 2013; Petry *et al.*, 2014).

Según Petry *et al.* (2015), en algunos países, los programas de fitomejoramiento ya han desarrollado y lanzado nuevas variedades de frijol común con concentraciones de hierro superiores a 94 μg g^{-1}, además, muestran una buena retención de dichos nutrientes después del procesamiento y un rendimiento agronómico igual o mayor que las variedades de frijol conocidas y comercializadas, lo que indica que el frijol común puede ser un cultivo prometedor para la biofortificación de hierro.

También es necesario conocer y evaluar previamente la diversidad genética que presenta el frijol, con un aproximado de 1400 variaciones en sus genotipos, además, muestra rangos muy amplios en el contenido de hierro (30–110 ppm) y zinc (25–60 ppm), a su vez, también surge la necesidad de conocer el contenido de diversos compuestos de interés que posee el frijol, esto permitiría seleccionar mejor los materiales a mejorar (Blair, 2013).

En el caso particular de México, el frijol es establecido como un producto estratégico en el desarrollo rural y social del país, ya que representa toda una tradición productiva y de consumo, cumpliendo diversas funciones de carácter alimentario (Secretaría de Economía, 2017). El frijol se considera un cultivo estratégico para biofortificar con Fe y Zn, debido a que es un alimento importante de la canasta básica ya que de este cultivo se obtiene la proteína de origen vegetal más utilizada por los diferentes estratos sociales. Para asegurar que la biofortificación con Fe y Zn resulte efectiva, deben establecerse las concentraciones en las cuales el grano de frijol pueda brindar al consumidor los niveles recomendados para evitar deficiencias nutricionales (Sánchez *et al.,* 2013).

Diversos estudios realizados en los últimos años demuestran que la adición de hierro y zinc ha incrementado las concentraciones de dichos elementos en los granos utilizados, además de que mejora el sistema antioxidante de esta planta (Sida-Arreola *et al.*, 2015; Morales-Morales *et al.*, 2016).

1.2.2.2. Biofortificación con Zinc. Una cuarta parte de la población mundial depende de dietas que contienen predominantemente cereales (arroz y trigo), legumbres y semillas, las cuales no aportan la cantidad necesaria de Zn para un correcto desarrollo (Bilski *et al.,* 2012; Ramesh *et al.,* 2014). Las concentraciones de minerales como el Zn en la solución del suelo se determinan por las reacciones de precipitación, formación de compuestos y adsorción específicas del suelo, y el factor más limitante en su absorción por las plantas es el pH. Para aumentar la disponibilidad de zinc, la fertilización de cultivos con las fuentes tradicionales de este nutriente representa una estrategia a corto plazo y complementaria, la cual es la necesaria para la captación o translocación de dicho elemento (Cakmak, 2008; White y Broadley, 2009).

La acumulación del zinc en porciones comestibles de plantas es muy variable según la especie; además, frecuentemente se ve reducida por los factores ambientales a los que estén expuestos los cultivos como las altas temperaturas, la alta intensidad de la luz, las concentraciones de CO_2 y el riego; también puede depender de las interacciones que se presenten con otros nutrientes, por ejemplo, se han encontrado correlaciones negativas entre el contenido de Fe y Zn en granos de sorgo, mientras que trabajos realizados en trigo han mostrado correlaciones negativas entre el contenido de P y Zn (McDonald *et al.*, 2008; White y Broadley, 2009).

Organizaciones a nivel mundial han realizado durante los últimos 10 años trabajos en más de 12 países, los cuales tienen el objetivo de aumentar la concentración de zinc en porciones comestibles de cultivos básicos importantes de cada región como lo son trigo, maíz, arroz y frijol. La mayor respuesta la encontraron en granos de trigo alcanzando el 83 % de incremento en este nutriente, seguido por el arroz con 27 %, siendo el maíz el que presentara la menor respuesta con un 9 % de incremento. En el caso del frijol, se encontró diferencia significativa en plantas a las que se le aplicaron Zn, obteniendo un incremento sustancial, además de que descubrieron que las proteínas son el sumidero de este nutriente en el grano (Ram *et al.*, 2016; Cakmak y Kutman, *et al.,* 2018).

1.2.2.3. Importancia del Zinc en la Planta. El zinc es un nutriente esencial para las plantas siendo un limitante en la producción de los cultivos. La concentración de este elemento en plantas está dentro de un rango de 25–150 ppm, mientras que una concentración inferior de 15-20 ppm en tejidos de hoja seca conduce a la deficiencia de este. Los síntomas de deficiencia de Zn incluyen hojas cloróticas, senescencia temprana y retraso en el crecimiento, lo que puede llegar a afectar severamente el rendimiento (Marschner, 2011; Mitra, 2015).

Tanto en plantas como en otros sistemas biológicos, el Zn solo existe como Zn^{2+} y sus funciones metabólicas se basan en su fuerte tendencia a formar complejos tetraédricos con N^- y O^-, por lo que el papel más importante del zinc en la planta es su rol como cofactor enzimático, participando en las reacciones de enzimas claves como lo son superóxido dismutasa, alcohol deshidrogenasa, anhidrasa carbónica, fosfatasa alcalina, entre otras. También participa en los procesos de transcripción y en el metabolismo de las proteínas, específicamente en la interacción proteína-proteína, actúa como regulador de la expresión génica y la transcripción de proteínas, además de que contribuye en la formación y fertilidad del polen, la síntesis de carbohidratos durante la fotosíntesis y tiene un rol en el metabolismo hormonal al regular la síntesis de auxinas, las cuales están relacionadas con el crecimiento de las plantas. Estas funciones tienen efectos significativos en el rendimiento y maduración del grano (Marschner, 2011; Mitra, 2015, Cakmak *et al*., 2017).

1.2.2.4. Absorción, Movilidad y Almacenamiento del Zinc. El zinc es absorbido en forma de catión divalente Zn^{+2} y en condiciones poco favorables también puede ser tomado como $Zn(OH^{+})$, su absorción vía edáfica depende de muchos factores como son el pH, textura del suelo, temperatura, materia orgánica, variedad del cultivo, entre otros; además, diversos ácidos orgánicos forman complejos con este elemento facilitando su absorción por las raíces (Marschner, 2011; Mitra, 2015).

Una vez dentro de las plantas, el zinc penetra la membrana plasmática gracias a transportadores ZIP (Permeasas Zinc-Hierro), los cuales son el principal medio de transporte activo en las plantas. El zinc se mueve vía simplástica mediante los plasmodesmos hasta alcanzar el xilema. Este movimiento puede ser afectado por la cantidad de nutriente suministrado y si bien su movimiento no coincide con el del agua, por lo general está relacionada con un buen suministro de esta. Una vez alcanzada la parte superior, se distribuye según las demandas de los distintos órganos vía floema (Marschner, 2011; Rehman *et al.,* 2012; Mitra, 2015).

La aplicación de fertilizantes como fuente de Zn, tanto de manera edáfica como foliar, aumenta la cantidad de Zn disponible para la planta. Sin embargo, para este nutriente, estudios previos demuestran que la aplicación en forma de aspersión al follaje resulta más eficiente que una aplicación al suelo, debido a que evita las pérdidas de nutrientes durante la absorción por la raíz (Gupta *et al.*, 2016).

La fertilización foliar se ha convertido en una forma rápida y eficaz para el desarrollo sustentable y productivo de los cultivos. En la fertilización foliar, la planta absorbe el ion Zn^{2+}. Estos iones ingresan a la planta hacia el apoplasto de la hoja a través de las estomas, específicamente por los poros que generan estas células, sin embargo, la absorción también se puede dar a través de la superficie cuticular, tricomas y lenticelas (Fernández *et al.*, 2013).

En la mayoría de las especies vegetales, con frecuencia el Zn es más móvil que la mayoría de los micronutrientes, por lo que una correcta aplicación foliar aumenta la concentración de este elemento en el floema de la hoja, desde donde se puede trasladar directamente a sumideros en crecimiento, principalmente a los frutos. Este proceso, ocurre durante la etapa de senescencia de las plantas y en condiciones de buen desarrollo puede alcanzar hasta un 20 % de translocación del total de Zn acumulado en las hojas. Esto puede considerarse como una baja movilidad, sin embargo, factores como la mala aplicación, la fuente utilizada y la fuerte unión del Zn a los tejidos foliares pueden modificar esos valores. Una vez en el floema, la movilidad de Zn aumenta como resultado de la quelación de este por metabolitos orgánicos (Zou *et al.,* 2012; Fernández *et al.,* 2013; Gupta *et al.,* 2016).

En el caso de los programas de biofortificación, la fertilización de zinc más un compuesto nitrogenado se utiliza frecuentemente debido a que mejoran el desarrollo de la planta además de que aumenta la cantidad de zinc y proteínas en el fruto; esto se debe a la sinergia positiva que existe entre estos dos nutrientes, por lo que un correcto desarrollo del metabolismo del nitrógeno en la planta genera un aumento en la síntesis de proteína y diversos estudios explican que la mayoría del zinc en la planta se encuentra unido a cuerpos proteicos, por lo que esta relación resulta en una mayor concentración de zinc en los frutos (Kutman *et al.,* 2010; Guo *et al.,* 2016).

1.2.3. La Nanotecnología en la Agricultura

Anteriormente, la agricultura era el centro de atención para la aplicación de nuevas tecnologías, siendo el predecesor de revoluciones industriales por más de 90 siglos, sin embargo, en el caso de la nanotecnología hace más de medio siglo que sus beneficios se están utilizando en la rama industrial y medica; mientras que, en la agricultura, la investigación de sus posibles beneficios sigue siendo minúscula, ya que menos del 5 % del total de investigaciones relacionadas con este tema pertenecen al área agrícola. A pesar de eso, la tendencia al alza sugiere que en los próximos 10 a 15 años la aplicación de productos basados en nano materiales en los campos agrícolas será una práctica regular. La Agencia de Protección Ambiental de los E.U.A define la nanotecnología como la ciencia de estudio y comprensión de la materia en dimensiones en el rango de 1 a 100 nm (Ditta, 2012; Mukhopadhyay, 2014).

La nanotecnología surge como una alternativa, debido a que como ciencia emergente está dirigida a comprender y crear materiales, dispositivos y sistemas que exploten las propiedades a nanoescala de diversos compuestos. En la agricultura, la nanotecnología ha comenzado a desarrollar productos que ayudan a mejorar la fertilidad de suelo, reducir enfermedades e incrementar la calidad y producción de los cultivos, pasando de una agricultura convencional a una agricultura de precisión. Ejemplos como nanotubos de carbono, Cu, Ag, Mn, Mo, Zn, Fe, Si, Ti, o en sus formas de óxidos, además de nanoformulaciones de fósforo, urea y azufre; son considerados como las futuras fuentes de fertilización agrícola (Solanki *et al*., 2015; Chhipa, 2017).

1.2.3.1. Nanofertilizantes. La fertilización de los cultivos es de suma importancia a la hora de producir alimentos, sin embargo, los fertilizantes convencionales presentan una baja efectividad,, llegando a ser menor del 25 %, además, de que su aplicación de manera excesiva tiene un impacto ambiental negativo, ya que causa deterioro en los suelos, contaminación en los mantos acuíferos y genera contaminación aérea, los estudios recientes se centran en encontrar alternativas menos dañinas para el medio ambiente, sin afectar la calidad y producción de los cultivos (Naderi y Danesh-Shahraki, 2013; Raliya et al., 2017).

En la última década, las investigaciones respecto a los nanofertilizantes ha crecido de manera exponencial, siendo el objetivo de diversas comunidades como lo son, American Chemical Society, Society of Environmental Toxicology and Chemistry y Crop Chemical Europe, además de agencias reguladoras como el Departamento de Agricultura de E.U. (USDA), llegando a causar gran impresión sobre la comunidad científica; sin embargo, en la rama industrial existe muy poca información de productos basados en la nanotecnología, siendo los pesticidas a base de nanopartículas los productos más comunes en el mercado, pero se sigue considerando como un sueño no muy lejano (Kah, 2015).

La nanotecnología brinda diferentes aspectos con potencial aplicación en diferentes campos de la agricultura y la biotecnología para dar solución a los problemas ambientales y las demandas biológicas con mayor precisión. El uso de estos nanofertilizantes provoca un aumento en su eficiencia, reduce la toxicidad del suelo, minimiza la aplicación excesiva y sus potenciales efectos tóxicos. Por lo que ofrece una oportunidad para que la nanotecnología tenga una influencia significativa en la energía, la economía y el medio ambiente (Naderi y Danesh-Shahraki, 2013).

Se consideran nanofertilizantes a todas aquellas partículas que contengan nutrientes y que su dimensión sea menor a los 100 nm, preferentemente entre 30 y 40 nm, además, dentro de sus características destaca una superficie de contacto del ion nutriente muy alta y una liberación más lenta y precisa que los fertilizantes tradicionales, esto se da debido a la reducción del tamaño de las partículas, lo que provoca que la superficie específica aumente y, como consecuencia, el área de contacto con las plantas también, con esto las plantas pueden absorberlas con una dinámica diferente, lo que presenta una ventaja adicional en comparación con las fuentes tradicionales de fertilización (Subramanian *et al.,* 2015; Subbaiah *et al.,* 2016).

Los nanofertilizantes se clasifican en 3 categorías: a) aquellos basados en macronutrientes, b) nanofertilizantes basados en micronutrientes y c) nanomateriales que actúan como transportadores de nutrientes. Diversos estudios comparan los efectos de las diferentes categorías de nanomateriales contra los fertilizantes tradicionales, obteniendo una eficacia media de los nanofertilizantes sobre los fertilizantes convencionales de 19, 18 y 29% para las categorías 1, 2 y 3, respectivamente (Kah *et al.,* 2018).

Debido a lo anterior, las nano formulaciones de insumos agrícolas de ingrediente activo permiten una reducción significativa en los problemas ambientales que causa el exceso de fertilización como lo son la eutrofización. El uso de nanofertilizantes como las nanopartículas de Ca, hidroxiapatita de P, Fe^{+2}, ZnO, TiO2, Ag y nanotubos de carbono pueden usarse como una alternativa a los insumos agrícolas convencionales (Chhipa, 2017; Kah *et al.,* 2018).

A pesar del prometedor panorama que presenta el uso de nanofertilizantes, los estudios que se han presentado hasta el momento aún no son suficientes para determinar que el uso de esta nueva tecnología es superior al uso de los fertilizantes tradicionales, además de que los estudios deben también enfocarse en establecer los efectos que generan estos materiales sobre el desarrollo de las plantas (Subramanian *et al.,* 2015; Kah *et al.,* 2018).

1.2.3.2. Nanofertilizantes de Zinc. Existen diversas formas de nanopartículas que contienen Zn, como lo son ZnS y ZnSe, o puntos cuánticos de CdSe/ZnS, que actualmente son utilizadas en diversos campos de la ciencia. Sin embargo, la forma más utilizada de zinc a manera de nanopartículas continúa siendo el óxido de zinc (ZnO), debido a su amplia gama de propiedades positivas, tales como su alta conductividad eléctrica y térmica, su estabilidad a altas temperaturas y un pH neutro, además de efectos antimicrobianos leves. También su uso está relacionado con la fácil disponibilidad y su bajo precio de obtención (Sturikova *et al.,* 2018).

Se estima una producción cercana a las 1000 toneladas por año de ZnO, las cuales se utilizan comúnmente en materiales de plástico, vidrio, cerámica, cemento y caucho, así como en pigmentos, pinturas y en bloqueadores solares (Piccinno *et al.,* 2012). En la agricultura, aplicaciones de nanopartículas de Zn, a concentraciones bajas, han demostrado tener efectos positivos en la germinación, el crecimiento vegetativo, el contenido de clorofila, carotenos y aumentos significativos en el rendimiento (Rossi *et al.*, 2019; Siddiqui *et al*., 2019).

Se ha comprobado que el uso de esta tecnología, aplicada de manera foliar mejora la calidad del fruto, aumentando la absorción, traslocacion y acumulación del Zn en éstos, convirtiéndola en una herramienta novedosa para los programas de biofortificación (Davarpanah *et al.,* 2016; Subbaiah *et al.,* 2016; El-Ramady *et al.,* 2018). Por otro lado, aplicaciones en altas concentraciones tienen efectos estresantes que pueden inhibir el crecimiento de las raíces, retrasar el desarrollo de los cultivos y afectar la síntesis de proteínas y ADN (Ma *et al*., 2015; Sturikova *et al.,* 2018).

1.2.4. Quitosano

En los últimos años, se han hecho muchos intentos para sustituir los productos derivados del petróleo en el desarrollo de materiales, por lo que se han investigado un gran número de biopolímeros como almidón, celulosa, colágeno, gelatina, alginato, quitina y quitosano, debido a que poseen una funcionalidad aplicable en el desarrollo ambiental sustentable. Lo más complicado de la aplicación de estas nuevas tecnologías es obtener materiales con propiedades equivalentes a las de los productos totalmente sintéticos, y que además conserven su funcionalidad. Dentro de este grupo destaca el quitosano, el cual tiene propiedades intrínsecas únicas y valiosas que no posee un equivalente petroquímico. El quitosano es un polímero lineal cristalino y una forma desacetilada de quitina, que es un copolímero lineal de 2-acetamido-2-desoxi-β-D-glucopiranosa y 2-amino-2-desoxi-β-D-glucopiranosa. Siendo el segundo polímero más abundante en la naturaleza, y puede ser encontrado en los exoesqueletos de los crustáceos, paredes celulares de hongos y en la cutícula de los insectos (Croisier y Jérôme, 2013; Piras *et al.,* 2014; Choudhary *et al.,* 2017).

Para convertirse en quitosano, la quitina debe tener al menos 60 grados de desacetilación, la cual se realiza por hidrólisis química en condiciones alcalinas severas o por hidrólisis enzimática en presencia de enzimas como la quitina desacetilasa. El quitosano se identifica en base a su grado de desacetilación, que puede variar entre 60 % y 90 % y peso molecular promedio entre 50 hasta 2000 KDa, estas variaciones se deben a sus diferentes maneras de obtención y producción. Su principal utilidad se debe a sus propiedades antimicrobianas, su densidad de carga y sus propiedades de formación de películas o coberturas (Riva *et al.,* 2011; Mármol *et al.,* 2011; Croisier y Jérôme, 2013).

Comúnmente el quitosano se ha utilizado en la industria biomédica, debido a sus efectos medicinales. También desde hace décadas, es muy conocido su uso en la industria alimentaria como espesante, gelificante, y emulsificante, además se utiliza en películas protectoras comestibles y en procesos industriales como la recuperación de proteína de desechos de ovoproductos. Además, una de las áreas de mayor relevancia es su uso como coadyuvante en el tratamiento de aguas, debido a sus características favorables con el medio ambiente; diversos estudios demuestran su papel como quelatador de metales pesados y pesticidas (Mármol *et al.,* 2011).

1.2.4.1. Uso del Quitosano en las Plantas. Debido a que es considerado como un compuesto amigable con el medio ambiente, por su rápida degradación, baja toxicidad y fácil obtención, el uso del quitosano en la agricultura ha aumentado en los últimos años. El mecanismo de acción de este polímero y sus derivados en las plantas es de alto impacto, debido a que pueden actuar como señalizadores, realizar simbiosis para la absorción de algunos nutrientes y por ende mejorar el crecimiento y desarrollo de estas. A su vez, pueden llegar a inducir el gen que regula la nodulación para fijar nitrógeno en leguminosas y ayudan a promover la división celular en ausencia de hormonas como auxinas o citoquininas (Van *et al.,* 2013; Vasconcelos, 2014; Deshpande *et al.,* 2017).

La aplicación de quitosano se ha estudiado en muchas especies de cultivos, incluidos cereales, plantas ornamentales, frutales y medicinales, y su efectividad depende de la estructura y concentración de este, a la especie de planta y la etapa fenológica en que se encuentre dicha planta. Diversos estudios han demostrado su propiedad como antitranspirante, además se le han atribuido propiedades antibacteriales y antifúngicas, a su vez se ha estudiado su efecto como promotor del crecimiento de plantas e impulsor del sistema de defensa ante situaciones de estrés (Abu-Muriefah, 2013; Pichyangkura y Chadchawan, 2015; Saharan *et al.,* 2016; Deshpande *et al.,* 2017).

Más específicamente, el quitosano produce efectos positivos en las plantas debido a que induce la actividad de enzimas involucradas en el metabolismo oxidativo como lo son superóxido dismutasa, peroxidasa y catalasa. Además de que aumenta los niveles de clorofila y mejora actividad fotosintética. También se le atribuyen propiedades benéficas en situaciones de escasez de agua debido a que induce el cierre estomal reduciendo así la transpiración y por ende la perdida de agua.

Otro efecto positivo que el quitosano produce en las plantas es la capacidad de activar los genes de defensa de esta mediante la ruta del octadecanoide aumentando la síntesis de ácido jasmónico, compuesto clave para las plantas para protegerse de ataques de patógenos, principalmente insectos (Pichyangkura y Chadchawan, 2015; Kashyap *et al*., 2015).

Es probable que los efectos benéficos que se le atribuyen al quitosano, sobre el desarrollo de las plantas, se deban a un posible rol como acarreador de nutrientes y a su poder de quelatación de metales. Este fenómeno se presenta debido a que la estructura de este compuesto facilita la unión de iones metálicos mediante intercambio iónico, formación de complejos y un encapsulamiento intra e intermolecular (Liu *et al.,* 2014; Vasconcelos, 2014; Deshpande *et al.,* 2017).

1.3. Hipótesis

La aplicación foliar de nanofertilizantes incrementará el contenido de zinc, la calidad y el desarrollo fisiológico de las plantas de frijol, en comparación con una fuente comercial de zinc.

La adición foliar de quitosano mejorará la absorción de nutrientes y ayudará a la asimilación de zinc en sus distintas formas acarreando un mejor desarrollo fisiológico en la planta.

1.4. Objetivo General

Biofortificar, promover el desarrollo y aumentar la calidad nutrimental del frijol mediante la aplicación foliar de nanopartículas de zinc más quitosano.

1.5. Objetivos Específicos

1. Evaluar la aplicación foliar de nanopartículas de zinc más quitosano con relación al rendimiento y el desarrollo fisiológico de las plantas de frijol ejotero.

2. Comparar el uso de nanopartículas de Zn contra un fertilizante a base de Zn ampliamente reconocido en el mercado en plantas de frijol ejotero.
3. Observar el efecto de la aplicación foliar de nanopartículas de zinc más quitosano sobre las variables relacionadas con el metabolismo nitrogenado de las plantas de frijol ejotero.
4. Evaluar el efecto de la aplicación foliar de nanopartículas de zinc más quitosano en el contenido de zinc, grado de biofortificación y patrón de distribución de este elemento en plantas de frijol ejotero.
5. Cuantificar el contenido mineral de plantas de frijol ejotero bajo la aplicación de nanopartículas de zinc más quitosano.
6. Estudiar el efecto de la aplicación de quitosano, en combinación con las distintas fuentes de Zn sobre el desarrollo de plantas de frijol.

1.6. Sección Integradora

1.6.1. Artículo 1. Biofortificación con Zinc: Uso de Nanopartículas como Alternativa para mejorar la Calidad Nutricional de los Cultivos.

Artículo de revisión donde el objetivo general fue describir la acción de las nanopartículas en trabajos de biofortificación, haciendo énfasis en el uso de nanopartículas de Zn y sus efectos en las plantas. Este capítulo nos permite abordar el tema de la biofortificacion y su importancia para solucionar los problemas de la deficiencia de micronutrientes en la población, además nos permite conocer el motivo por el cual el uso de la nanotecnología se presenta como una opción novedosa y sustentable para futuros trabajos de biofortificación.

1.6.2. Artículo 2. Uso de Compuestos Bioestimulantes en la Agricultura: el Quitosano como Opción Sustentable para el Desarrollo de Plantas.

Artículo de revisión en el cual el objetivo general fue describir el rol de los bioestimulantes en la agricultura haciendo énfasis en el uso del quitosano y sus efectos en las plantas, además de la relación e interacción que presenta con micronutrientes claves en la nutrición vegetal como el hierro y particularmente el zinc. Este capítulo de revisión nos permite sentar las bases fisiológicas y bioquímicas del uso del quitosano y su actual papel como un potencial bioestimulante que coadyuva a la biofortificación con micronutrientes en cultivos agrícolas de interés alimenticio.

1.6.3. Artículo 3. Impacto de la Aplicación Foliar de Nanopartículas de Zinc más Urea y Quitosano sobre la Asimilación de Nitrógeno, Actividad Fotosintética y Producción de Frijol Ejotero.

Artículo de investigación que corresponde al cumplimiento del objetivo general y específicos número 1, 3 y 6, en el cual se evaluó la eficiencia de la aplicación foliar de nanopartículas de óxido de zinc contra nitrato de zinc acomplejado con quitosano sobre la asimilación de nitrógeno, actividad fotosintética y producción de frijol cv. Strike. Los resultados obtenidos indican que la aplicación foliar de nanopartículas de Zn a 25 ppm y nitrato de zinc a 50 ppm fueron las dosis más eficientes para favorecer la acumulación y producción de biomasa. La adición de quitosano favoreció la biomasa, la producción y los parámetros relacionados con la fotosíntesis, especialmente cuando se combinó con nitrato de zinc mientras que la combinación con nanopartículas de ZnO presentó una posible quelación de las nanopartículas, retrasando la liberación de Zn. Finalmente, el mejor tratamiento con nanopartículas fue ZnO a 25 ppm, que tuvo resultados similares al tratamiento con 50 ppm de NZN. Esto indica que las nanopartículas de ZnO pueden reducir la cantidad de fertilizante que se utilizará sin afectar el rendimiento de los cultivos.

1.6.4. Artículo 4. Biofortificación con Nanopartículas y Nitrato de Zinc más Quitosano en Frijol Ejotero: Efectos en el Rendimiento y Contenido Mineral

Artículo de investigación que corresponde al cumplimiento del objetivo general y específicos número 2, 4, 5 y 6. Se evaluó el contenido de zinc (biofortificación) en fruto y su patrón de distribución en la planta, además del contenido mineral y contenido de proteína. Los resultados obtenidos indican que la aplicación de zinc favoreció el proceso de biofortificación, encontrando incrementos para todos los tratamientos utilizados. Los tratamientos que destacaron fueron nitrato de zinc más quitosano a 50 y 100 mg kg^{-1}, que incrementaron el contenido de zinc en frutos en más del 110%. La aplicación de nanopartículas de Zn a 25 mg kg^{-1} y de nitrato de zinc a 50 mg kg^{-1} favoreció la acumulación y producción de biomasa. Además, la adición de quitosano ayudó a la biomasa y al rendimiento, especialmente cuando se combinó con nitrato de zinc.

2. BIOFORTIFICATION WITH ZINC: USE OF NANOPARTICLES AS AN ALTERNATIVE TO IMPROVE THE NUTRITIONAL QUALITY OF CROPS

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Biofortification with zinc: use of nanoparticles as an alternative to improve the nutritional quality of crops.

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Abstract

About 12% of the world's population was affected by severe food insecurity in 2020 and according to United Nations estimates, the world's population will increase by approximately 2.2 billion by 2050. An estimated 33% of the world's population is affected or at risk of zinc (Zn) deficiency. It has been reported that lower than recommended levels of Zn intake can cause problems in pregnancy development, growth in children, immune system related diseases, as well as poor neurobehavioral development. An innovative strategy to the problem of micronutrient deficiency in the diet is biofortification, which has been defined as the process of increasing the bioavailable concentrations of essential elements in edible portions of cultivated plants through agronomic management. The use of nanotechnology, in the form of nanofertilizers, emerges as an option to achieve optimal plant development in a sustainable and precise manner; given their size, plants can absorb them with a different dynamic. It has been proven that the use of this technology, applied foliarly, improves fruit

quality, increasing the absorption, translocation and accumulation of Zn in the fruit, which makes it a novel tool for biofortification programs. The general objective of this review is to describe the action of nanoparticles in biofortification works, emphasizing the use of Zn nanoparticles and their effects on plants.

Key words: *nanotechnology, micronutrients, sustainability, food safety.*

1. Introduction

About 12% of the world's population was affected by severe food insecurity in 2020 and according to United Nations estimates, the world population will increase approximately 2.2 billion by 2050 (FAO *et al.*, 2021). In addition, the problem of malnutrition affects one in three people, of which the main problems they suffer from are: wasting, stunting, underweight, and micronutrient deficiencies (WHO, 2021).

Micronutrients are essential elements for the formation of healthy bones, brains and bodies, which are required in small amounts. These nutrients should be obtained from food, ideally from a balanced diet. Deficiencies in iodine, iron and zinc are the most important problems and pose a threat to the health and development of populations worldwide, particularly for children and pregnant women in underdeveloped countries (Dahdouh *et al.*, 2019; WHO, 2018).

In the case of zinc (Zn), it is estimated that 33% of the world's population is affected or at risk of suffering from a deficiency of this microelement. It has been reported that levels of Zn intake below the recommended levels can cause problems in the development of pregnancy, growth in children, diseases related to the immune system, as well as poor neurobehavioral development. In turn, severe Zn deficiency causes pustular dermatitis, alopecia, chronic diarrhea, emotional disorder, weight loss, recurrent infections, hypogonadism in men, neuro-sensory disorders and difficulty in ulcer healing; problems that could become fatal if not recognized and treated in time (Dahdouh *et al*., 2019; Sida-Arreola *et al*., 2017; Prasad, 2012; Wessells *et al*., 2012).

An innovative strategy to the problem of micronutrient deficiency in the food diet is "biofortification", which has been defined as the process of increasing the bioavailable concentrations of essential elements in edible portions of cultivated plants through agronomic management (Pachón, 2010; White and Broadley, 2005). The exogenous application of such nutrients through fertilization is necessary to carry out the process of increasing the concentration of minerals in plant tissues (Blasco, 2015).

Currently, the main objective of agricultural production systems is to increase the profits of farmers and agricultural industries, leaving product quality and human health issues in the background (Bulgari et al., 2015). In addition, resources such as water and soil are becoming increasingly scarce, as well as climate change which is progressively more severe and frequent, aggravating the situation (Singh and Prasad, 2014; Rouphael and Colla, 2018). The use of nanotechnology, in the form of nanofertilizers, emerges as an option to achieve optimal plant development in a sustainable and precise manner; given their size, plants can absorb them with a different dynamic, which presents an additional advantage. Several studies suggest that the use of nanofertilizers increases by 20-30% the yield and nutritional quality of plants in relation to conventional fertilizers (Raliya *et al*., 2017; Kah *et al*., 2018).

Applications of Zn nanoparticles, at low concentrations, have been shown to have positive effects on germination, vegetative growth, photosynthetic pigment content and significant increases in yield (Rossi et al., 2019; Siddiqui et al., 2019). It has been proven that the use of this technology, applied foliarly improves fruit quality, increasing the absorption, translocation and accumulation of Zn in these, which makes it a novel tool for biofortification programs (Subbaiah *et al.*, 2016; Davarpanah *et al.*, 2016; El-Ramady *et al.*, 2018). Based on the above, the general objective of this review is to describe the action of nanoparticles in biofortification works, emphasizing the use of Zn nanoparticles and their effects on plants.

2. Malnutrition

The word malnutrition refers to nutritional imbalances and deficiencies or excesses in a person's caloric intake, causing decreased physical and mental activity that in the long term deteriorates the clinical condition of the individual (WHO, 2021; Cederholm *et al.*, 2015).

The concept of malnutrition encompasses both undernutrition and overweight and obesity disorders which, despite manifesting as distinct physical problems, various studies indicate that they share symptoms such as altered metabolism, chronic inflammation and intestinal dysfunction. There is also a correlation between excessive energy and macronutrient intake and micronutrient deficiencies in overweight individuals. In addition, early undernutrition increases the risk of obesity in adulthood leading to clinical problems such as high percentage of adipose tissue, high circulating levels of triglycerides and high blood pressure (WHO, 2021; Bourke *et al.*, 2016).

Globally, malnutrition affects more than 2 billion people around the world, with women and children being the sector of the population with the most alarming data. It is estimated that, in 2020, 29.9% of women aged 15 to 49 years will suffer from anemia, while 22% of children under five years of age will be stunted, 6.7% will be wasted and 5.7% will be overweight (FAO *et al.*, 2021). UNICEF (2021) reports that 2 out of every 3 children between 6 and 23 months do not have a sufficiently balanced diet for healthy growth, and only 52% of children under 2 years of age have access to the recommended frequency of meals. Potential causes of malnutrition include poor nutrition, mental health problems, mobility problems, digestive disorders, stomach disorders and alcoholism (Ahmed *et al*., 2022).

Despite global efforts to address food insecurity, malnutrition continues to be a growing problem and is a key factor in increased infectious mortality, neurological disability, and limited mental and physical growth. In turn, it has been linked to an increase in cardiovascular disease, cancer, stroke, and diabetes (FAO *et al*., 2021; Wakeel *et al*., 2018). More recently, malnutrition has been associated with an increased risk of SARS-CoV-2 infection and people with this condition present a more severe clinical picture, reaching a higher number of hospitalizations requiring mechanical ventilation. In addition, the side effects of SARS-CoV-2 could be leading to malnutrition or impairments in related physiological processes, as a higher incidence of malnutrition was found in older adults with severe SARS-CoV-2 infection (Kurtz *et al*., 2021; Huizar *et al*., 2020).

2.1 Micronutrient deficiency

The concept of malnutrition refers to three major conditions, the most studied being undernutrition and obesity; however, there is also malnutrition related to micronutrient imbalances, which generally has invisible health implications and is therefore called "hidden hunger", since its development is progressive over time and its effects are not noticeable until it has caused irreversible damage (Praharaj *et al*., 2021; WHO, 2021, UNICEF, 2021b).

Although humans require micronutrients in minimal amounts, approximately 3 billion people are deficient in micronutrients, making it a problem with a negative global socioeconomic impact. The problem mainly affects women, children, and the elderly, especially those in low-income countries whose diet is cereal-based or poorly balanced (Kiran *et al.*, 2022; Praharaj *et al.*, 2021; Peña-Rosas *et al.*, 2019). However, micronutrient deficiency is not only caused by incorrectly chosen diets or limited access to nutrient-rich foods, but also by altered metabolism leading to changes in nutrient absorption, distribution or excretion because of systemic inflammation caused by obesity. This problem occurs mainly in developed countries where people obtain more than 30% of their daily calories from nutrient-poor foods (Kobylińska *et al.*, 2022).

Among the characteristic symptoms of micronutrient deficiency are anemia, blindness, birth defects, growth retardation, cognitive delay, immune weakness and constant infectious diseases, which are the main causes of morbidity and mortality in low-developed countries (Peña-Rosas et al., 2019; Tulchinsky, 2010). In turn, Song et al. (2015), relate micronutrient deficiency with a higher rate of suffering from depression, finding that people suffering from this problem suffer from anxiety and chronic sadness.

Deficiencies in nutrients such as iron and vitamin B12 generate an important problem such as anemia, which in addition to affecting a significant number of women of childbearing age, is estimated to reduce the mental and work capacity of people by 20%, and increases the risk of maternal mortality, premature births and low birth weight. Also, iron deficiency during the first years of life has been linked to problems in growth, neurological development, cognitive performance and an increased risk of contracting infections (Peña-Rosas et al., 2019; Wakeel et al., 2018; Ritchie and Roser, 2017).

Vitamin A deficiency is the main cause of preventable blindness in children, but progresses to permanent blindness in more severe cases, also in lactating women and infants it potentiates serious infections increasing mortality rates. Furthermore, this deficiency has been linked to epithelial defects in the respiratory, urinary, gastrointestinal tracts and glandular ducts, causing alterations in organ growth and development and thyroid dysfunction (Timoneda et al., 2018; Ritchie and Roser, 2017). Whereas iodine deficiency is the main cause of brain damage in children, it is also related to thyroid problems and can cause a loss of about 13 IQ points, generating mental retardation (Yadav and Pandav, 2018). There are also other essential nutrients that cause various problems in human health such as folic acid and zinc (Ritchie and Roser, 2017).

2.1.1 Zinc deficiency in humans

Zn is an essential nutrient for almost all organisms and has a fundamental role as a metal cofactor in more than 300 proteins within the human body. In addition, it plays an essential role as a stabilizer and receptor for steroid hormones, thyroid hormones, retinoids and cell membranes. It also acts as a regulator and is essential for the synthesis of biomolecules such as DNA and binds to nuclear proteins and forms complexes called "zinc fingers". These functions of Zn are related to its ability to bind to histidine and cysteine, stabilize active sites and intervene in maintaining the integrity of the immune system (Taboada-Lugo, 2017; Caro *et al*., 2016).

The adult requirement for Zn is 11 mg day^{-1} and it is present in the human body in an amount of 2 to 3 g and is second only to iron as the most abundant micronutrient. Zinc deficiency is associated with impaired immune responses and leads to an increased risk of respiratory viral infections, particularly in the elderly. In addition, it generates a decrease in antibody production through altered immune cell function and number (Gorji and Ghadiri, 2020). This problem is very common in countries where the diet is unbalanced, mainly in places with cereal-based diets and low protein intake (Bilski *et al.*, 2012). Zinc deficiency, unlike anemia, can occur interchangeably in men, women and children. In 2014, approximately 33% of the world's population was at risk for such deficiency, accounting for 5% of deaths in children under 5 years of age in developing countries, representing the fifth leading cause of disease and mortality (Reed *et al.*, 2015; Singh and Prasad, 2014).

This deficiency can affect the central nervous, gastrointestinal, immune, epidermal, reproductive and skeletal systems. It has been reported that Zn levels below the recommended levels can cause problems in the development of pregnancy and growth in children, difficulties with the immune system, as well as poor neurobehavioral development. In turn, a severe deficiency of this microelement causes pustular dermatitis, alopecia, chronic diarrhea, emotional disorder, weight loss, recurrent infections, hypogonadism in men, neuro-sensory disorders and ulcer healing problems, conditions that could become fatal if not recognized and treated in time (Dahdouh *et al.*, 2019; Sida-Arreola *et al.*, 2017; Prasad, 2012; Wessells *et al.*, 2012).

Zinc is considered essential in recovery after any illness or injury, so deficiencies of this element can increase the time of this process, due to increased susceptibility to diseases and infections. In addition to the fact that it can reduce mental capacity and increase the prevalence of maternal, neonatal and infant complications (Ritchie and Roser, 2017). Recent studies place Zn supplementation as an alternative to decrease the effects caused by SARS-CoV-2 infections, finding that Zn enhances the antiviral potential in mammalian cells by improving the immune system (Sharma *et al.*, 2021; Joachimiak, 2021).

3. Biofortification: History and advances

An innovative strategy to the problem of micronutrient deficiency in the diet is biofortification. Although there is still no consensus on the term, biofortification has been defined as the process of increasing the bioavailable concentrations of essential elements in edible portions of cultivated plants through agronomic management (fertilization) or genetic improvement (Gupta *et al*., 2021; Velu *et al*.,2014; White and Broadley, 2005). Biofortification of staple crops is a recent strategy, in addition to others such as food fortification through industrial processes, the addition of supplements at the time of consuming a food or consuming a balanced diet (Pachón, 2010; FAO, 2014). Through its different alternatives, biofortification attempts to be a tool that seeks to improve the nutritional content of staple foods consumed mainly by low-income people, providing a medium- and long-term solution to the serious problem of malnutrition suffered by these populations, and at the same time it is presented as a relatively inexpensive, profitable and sustainable solution (Bouis, 2018a; Bouis *et al*., 2011).

Since 2004, the HarvestPlus project, coordinated by the International Food Policy Research Institute (IFPRI) and the International Center for Tropical Agriculture (CIAT), has been conducting programs aimed at increasing the nutrient content of staple crops in developing countries (FAO and HarvestPlus, 2019; Pachón, 2010). In 2008, expert economists ranked biofortification among the most cost-effective and sustainable solutions to address the global issue of poor nutrition and the problems it causes. Also in 2016, the World Food Prize was awarded to Howarth Bouis of the HarvestPlus organization and Maria Andrade, Robert Mwanga and Jan Low of IPC (International Potato Center), for their work related to food biofortification (Andersson *et al*., 2017; Bouis, 2018b).

According to the World Bank, biofortification programs have proven to have excellent cost-benefit, finding that for every $1 invested in biofortification, up to $17 in benefits can be realized, since for most crops only a one-time investment is required for the propagation of varieties with the elevated nutrient levels for this to become self-sustaining. However, a concern for markets could be changes in colorations and image of biofortified fruits so awareness campaigns among the population are required (Bouis, 2018b; De Steur *et al.*, 2017).

For biofortification to have an impact on society, it is necessary to thoroughly evaluate the crop to be used, because the amount of nutrient that can be increased depends on it, as well as the population that will be benefited and the access to such product. A biofortifiable crop is one that has high yields, tastes good, is easy to cook and is considered a good source of the nutrient to be biofortified. Crops such as rice (*Oryza sativa* L.), sweet potato (*Ipomoea batatas* L.), beans (*Phaseolus vulgaris* L.), maize (*Zea mays* L), wheat (*Triticum* spp.) or cassava (*Manihot esculenta* L.) are of great importance in the diet of most developing countries, so the focus of biofortification has centered on studies on these crops (Table 1) (Ghosh *et al.*, 2019; De Steur *et al.*, 2017; Pachón, 2010).

Crop	Biofortification from	Results	Reference
Rice	Genetic modification with overexpression of ferritin, NAS and OsYSL2.	The Fe concentration of the modified seeds increased up to six times in the greenhouse crop and 4.4 times in the field crop.	Masuda *et al.*, 2012
	Genetic modification by OsVIT gene silencing.	Increased iron translocation and a 1.8-fold increase in grain Fe concentration.	Bashir *et al.*, 2013

	Foliar and soil application of Zn.	Yield increases of 7% and grain Zn content increases of 58 to 176%.	Gómez-Coronado *et al.*, 2016
	Foliar application of selenium (Se) in the form of selenite and selenate.	Se content in the fruit increased up to 884% without yield change in different rice cultivars.	Lidon *et al.*, 2019
Bean	Genetic improvement through the crossing of varieties.	Obtaining varieties with greater storage of Fe in grain, reaching up to 99 ppm per gram.	Blair and Izquierdo, 2013
	Application of silicon to the nutritive solution through fertirrigation.	Significant increases in the content of silicon in the fruit both in its raw and cooked form.	Montesano *et al.*, 2016
	Foliar application of Zn sulfate in 31 different production locations.	14.7% increase in Zn content in grains.	Ram *et al.*, 2016
	Addition of Iodine (I) in the nutritive solution applied in the form of fertigation.	Increase of 0.6 ppm of I in the fruit and without affectation in the development of the plant.	Dobosy *et al.*, 2020
	Edaphic and foliar application of Zn	The results indicate that the foliar application increases the concentration of Zn in a better way than the edaphic one, while the latter helps in the increase of amino acids and proteins.	Kachinski *et al.*, 2022
Wheat	Foliar application of Fe citrate, Fe sulfate and Zn sulfate	Increases in the content of Fe and Zn in the treatments applied both in grain and in flour. The increase in Zn being more promising.	Zhang *et al.*, 2010

	Inoculation with *Providencia sp.* *Anabaena sp* and *Calothrix sp.*	Increase in the content of protein, Fe, Mn and Cu in grain.	Rana *et al.*, 2012
	Foliar and edaphic application of different types of fertilizers with Se.	Increases in the concentration of Se in grains, especially when it was applied in an edaphic way and without another nutrient.	Ramkissoon *et al.*, 2019
	Mn application in the form of seed coverage, priming, soil application and foliar application.	The content of Mn increased when it was applied in a foliar way in 29% in relation to the control without application.	Zulfiqar *et al.*, 2021
Maize	They used a partial diallel mating design to develop 36 maize lines with provitamin A production.	They found that the development of hybrid varieties may be the appropriate approach for biofortification programs related to carotenoid content.	Halilu *et al.*, 2016
	Foliar application of Zn in different forms.	Increase in growth, yield and zinc content in grain.	Subbaiah *et al.*, 2016
	Edaphic application of N, Fe, Zn and Se.	Increase in the content of the four elements applied without finding significant effects on biomass production.	Grujcic *et al.*, 2021

Table 1. Biofortification with micronutrients in staple crops.

3.1 Types of biofortification

Several studies indicate that the main forms of biofortification are genetic improvement and agronomic management. The former includes conventional plant breeding, the application of molecular tools such as gene editing and even the use of genetically modified organisms.

Generally, the institutions in charge of carrying out biofortification programs prefer the use of traditional breeding techniques, using the crossing of varieties until the expected results are obtained, as they consider that this is the simplest way to bring biofortified products to consumers, since it does not have the same regulations as the use of more modern techniques (Cakmak and Kutman, 2018; Bouis and Saltzman, 2017).

One of the most promising techniques is genetic modification, which is an alternative when conventional breeding does not achieve the proposed objectives. This technique focuses on achieving greater absorption of the desired nutrient, increased translocation from the leaf or root to the grain, specialization of storage in the endosperm, reduction of compounds that interfere with the absorption of the nutrient, and increased bioavailability at the time of consumption (Ludwig and Slamet-Loedin, 2019). Several studies report the efficiency of this technique, reaching the desired biofortification levels for nutrients such as Zn, Fe, vitamin A, protein and selenium in crops such as rice, wheat, among others. However, its use presents many questions as it faces numerous regulatory processes and biosafety standards (Saini *et al*., 2020; Ludwig and Slamet-Loedin, 2019; Bouis and Saltzman, 2017).

For its part, agronomic improvement consists of applying various sources of fertilization or beneficial compounds through foliar or edaphic application (Figure 1), being mainly used when dealing with micronutrients (Bouis and Saltzman, 2017), likewise, it is a simple tool to apply and is very cost-effective, besides being the most effective when there are problems in the soil that limit the absorption and mobility of nutrients. Agronomic management potentiates its effects depending on the method and timing of application, agroclimatic conditions and crop variety. Also, it has the advantage that in addition to increasing the content of a nutrient, it can increase crop yields, so it is considered a promising technique and a short-term solution to the problem of poor food quality of staple crops (Praharaj *et al*., 2021; Ludwig and Slamet-Loedin, 2019).

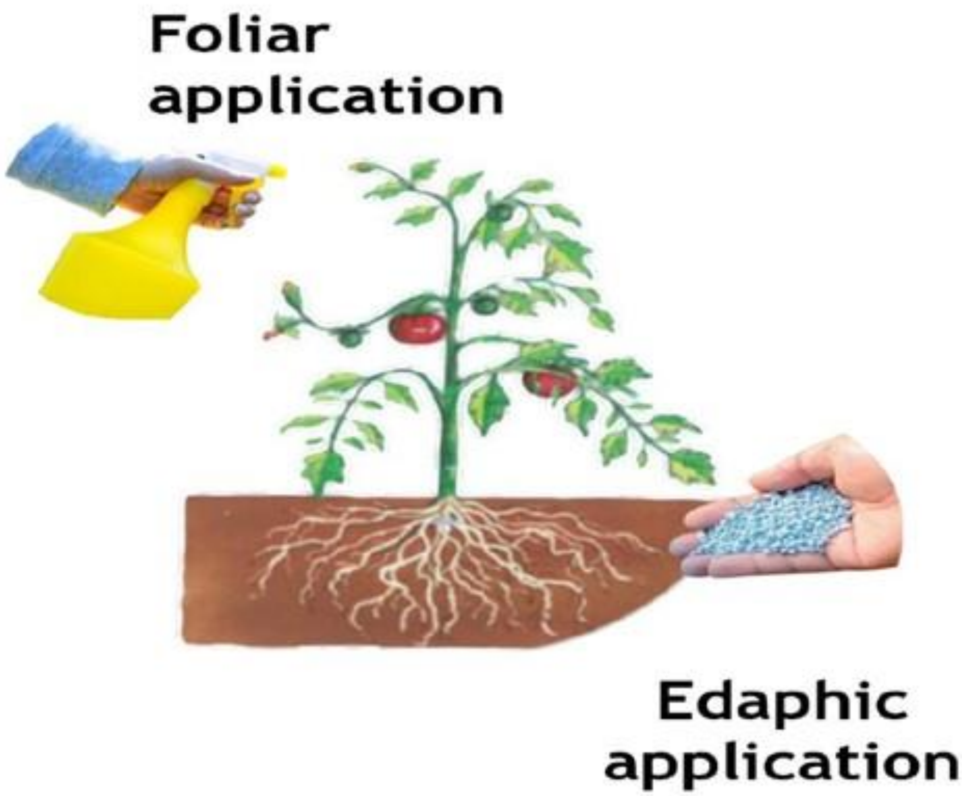

Figure 1. Forms of fertilization used in agronomic biofortification.

In recent years, micronutrient deficiency has become very important worldwide, and various strategies to improve the nutritional quality of crops have focused on it. Key micronutrients for human nutrition such as Fe, Zn, I and Se, have been the subject of study because they are the main deficiency problems in the diet, especially in resource-poor populations (Jha, and Warkentin, 2020; Wakeel *et al*., 2018). Biofortification, because of its advantages and benefits has gained momentum in the last decade, finding a balanced way to overcome deficiencies of these micronutrients (Jha and Warkentin, 2020).

3.2 Biofortification with zinc

A quarter of the world's population relies on diets containing predominantly cereals, legumes and seeds, which do not provide the necessary amount of Zn for proper physiological development (Ramesh *et al*., 2014; Bilski *et al*., 2012). Zinc accumulation in edible portions of plants is highly variable depending on the species and is frequently reduced by the environmental factors to which the crops are exposed. In addition, it may depend on the interactions that may occur with other nutrients; for example, negative correlations have been found between Fe and Zn content in sorghum grains, and negative correlations between P and Zn content were also found in wheat (White and Broadley, 2009; McDonald *et al*., 2008).

During the last 10 years, organizations worldwide have carried out work in more than 12 countries, with the objective of increasing the concentration of zinc in edible portions of important staple crops in each region. The greatest response was found in wheat grains, with an 83% increase in this nutrient, followed by rice with 27%, and corn with the lowest response, with a 9% increase. In the case of beans, in plants that were applied Zn, a substantial increase in its content in the fruit was obtained, in addition to the fact that they discovered that proteins are the ones that store zinc in the grain (Cakmak and Kutman, 2018; Ram *et al*., 2016).

4. Nanotechnology: History and concepts

Nanotechnology is defined as "the design, synthesis, characterization and application of materials, devices and systems, while controlling size and shape at the nanoscale". It can also be defined, as the practical application of the theory of nanoscience, which is defined as the study of materials and/or substances with a dimension between 1 and 100 nm (Rafique *et al*., 2020). In consensus, nanotechnology is an emerging engineering discipline that applies nanoscience methods to create usable, marketable, and economically viable products by manipulating and characterizing materials between molecular and micrometer size. The characteristics offered by nanoparticles are attributed to the principles of quantum mechanics and the combination of multiple scientific disciplines, including biology, physics, chemistry, medicine, and engineering (Bhagyaraj and Oluwafemi, 2018; Sudha *et al.*, 2018).

The history of nanotechnology dates to Mesopotamian and Roman civilizations, which used gold, silver and copper nanoparticles in the vessels and cups they manufactured with the purpose of giving a shiny effect on the surface and making the color change when exposed to light, this fact was confirmed until 1990 when scientists subjected these vessels to analysis with a transmission electron microscope (Bayda *et al.*, 2019; Sudha *et al.*, 2018). Whereas, Englishman John Utynam, in 1449, patented glass based on gold nanoparticles. In the 16th century, the Swiss physician Theophrastus von Hohenheim used gold nanoparticles to treat patients suffering from various ailments. The Austrian chemist Richard Zsigmondy, winner of the Nobel Prize for chemistry in 1925, was the first to introduce the term nanometer in his research on the measurement of particles such as colloidal gold (Rafique *et al.*, 2020).

The concept of nanotechnology was imagined by the American physicist and Nobel laureate Richard Feynman in 1959, considered the father of nanotechnology, he described a vision of using machines to build smaller machines and so on down to the molecular level. Fifteen years later, Japanese scientist Norio Taniguchi was the first to define the concept of nanotechnology to refer to the separation, consolidation and deformation processing of materials by an atom or molecule. Another important point in the history of nanotechnology was the introduction of the concepts of molecular nanotechnology by the American scientist Eric Drexler in 1977, in addition to the invention of the tunneling microscope in 1981, by physicists Gerd Binnig and Heinrich Rorher at the IBM Zurich research laboratory, from this the growth of this emerging science was exponential (Bayda *et al.*, 2019; Bhagyaraj and Oluwafemi, 2018).

The advantages offered using nanotechnology compared to other technologies are typically since nanoparticles have a larger surface area by weight than larger particles, which makes them more reactive with other molecules. These properties have allowed the use of this technology in various fields such as energy storage, water and contaminated soil treatment, food protective films, nanocarriers that work for greater efficiency in drug intake, among many other functions that allow its application in the fields of medicine, mining, electronics, computing, robotics, in resource conservation processes, energy generation and in the pharmaceutical, food industries, among others (Chakraborty *et al.*, 2020; Pathakoti *et al.*, 2018).

4.1 Nanotechnology in agriculture

In the past, agriculture was the center of attention for the application of new technologies, being the predecessor of industrial revolutions for more than 90 centuries, however, in the case of nanotechnology, its benefits have been used in the industrial field and in the health sciences for more than half a century; whereas, in agriculture, research on its potential benefits is still minuscule, since less than 5% of the total research related to this topic pertains to the agricultural area. Despite that, the upward trend suggests that in the coming years the application of nanoparticle-based products in agricultural fields will be a regular practice (Mukhopadhyay, 2014; Ditta, 2012).

Nanotechnology emerges as an alternative, because as an emerging science it is aimed at understanding and creating materials, devices and systems that exploit the nanoscale properties of various compounds. In agriculture, nanotechnology has begun to develop products that help improve soil fertility, reduce diseases and increase crop quality and production, moving from conventional agriculture to precision agriculture. Examples such as carbon nanotubes, Cu, Ag, Mn, Mo, Zn, Fe, Si, Ti, or in their oxide forms, in addition to nanoformulations of phosphorus, urea, sulfur; are considered as the future sources of agricultural fertilization (Chhipa, 2017; Solanki *et al.*, 2017).

4.2 Nanofertilizers

Crop fertilization is of utmost importance when producing food, however, the low effectiveness that they present to be used by crops, reaching less than 25%, in addition to their excessive application, has a negative environmental impact causing soil deterioration, contamination in aquifers and air pollution, recent studies focus on finding less harmful alternatives for the environment, without affecting the quality and production of crops (Raliya *et al.*, 2017; Naderi and Danesh-Shahraki, 2013). In the last decade, research on nanofertilizers has grown exponentially, being the target of various communities such as ACS, SETAC and Crop Chemical Europe, as well as regulatory institutions such as the USDA, causing great impact on the scientific community; however, in the industrial branch there is very little information on nanotechnology-based products, being nanoparticle-based pesticides the most common products on the market, but it is still considered as a dream not too far away (Kah, 2015).

Nanotechnology offers different aspects with potential application in different fields of agriculture and biotechnology to solve environmental problems and biological demands with greater precision. The use of these nanofertilizers causes an increase in their efficiency, reduces soil toxicity, minimizes over-application and its potential toxic effects. Thus, there is an opportunity for nanotechnology to have a significant influence on energy, economy and environment (Naderi and Danesh-Shahraki, 2013).

Nanofertilizers are all those particles that contain nutrients and whose dimension is less than 100 nm, preferably between 30 and 40 nm. In addition, within their characteristics, a very high contact surface of the nutrient ion and a slower and more precise release than traditional fertilizers stand out, this is due to the reduction of the particle size, which causes the specific surface to increase and, as a consequence, the contact area with the plants as well, with this the plants can absorb them with a different dynamic, which presents an additional advantage compared to traditional sources of fertilization (Subbaiah *et al.*, 2016; Subramanian *et al.*, 2015).

Nanofertilizers are classified into three categories: 1) nanofertilizers based on macronutrients, 2) nanofertilizers based on micronutrients and 3) nanomaterials that act as nutrient carriers. Several studies compare the effects of different categories of nanomaterials against traditional fertilizers, obtaining an average efficacy of nanofertilizers over conventional fertilizers of 19, 18 and 29% for categories 1, 2 and 3, respectively (Kah *et al.*, 2018). Because of this, nanoformulations of active ingredient agricultural inputs could cause a significant reduction in environmental problems caused by over-fertilization such as eutrophication. The use of nanofertilizers such as Ca nanoparticles, P-hydroxyapatite, Fe+2, ZnO, TiO2, Ag and carbon nanotubes can be used as an alternative to conventional agricultural inputs (Kah *et al.*, 2018; Chhipa, 2017).

Despite the promising outlook for the use of nanofertilizers, the studies that have been presented so far are not yet sufficient to determine that the use of this new technology is superior to traditional fertilizers, in addition to the fact that studies should also focus on establishing the effects generated by these materials on plant development (Kah *et al.*, 2018; Subramanian *et al.*, 2015).

4.2.1 Zinc nanoparticles

There are several forms of Zn-containing nanoparticles, such as ZnS and ZnSe, or CdSe/ZnS quantum dots, which are currently used in various fields of science. However, the most widely used form of zinc as nanoparticles continues to be zinc oxide (ZnO), due to its wide range of positive properties, such as high electrical and thermal conductivity, high temperature stability and neutral pH, as well as mild antimicrobial effects. Also, its use is related to easy availability and low procurement price (Sturikova *et al.*, 2018). A production of close to 1000 tons per year of ZnO is estimated, which are commonly used in plastic, glass, ceramic, cement and rubber materials, as well as in pigments, paints and sunscreens (Piccinno *et al.*, 2012). In agriculture, applications of Zn nanoparticles, at low concentrations, have been shown to have positive effects on germination, vegetative growth, chlorophyll content and significant increases in yield (Rossi *et al.*, 2019; Siddiqui *et al.*, 2019).

It has been proven that the use of this technology, applied foliarly, improves fruit quality, increasing the absorption, translocation and accumulation of Zn in fruits, making it a novel tool for biofortification programs (Subbaiah *et al.*, 2016; Davarpanah *et al.*, 2016; El-Ramady *et al.*, 2018). On the other hand, applications at high concentrations have stressful effects that can inhibit root growth, delay crop development, and affect protein and DNA synthesis (Ma *et al.*, 2015; Sturikova *et al.*, 2018).

5. Biofortification with nanofertilizers

Despite the positive results with biofortification work, there is a serious problem caused by the excess fertilization required to reach the levels of the nutrient to be biofortified. Among the most common problems caused by excess fertilization are an over-accumulation of heavy metals in soil and water, eutrophication and soil degradation. In addition, the success of biofortification depends on the form, uptake, mobilization and accumulation of the nutrient, so there is a need for alternative, sustainable and accurate strategies to deliver the nutrient to the plant (Khan *et al*., 2021; Kulkarni, and Goswami, 2019).

Modern technologies, such as nanotechnology, emerge as an efficient alternative for increasing production and improving food quality, being a technique that can be progressively applied for biofortification programs (Solanki and Laura, 2018). Nanotechnology in the form of nanofertilizers, as mentioned above, present advantages over the application of traditional fertilizers, finding that in addition to increasing yield, it increases the activity of nitrogen metabolism, helps to mitigate stress situations, an increase in the photosynthetic rate and above all, several studies have found a greater translocation of the nutrient from the roots and leaves to the grain (Khan *et al*., 2021; Lv *et al*., 2019; Zulfiqar *et al*., 2019).

Biofortification projects are successfully using three main forms of nanofertilizers: I) nanoscale coating fertilizers, in which conventional fertilizers are enclosed in nanoparticles or intercalated in nanopores of zeolites and/or nanometer clays to increase nutrient availability, postpone release, or supplement with an extra nanoscale element; II) fertilizers with nanoscale additions, in which traditional fertilizers are supplemented with nutrient nanoparticles and III) nanoscale fertilizers, which are nanoparticles, less than 100 nm in size that contain nutrients and are used as fertilizer directly (Khan *et al*., 2021).

In addition to the forms of nanofertilizers, the manner of application greatly influences the achievement of biofortification. There are mainly three methods of application, which are seed priming, soil application and foliar application. Also, the chemical composition, stability, concentration and size of the nanoparticles must be considered for nutrient absorption, translocation and accumulation to take place. Numerous studies indicate that, like traditional biofortification, biofortification with nanoparticles focuses mainly on the application of micronutrients, such as Zn, Fe, I, Cu and Se. Among these micronutrients, zinc stands out, which presents the largest number of works in biofortification programs (Dhaliwal et al., 2022; El-Ramady et al., 2021; Szerement et al., 2021).

5.1 Biofortification with zinc nanofertilizers

In recent years, the use of Zn nanoparticles to improve plant uptake has become more popular. This is because decreasing the particle size results in a higher number of particles per unit of Zn applied, in addition to increasing the specific surface area of a fertilizer, which will increase the dissolution rate of fertilizers with low water solubility, such as Zn oxide (ZnO), so gradual increases in Zn uptake can be observed as particle size decreases (Solanki and Laura, 2018).

In relation to the above, Du et al. (2019), examined the effects of ZnO nanoparticles and zinc sulfate foliarly on wheat (*Triticum aestivum* L.) growth at the same doses, the highest Zn content in grain was found with 100 ppm ZnO, which was almost 30% higher compared to the application of 2000 ppm zinc sulfate. Similarly, Bautista-Diaz et al. (2021) reported significant differences in Zn content in bean fruits when ZnO nanoparticles were foliar applied compared to Zn sulfate application, also finding increases in leaves and roots. Similar results were published by Mahdieh et al. (2018), who applied on two occasions in a foliar manner in pinto bean plants, zinc nanoparticles in the form of ZnO and compared it with Zn sulfate and chelate at twice the concentration, obtaining an increase of 28.5% in the zinc content in grains, compared to the application of Zn sulfate and 11.3% compared to chelate.

In recent years, studies with ZnO nanoparticles have grown exponentially, because their physicochemical properties make them a potential solution to counteract Zn deficiency in the human diet, and these same properties allow them to dissolve in the soil, thus avoiding soil contamination from excess fertilization that could be present in biofortification programs. In addition, ZnO nanoparticles can produce a longer lasting release of Zn^{2+}, which is the form of Zn that can be more easily absorbed by plants (Doolette *et al.*, 2020; Du *et al.*, 2019). Almendros et al. (2022), found a higher translocation factor of Zn within the plant when applying ZnO nanoparticles and pointed out that the translocation of Zn to the fruits is influenced by the concentration of the same, finding that the roots when having high doses of Zn avoid its translocation to avoid toxicity in other organs of the plant, so that low applications of ZnO in the form of nanoparticles could stimulate to a greater extent the movement of Zn within the plant.

Umar et al. (2021) found the maximum Zn concentration in corn grains when they applied ZnO nanoparticles foliarly and reported that Zn absorption and translocation is comparable to when Zn sulfate is applied. Similarly, Subbaiah et al. (2016), achieved a 37% increase in Zn content in corn grains when applying 100 ppm of ZnO foliarly. Velázquez-Gamboa et al. (2020) found a 240.31% increase in Zn content in Stevia plants when ZnO nanoparticles were applied, showing that this form of Zn is more assimilable than Zn sulfate. Meanwhile, de Souza et al. (2021) found that the application of ZnO in particulate form increased the Zn content in cowpea beans, however, they found no differences with the application of ZnO in conventional form. Several studies indicate the effects of the use of ZnO in biofortification (Table 2).

Reference	Crop	Dose	Application method	Results
Subbaiah *et al.*, 2016	Maize	50, 100, 200, 400, 600, 800,1000, 1500 and 2000 ppm.	Foliar application	It increased by 37% the content of Zn in grain with the dose of 100 ppm in relation to the control without application.
Mahdieh *et al.*, 2018	Pinto beans	0.05%, 0.1% and 0.15% p/v of ZnO	Aplicación en semillas y aplicación foliar en dos etapas de desarrollo del cultivo.	Increase of 28.5% in the content of zinc in grains with the dose of 0.15%, compared to the application of Zn sulfate and 11.3% compared to the chelate at twice the concentration.
Ponce-García *et al.*, 2019	Green beans	25, 50 and 100 ppm	Edaphic application in nutrient solution	The concentration of Zn in the fruit increased in the 3 treatments in relation to the control.
Du *et al.*, 2019	Wheat	0, 10, 20, 50, 100, 200 and 1000 ppm	Edaphic application in nutrient solution	Increases of 48, 138, 175, 186 and 230% in the doses from 20 to 1000 ppm, respectively.
Velázquez-Gamboa *et al.*, 2020	Stevia	50, 75 and 100 ppm	Edaphic application in nutrient solution	They found a 240.31% increase in Zn content in Stevia plants by applying ZnO nanoparticles compared to Zn sulfate.
Umar *et al.*, 2021	Maize	8 kg of $Zn.ha^{-1}$ in the edaphic way and a 2% solution in the foliar way.	Edaphic application in nutrient solution and foliar application	A relative increase of 82 and 59% in grain Zn concentration was recorded with soil and foliar application, respectively, in relation to the control.
de Souza *et al.*, 2021	Cowpea bean	0, 100, 300, 500 and 800 $mg.dm^{-3}$	Soil application	At the dose of 100 mg dm^{-3}, it represented an increase of 14%

				of Zn in grain compared to the control.
Aslam *et al.*, 2021	Mung bean	0.1% ZnO and 0.1% ZnO plus lysine	Foliar application	Increases of 33.81 and 40.87% in the content of Zn in grain, respectively.
Yang *et al.*, 2021	Rice	25 and 100 $mg.kg^{-1}$ at different stages of development: basal, tillering and panicle formation	Soil application	The application of ZnO increased the Zn concentration of the rice by 13.5, 19.8 and 39.4%, for each one of the stages compared to the traditional fertilization of the crop.
Dhaliwal *et al.*, 2021	Chickpea	0.5% ZnO and 0.5% ZnO + 0.5% Fe nanoparticles	Foliar application	12.7% increase in Zn concentration in the grain for both treatments compared to the control with the standard fertilization dose.
Palacio-Márquez *et al.*, 2022	Green bean	25, 50 and 100 ppm	Foliar application	Increases of 32.96, 97.77 and 103.35% in the content of Zn in the fruit in relation to the control without foliar application.

Table 2. Application of ZnO in the form of nanoparticles in biofortification work.

6. Conclusions

Advances in the use of Zn nanoparticles in biofortification works have been exponential in recent years, positioning them as a unique, efficient and sustainable product, capable of increasing the Zn content in various fruits. Zn nanoparticles, thanks to their specific properties, have the ability to be absorbed with a different dynamic, which presents an additional advantage compared to traditional sources of fertilization, improving efficiency and reducing the doses to be used, making them an alternative to the problem of environmental pollution caused by excessive fertilization. In addition, although their physiological effects are not completely proven, the benefits on growth and yield allow their use in biofortification works without affecting agricultural production. However, despite these positive effects, there are still many differences in terms of their synthesis, doses and forms of application, which generates discrepancies in the results, so more studies on the use of Zn nanoparticles in biofortification are needed to determine that the use of this new technology is superior to the use of traditional fertilizers.

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2. USE OF BIOSTIMULANT COMPOUNDS IN AGRICULTURE: CHITOSAN AS A SUSTAINABLE OPTION FOR PLANT DEVELOPMENT.

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Use of biostimulant compounds in agriculture: chitosan as a sustainable option for plant development

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Abstract

A novel and sustainable strategy to solve problems caused by stress in plants is the use of naturally prepared solutions called biostimulants. These products in the last decade have received attention by the scientific communities of greatest relevance in agricultural systems because they modify physiological processes to improve crop production and quality. Within this group, one of the biopolymers with the greatest number of beneficial properties is chitosan, a deacetylated form of chitin found in the exoskeletons of crustaceans, fungal cell walls and in the cuticle of insects. In many species of crops the application of chitosan is studied. Several studies have demonstrated its property as an antiperspirant, plant growth promoter and defense system booster in stressful situations. There is evidence that chitosan is one of the most suitable compounds to use together with macro and micronutrients, due to its wide range of characteristics that include biocompatibility, biodegradability, high permeability, cost-benefit ratio, low toxicity, and excellent film-forming capacity. that are used as covers, in addition to that their uses can be extended with pesticides, herbicides, genetic material and plant hormones. The general objective of this review is to describe the role of biostimulants in agriculture, emphasizing the use of chitosan and its effects on plants, in addition to the relationship and interaction it presents with key micronutrients in plant nutrition such as iron and zinc.

Keywords: chitosan; defense inducer; iron; nanoparticles; plants; zinc

Introduction

According to United Nations estimates, the world population will expand by approximately 2.2 billion by 2050. Therefore, the greatest problem worldwide is centered on how to ensure that the growing world population has enough food and that it is of a quality necessary for meet the nutritional needs of this population (FAO, 2019). In addition to the above, resources such as water and soil are increasingly scarce, in addition to

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the increasingly severe and frequent climatic changes that aggravate the situation (Singh and Prasad, 2014; Rouphael and Colla, 2018).

Unfortunately, the main objective of agricultural production systems is to increase profitability for farmers and agricultural industries, using for this the excessive and dependent application of inorganic fertilizers, leaving in the background what is related to the quality of the products and their effects in human health, making work difficult for agricultural scientists. However, the focus is now on the quality of the products and the sustainability of the production systems (Bulgari *et al.*, 2015; Reyes and Cortés, 2017; Méndez-Argüello and Lira-Saldívar, 2019).

Various types of stress caused by biotic factors (bacteria, fungi, or viruses) and abiotic factors (soil composition, salinity, pH, extreme temperatures, drought, pollution, humidity, wind or UV radiation) are the main responsible for quality losses. and production of agronomic crops. Despite the great advances in this area, the current understanding of the mechanisms involved and the strategies to mitigate these effects is limited (Yakhin *et al.*, 2017; Drobek *et al.*, 2019).

A novel and sustainable strategy to solve these types of problems caused by stress is the use of naturally prepared solutions called biostimulants. These products have begun to have a greater relevance in agricultural systems with the aim of modifying physiological processes and optimizing production and quality; receiving important attention in the last decade by and the scientific communities, significantly increasing the number of publications related to the mechanisms in which biostimulants help to face stress situations and increase the productivity of agricultural crops (Bulgari *et al.*, 2015; Yakhin *et al.*, 2017; Van Oosten *et al.*, 2017; Drobek *et al.*, 2019).

The most complicated part of the application of these new technologies is to obtain materials with properties equivalent to those of fully synthetic products, and which also retain their functionality. Within the group of biostimulants, the use of chitosan stands out, which is the deacetylated form of chitin, which in turn is the second most abundant waste material and comes mainly from the exoskeleton of crustaceans and insects. Different authors have reported positive effects on vegetative growth, concentration of photosynthetic pigments and performance in more than 20 plant species, and it has also shown antifungal effects and inducer of defense mechanisms in plants (Pichiyangkura and Chadchawan, 2015; Ibrahim and Ramadan, 2015; Choundary *et al.*, 2017).

Based on the above, the general objective of this review is to describe the role of biostimulants in agriculture, emphasizing the use of chitosan, its effects on plants and the relationship and interaction with key micronutrients in plant nutrition such as iron and zinc.

Biostimulants: concepts and definitions

The word biostimulant has been used, in horticulture, to describe substances that promote plant growth without being nutrients, soil improvers or pesticides. This concept was first used in 1997 by the Department of Crop and Soil Environmental Sciences of the Virginia Polytechnic Institute and Virginia State University, which defined biostimulants as "materials that, in minute amounts, promote plant growth", referring mainly to extracts of algae and humic acids (Du Jardin, 2015).

Over the years, the concept has undergone many modifications due to changes in the diversity of inputs that can be considered within this group, in addition, there are two industrial branches that have their own definitions about the term biostimulants (Calvo *et al.*, 2014). The European Council of the Biostimulant Industry (EBIC), defined biostimulants as follows: "Plant biostimulants contain substances and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to improve and/or benefit nutrient uptake, nutrient efficiency, abiotic stress tolerance, and crop quality. Biostimulants do not have direct action against pests and, therefore, do not fall within the regulatory framework of pesticides" (Rouphael and Colla, 2018).

For its part, the Coalition of biostimulants in the United States defines them as "substances, including microorganisms, that are applied to the plant, seed, soil or other growing media that can improve the ability of the plant to assimilate the nutrients applied or provide benefits for the development of the plant. Biostimulants are not nutrients for plants and, therefore, cannot make nutritional claims or guarantees" (Biostimulant Coalition, 2021). In more concise words, plant biostimulants are a diverse group of substances that can be added to crops and have positive effects on plant growth and nutrition (Figure 1), but also on abiotic and biotic stress tolerance, however, biostimulants are not considered as nutrients even though they facilitate their absorption (Van Oosten *et al.*, 2017).

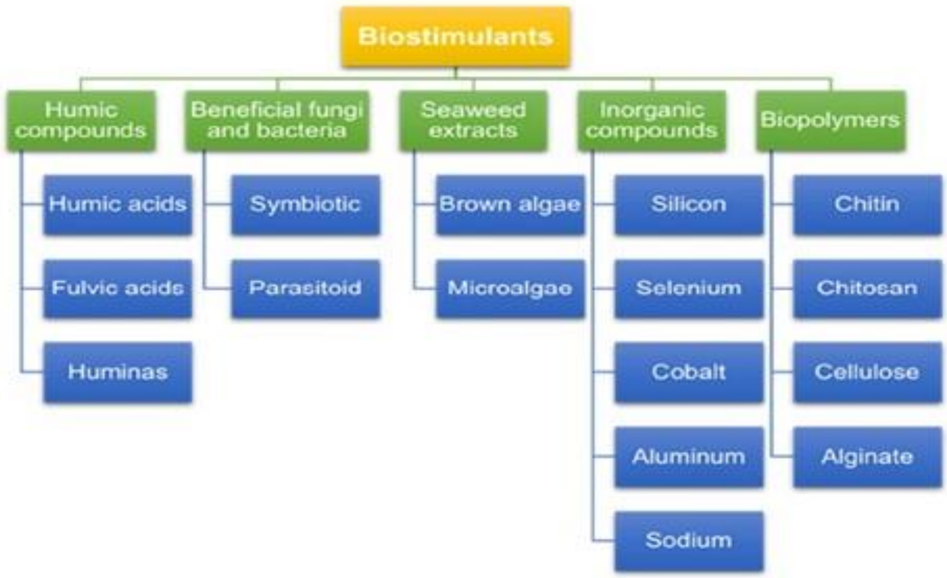

Figure 1. Types of biostimulants commonly used in agriculture

Use of biostimulants in agriculture and classification

The use and production of biostimulants is spreading from more developed countries to a larger number of countries. In Europe, the biostimulant market is expected to range between 1,500 and 2,000 million dollars in 2022, representing approximately half of the world market, with an annual growth rate of 10 to 12%. This is mainly due to the global trend to reduce the amount of fertilizer applied and the search for products that generate a lower environmental impact and are healthy to consume (Economic Overview of the European Biostimulants Market [EBIC], 2021).

Despite recent efforts to produce an international regulation on biostimulants, there is no legal definition of plant biostimulants anywhere in the world. This situation allows several substances and categories that can fall into this group. Despite this, some important categories are always included in various scientific works (Calvo *et al.*, 2014; Du Jardin, 2015; Yakhin *et al.*, 2017; Van Oosten *et al.*, 2017). The following section briefly details the categories that in our opinion represent the biostimulant market worldwide, with an emphasis later the compound of interest for this work.

Humic compounds

Humic compounds (HC) are found within the organic matter of the soil and are formed by the decompositions of plant and animal matter thanks to the action of microbial metabolism and represent the main reserve of organic carbon on the surface of the earth. The CH were classified into three groups: humins, humic acids and fulvic acids, originally categorized according to their molecular weights and solubility, being those with the lowest molecular weight the ones that tend to have the greatest positive biological effects on plants (Canellas *et al.*, 2015; Du Jardin, 2015; Halpern *et al.*, 2015).

HC can be extracted from many different sources, including soils, municipal waste, earthworms, coal deposits, peat moss, and leonhardite. HC have been shown to have a great variety of beneficial functions in the soil, such as controlling the availability of nutrients, the exchange of carbon and oxygen between the soil and the atmosphere; in turn, they are responsible for the transformation and transport of chemical substances that may become toxic to the microecosystems of the rhizosphere. These properties contribute to the regulation of many ecological and environmental processes that are crucial for the growth of plants and terrestrial life, they have also been reported to regulate the carbon and nitrogen cycle of the soil, in addition to improving the stabilization of the soil. soil structure (Calvo *et al.*, 2014; Canellas *et al.*, 2015; Halpern *et al.*, 2015).

More specifically Rose *et al.* (2014), concluded in an extensive analysis that in works where HC was applied, a total increase in dry weight of approximately 22% for the aerial part and 21% for the root part was achieved. For their part, Halpern *et al.* (2015) in their review emphasize various studies that mention the positive effect of HC, increasing the number of fruits and flowers in various crops. It also mentions the positive effects on the absorption of nutrients such as N, P, Zn and Fe, this is partly due to the positive effects it generates on the structure of the soil that was mentioned above but they also mention that CH can affect the morphology of the roots allowing greater absorption and greater activity of the H + -ATPase enzyme, as well as the enzymes responsible for assimilating nitrates. These changes in the morphology of the roots can be attributed to possible changes in the cellular energy metabolism that facilitate the proliferation of lateral roots (Jindo *et al.*, 2012). In turn, Van Oosten *et al.* (2017) emphasizes how humic acids help the plant to cope with situations of hydric and saline stress, activating defense mechanisms and increasing the compounds responsible for antioxidant activity, as well as the levels of proline and plant hormones specifically cytokinins.

These benefits can be easily exploited by farmers because HC can be very inexpensive, their price ranges from 40 to 800 dollars per ton, which compared to the prices of top-quality fertilizers can generate a greater cost-benefit, taking into account that it is a complementary product that due to its benefits will minimize the costs of applying fertilizers in the future (Quilty and Cattle, 2011; Rose *et al.*, 2014).

Beneficial fungi and bacteria (BFB)

The use of BFB in agriculture has its first known reports more than 100 years ago, when in 1909 a study showed that a consortium of *Pseudomonas radicicola* and *Azotobacter sp.* improved the growth of oats and barley, in addition to improving the absorption of N. Despite promising results over the years, it was not until 1979 when it gained relevance in agriculture, emphasizing the bacteria that promote plant growth by modifying the soil microflora (Ruzzi and Aroca, 2015). About this topic, two types of groups of functional and ecological bacteria have been studied; Mutualistic endosymbionts of the Rhizobium type and plant growth promoting bacteria (PGPB) found in the rhizosphere, these bacteria belong to several genera such as *Rhizobium, Bradyrhizobium, Azotobacter, Azospirillum, Pseudomonas* and *Bacillus* (Du Jardín, 2015; Van Oosten et al., 2017).

Bacteria have been shown to be multifunctional in terms of their benefits on plants, since they contribute to mitigating both abiotic and biotic stresses, including pathogen control, greater tolerance to salt, greater resistance to heavy metals and other toxins; as well as help increase growth and yield (Alavi *et al.*, 2013; Berg *et al.*, 2014; Du Jardín, 2015).

Among the ways in which bacteria interact with plants are parasitism, mutualism, penetration into the interior of cells, extending to the rhizosphere or rhizoplane and with the participation in biogeochemical cycles that modify the structure of the soil and plant cover. The modes of action are diverse depending on the species. The most common mechanisms presented by the application of bacteria to plants are changes in the hormonal content, increasing the contents of auxins or cytokinins, the production of volatile compounds, the increase in the availability of nutrients through the production of siderophores that facilitate the entry through the root and the production of organic acids that modify the pH, especially affecting the availability of micronutrients (Calvo *et al.*, 2014; Halpern *et al.*, 2015; Du Jardín, 2015; Rouphael and Colla, 2018).

Like bacteria, beneficial fungi also form parasitoid associations and mutualistic symbioses with plants; It is believed that approximately 5000 species of fungi can colonize roots and subsequently providing plants with soil nutrients (Behie and Bidochka, 2014). Within this large group of fungi, the mycorrhizal fungi stand out, which are a heterogeneous group of taxa that establish symbiosis with more than 90% of all plant species. Among the different forms of physical interactions, arbuscular mycorrhizal fungi (AMF) are a widespread type of endomycorrhizae, where the fungal hyphae of *Glomeromycota* species penetrate the cortical cells of the root. Various studies report that AMF contribute to the survival of plants in desert ecosystems and function in soils with low levels of organic matter and phosphorus (Du Jardín, 2015; Osuna-Ávila *et al.*, 2021).

It has also been reported that AMFs are very effective in improving the absorption of nutrients helping the growth of the plant, currently they are of great interest since they have been recognized as a biofertilizer that helps control both biotic and abiotic stress. In addition, mycorrhizal fungi can form hyphal connections between the root systems of two or more host plants, forming networks of mycorrhizae below the ground between various plants (Gilbert and Johnson, 2017; Hussain *et al.*, 2018).

Another genus of fungi that has become very important in the agricultural sector is *Trichoderma sp.* which was widely recognized for its function as a biological control, especially against phytopathogenic fungi. However, recent studies show a relationship between its association with the roots and a greater absorption of nutrients from plants because of a solubilization of macro and micronutrients. Several strains of *Trichoderma* have also been found to stimulate plant growth, improve yield and nutritional quality. Another recently discovered benefit is the ability of this genus to increase the photosynthetic capacity and in turn the content of photosynthetic pigments (Fiorentino *et al.*, 2018; Harman *et al.*, 2019; Lombardi *et al.*, 2020).

Seaweed extracts (SE)

The use of seaweed extracts as biostimulants has emerged in the last decade commercially as products that promote plant growth and as stress relievers, such as excess heat, salinity, and drought. However, its use in agriculture dates to ancient times since it was used as compost to improve the soil and directly to improve the productivity of crops, being used commonly by the Roman Empire, Japan, China, France, Spain and the former Great Britain (Craigie, 2011; Van Oosten *et al.*, 2017; Mukherjee and Patel, 2020).

The use of SE has taken on commercial relevance, especially as biofertilizers or purifying the metabolites that make up these extracts, these compounds include the polysaccharides laminarin, alginates and carrageenan's and their degradation products, other components such as sterols and compounds that contain N such as betaines and hormones. Metabolites such as phlorotannin's, fatty acids, halogenated compounds, alkaloids, terpenoids and lectins have also been isolated; many of these being exclusive to its source of algae (Du Jardin, 2015; Mukherjee and Patel, 2020).

There are 2 main groups of algae on the market: macroalgae and microalgae and there are currently more than 47 companies that produce and market various extracts of algae for agricultural use. Most of the formulations come from the brown algae of the *Ascophyllum nodosum* species that are mainly harvested from marine waters, but its quality as a biofertilizer varies according to the availability of nutrients, the harvest time, and the place where it is harvested, so its effectiveness is not as constant as you would like. Based on this, the scientific community has found a promising alternative towards the standardization of the raw material and the reduction of costs in the production of algal biomass with the use of microalgae, which are already on the market in some species, such as *Chlorella* spp., *Dunaliella* spp., *Haematococcus* spp., *Isochrysis* spp., *Nannochloropsis* spp., *Porphyridium* spp., and *Spirulina* spp. but they are only sold as supplements to traditional fertilization (Van Oosten *et al.*, 2017; Chiaiese *et al.*, 2018).

The effects of the application of algae extracts can be seen both in the soil and in the plant itself. In an edaphic way, the application of these extracts as solid or liquid compost, modify the aeration of the soil and its water retention capacity, it also modifies the cation exchange capacity and some of the compounds have the capacity to capture heavy metals. that could become toxic to plants (Du Jardin, 2015). For their part, SEs have effects on plant growth, increase in photosynthetic activity and chlorophyll content, increases in the activity of enzymes related to nitrogen metabolism, which leads to a higher content of proteins and amino acids. Effects on flowering and the production of compounds related to antioxidant capacity have also been reported. In addition, impacts on germination, seedling establishment and development have been reported that are related to hormonal effects, which have been identified in algae extracts as cytokinin's, auxins, abscisic acid, gibberellins, and other classes of hormone-like compounds, such as sterols. and polyamines (Stirk *et al.*, 2013; Wally *et al.*, 2013; Mukherjee and Patel, 2020).

Another of the positive effects of using SE is the effect it has on the mechanisms responsible for the defense of the plant before stressful situations, several studies show that extracts of seaweed that contain betaines and cytokinin's can help to maintain the stability of the proteins. embedded in the cell wall and keep cells plump for longer in situations of lack of water or excessive heat. Effects on stomatal conductivity and water potential in the leaf have also been reported due to changes in potassium flux in cells (Calvo *et al.*, 2014; Saa *et al.*, 2015; Mukherjee and Patel, 2020).

Inorganic compounds

Inorganic biostimulants are all those elements that can be beneficial for plants but do not fall under the rule of essentiality. The five main beneficial elements are Al, Co, Na, Se and Si, which can be found in the soil solution as inorganic salts and recently being applied as commercial products (Marschner, 2011; Bhupenchandra *et al.*, 2020).

Through the years, these elements have been recognized for their effects on the growth, quality and protection of crops. Its effects on abiotic stress situations have recently been studied and positive effects have been found on cell wall rigidity, osmoregulation, reduction in perspiration under water stress, thermal regulation against temperature stress, antioxidant protection, interactions with symbionts, protection against heavy metal toxicity, in addition to the synthesis and signaling of plant hormones (Du Jardin, 2015; Bhupenchandra *et al.*, 2020).

Among this group of beneficial elements, the one most used as a biostimulant is silicon (Si), which is the second most abundant element on the earth's surface. Plants take Si in the form of silicic acid [$Si(OH)_4$] from the soil solution and it is easily transported to the different organs of the plant, its essentiality for plants is still under discussion, however, its positive effects on growth and productivity are widely demonstrated (Marschner, 2011; Tubana *et al.*, 2016). One of the most important effects of Si is that it affects the chemical and biological properties of some micronutrients such as P, Al, Fe, Mn and the mobility of heavy metals, microbial activity, the stability of soil organic matter and the formation of polysilicic acids, which have a significant effect on soil texture, water retention capacity, adsorption capacity and stability of soil erosion (Swain and Rout, 2017).

Regarding the effects on plants, there are increased resistance to abiotic stress and resistance to pathogens and diseases. Effects on the absorption of nutrients such as P, Ca, K and N have also been demonstrated, especially when these are found in low amounts in the soil. Another positive effect when applying Si is that they increase the vigor and rigidity of the leaves, thus increasing light absorption and photosynthesis. It has been found that they also modulate the mobility of water, apparently due to the formation of silica gel in the cell walls, which reduces perspiration, thus avoiding the problems caused by water stress (Guntzer et al., 2012; Liang, et al. al., 2015; Albrecht, 2019).

Biopolymers

In recent years, many attempts have been made to substitute petroleum products in materials development, so many biopolymers such as starch, cellulose, collagen, gelatin, alginate, chitin and chitosan have been investigated, due to that they have a functionality applicable in sustainable environmental development (Croisier and Jérôme, 2013).

In agriculture, alternatives are being sought for the use of agrochemicals through green technology or renewable nanomaterials. Synthetic polymers such as polycaprolactone, polyethylene, polyvinyl alcohol, and acrylate-based polymers are used to achieve slow release of fertilizers that allow better absorption and improvement of the soil; However, these synthetic polymers are not biodegradable, and their residues can remain in the soil for a long time, helping to pollution. It has been reported that the biopolymers, mentioned above, due to their properties have been used as covers for fertilizers and slow-release pesticides, in addition to their use as hydrogels for moisture retention in places where the climate is very arid (Sampathkumar *et al.*, 2020).

The term biopolymer is generally understood as an organic polymer that is produced naturally by living organisms or that is synthesized through waste from living things. These compounds have their main advantage in their easy degradation and that they transform into compounds that are easily found in the environment such as water, carbon dioxide or methane (Tănase *et al.*, 2014). Various studies have proven that they act as inducers that can activate the defense responses of plants and induce plants to produce disease-resistant compounds, they have also been shown to have various properties as protectors against abiotic stress. Within this group, one of the biopolymers with the greatest number of beneficial properties is chitosan (Du Jardin, 2015; Zheng *et al.*, 2020).

Chitosan

Origin and uses of chitosan

Chitosan is a crystalline linear polymer and a deacetylated form of chitin (Figure 2), which is a linear copolymer of 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D- glucopyranose. Being the second most abundant renewable polymer in nature, after lignocellulosic biomass, it can be found in the exoskeletons of crustaceans, fungal cell walls and in the cuticle of insects (Croisier and Jérôme, 2013; Piras *et al.*, 2014; Malerba and Cerana, 2016; Choudhary *et al.*, 2017).

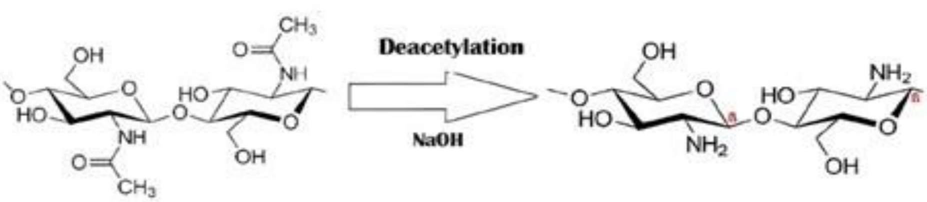

Figure 2. Conversion of chitin to chitosan

To become chitosan, chitin must have at least 60 degrees of deacetylation, which is done by chemical hydrolysis under severe alkaline conditions or by enzymatic hydrolysis in the presence of enzymes such as chitin deacetylase. Chitosan is identified based on its degree of deacetylation, which can vary between 60% and 90% and average molecular weight between 50 and 2000 KDa, these variations are due to its different ways of obtaining and production (Riva *et al.*, 2011; Mármol *et al.*, 2011; Croisier and Jérôme, 2013). These variations generate chitosan preparations in terms of the degree of deacetylation, molecular mass, degree of polymerization, viscosity, pKa value, so when talking about chitosan we do not speak of a single compound but of a group of biopolymers that are currently found. commercially available. In addition, the presence of (acetyl) amino groups within chitosan structures has been reported that would be very beneficial for chemical modifications to build sophisticated molecular architectures with wide potential for use in industry (Malerba and Cerana, 2016; Zheng *et al.*, 2020).

Chitin was first isolated in 1811, when Professor Henri Braconnot obtained it from a fungus and named it fungin. After that, Auguste Odier in 1821, gave it the name chitin after isolating it from the exoskeleton of insects. For its part, chitosan was discovered in 1859 by Charles Roughet, when he made chitin soluble in water, however, the term was used until 1870 and the great discoveries about these biopolymers did not arrive until 1930 and despite these Discoveries the true interest of the industry came in the 70s, when alternatives were sought to better use the biological waste of marine crustaceans (Crini, 2019; Amine *et al.*, 2021).

Its main utility is due to its antimicrobial properties, its charge density and its film-forming or coating properties. Currently, chitosan is used in the form of solutions, suspensions, and particles, for example nanoparticles, beads, resins, sponges, hydrogels, foams, protective films, fibers, and microscopic threads. Chitosan has been commonly used in the biomedical industry, due to its proven medicinal effects. Also, for decades, its use in the food industry is well known as a thickener, gelling agent, and emulsifier, it is used in edible protective films, as a functional ingredient that provides dietary fiber due to its hypocholesterolemic effect and in industrial processes. In addition, one of the most relevant areas is its use as an adjuvant in water treatment, due to its favorable characteristics with the environment; Various studies demonstrate its role as a chelator of heavy metals and pesticides (Mármol *et al.*, 2011; Morin-Crini *et al.*, 2019).

Chitosan applications in agriculture

Because it is a friendly compound with the environment, due to its rapid degradation, low toxicity and easy obtaining, the use of chitosan in agriculture has increased in recent years. The use of chitosan in agriculture began approximately in the 90's, since bactericidal properties were found, or in some cases it hindered the growth of bacteria, delaying their effects on plants. Also, due to its chelating activity, it has been proven as an excellent fungicide and antiviral. Although its main use was to retard the release of fertilizers and pesticides (Vasconcelos, 2014; Morin-Crini *et al.*, 2019).

The application of chitosan has been studied in many species of crops, including cereals, ornamental, fruit and medicinal plants, and its effectiveness depends on the structure and concentration of this, the plant species and the phenological stage in which plant is found. Several studies have demonstrated its property as an antiperspirant, in turn its effect as a plant growth promoter and defense system promoter in stress situations has been studied (Abu-Muriefah, 2013; Pichyangkura and Chadchawan, 2015; Saharan *et al.*, 2016; Deshpande *et al.*, 2017).

Chitosan and its derivatives are used in agriculture in various ways (Table 1); One of these uses is its role as a seed coating in which its antifungal role generates the ability to protect the seedlings, they also have a positive effect on the germination rate, the growth parameters, and the vigor of these. Another of its uses is as an amendment to improve the structure of soils and it was found that chitosan successfully reduces *Fusarium* wilt and against infections by *Cylindrocladium floridanum*, *Alternaria solani*, and *Aspergillus flavus*. Its uses also include the application as a foliar spray, supplement in hydroponic solutions and as a supplement in plant tissue culture medium, finding improvement in crop performance, induction of the defensive system and promotion of plant growth (Orzali *et al.*, 2016; Morin-Crini *et al.*, 2019).

Physiological and biochemical effects of chitosan on crops

As mentioned above, various studies have shown that chitosan is a natural molecule that induces numerous physiological responses in plants, however, these responses depend on its structure and concentration when applied, in addition to the species and stage of development. of the plant. The mode of action of chitosan is not completely revealed, various authors suggest that the physiological effects of the application of chitosan in plants are the result of the ability of this polycationic compound to bind to a wide range of cellular components such as DNA, the plasma membrane and the constituents of the cell wall, but also to bind to specific receptors involved in the activation of defense genes, in a similar way to the defense inducers of plants (Du Jardín, 2015; Malerba and Cerana, 2016).

Table 1. Uses and benefits of the application of chitosan in agricultural crops

Crops	Application from	Results	References
Bell pepper (*Capsicum annuum* L.) Cv. 'Yolo Wonder'	Foliar in leaves and fruits	Increase in weight, diameter and fruit yield	Mahmood *et al.*, 2017
Bell pepper (*Capsicum annuum* L.) Cv. 'California Wonder'	Priming in seeds	Acceleration of germination and lower incidence of fungal attacks	Samarah *et al.*, 2016
Chilli pepper (*Capsicum annuum* L.)	Foliar	Increase in number of fruits, leaves and chlorophyll content. In addition, it reduced the incidence of attacks by *Phytophthora capsici*	Esyanti *et al.*, 2019
Tomato (*Lycopersicon esculentum* L.) Cv. 'PKM1'	*In vitro*	Protection against *Alternaria solani*	Sathiyabama *et al.*, 2014
Tomato (*Solanum lycopersicum* L.) Cv. 'Marglobe'	Soil irrigation	Increase in root colonization by *Pochonia chlamydosporia*, a parasitic fungus of *Meloidogyne spp.*	Escudero *et al.*, 2017
Tomato (*Solanum lycopersicum* L.) Cv. 'BINAtomato-6'	Foliar	Increase in variables related to the yield and activity of the nitrate reductase enzyme	Mondal *et al.*, 2016
Tomato (*Lycopersicon esculentum* L.)	Foliar	Increase in biomass, yield and chlorophyll content, low water stress	Hassnain *et al.*, 2020
Cucumber (*Cucumis sativus* L.)	Postharvest, as a protective cover	Maintained quality and reduced carbon dioxide production	Olawuyi *et al.*, 2019
Cucumber (*Cucumis sativus* L.) Cv. 'Celebrity F1'	Foliar	Increased quality, yield and vegetative growth for two consecutive years	Shehata *et al.*, 2012
Cucumber (*Cucumis sativus* L.)	Foliar	Maintained growth and production rates under temperature stress	Ali *et al.*, 2020
Bean (*Phaseolus vulgaris* L.)	Foliar	Increase in variables related to growth and yield under water stress	Abu-Muriefah *et al.*, 2013
Bean (*Phaseolus vulgaris* L.) Cv. 'Giza 3'	Priming	Reduced the incidence of damping-off and root rot	El-Mohamedy *et al.*, 2017
Bean (*Phaseolus vulgaris* L.) Cv. 'Nebraska'	Foliar	Inconsistent results on yield parameters under temperature stress	Ibrahim and Ramadan, 2015
Potato (*Solanum tuberosum* L.) Cv. 'Agria'	*In vitro*	Increased fresh tuber weight and yield	Amini, 2015
Potato (*Solanum tuberosum* L.) Cv. 'Spunta'	*In vitro* and in hydroponic solution	Significantly reduced the attack of various species of *Fusarium*	Mejdoub-Trabelsi *et al.*, 2020
Watermelon (*Citrullus lanatus* L.) Cv. 'Jubilee'	Hydrogel on the substrate	Increased root size and stoma width	Gonzalez-Gomez *et al.*, 2017
Maize (*Zea mays* L.) Cv. 'Suria local'	*In vitro* and foliar	Increase in antioxidant activity and variables associated with yield	Choudhary *et al.*, 2017b
Maize (*Zea mays* L.) Cv. 'Pioneer 3906' and 'SR03'	Foliar	Reduced the effects of salt stress	Al-Tawaha *et al.*, 2018
Maize (*Zea mays* L.) Cv. 'Giza 9'	Priming and foliar	Increased chlorophyll content and yield, low salt stress	ALKahtani *et al.*, 2020
Rice (*Oryza sativa* L.)	Edaphic solution	Increase in yield	Nguyen and Tran, 2013
Rice (*Oryza sativa* L.)	*In vitro*	*Rhizoctonia solani* growth reduction	Liu *et al.*, 2012

More specifically, chitosan produces positive effects in plants because it induces the activity of enzymes involved in oxidative metabolism such as superoxide dismutase, peroxidase, and catalase; in addition to increasing chlorophyll levels and in turn a better photosynthetic activity (Pichyangkura and Chadchawan, 2015). It is also attributed beneficial properties in situations of water scarcity because it induces stomal closure, thus reducing perspiration and therefore water loss (Kashyap *et al.*, 2015).

Effects of chitosan on plant defense inducers

For the induction of defense responses in plants, chitosan can affect gene expression by generating changes in the interaction with chromatin, and it can also bind to specific receptors. One of these receptors is a binding protein belonging to the lectin family of glycoproteins. The presence of chitosan receptors is also suggested by the rapid activation of the plasma membrane H^+-ATPase. Once recognized by a receptor, the signal is translated by a secondary messenger and physiological responses begin to activate (Malerba and Cerana, 2016; Hidangmayum *et al.*, 2019).

Among the best-known responses of chitosan to cell receptors, the accumulation of hydrogen peroxide, reactive oxygen species (ROS), nitric oxide (NO) and an increase in the content of Ca^{2+} inside the cell have been demonstrated (Figure 3). The production of these compounds triggers a series of responses related to defense mechanisms in plants (Du Jardin, 2015; Pichyangkura and Chadchawan, 2015; Malerba and Cerana, 2016).

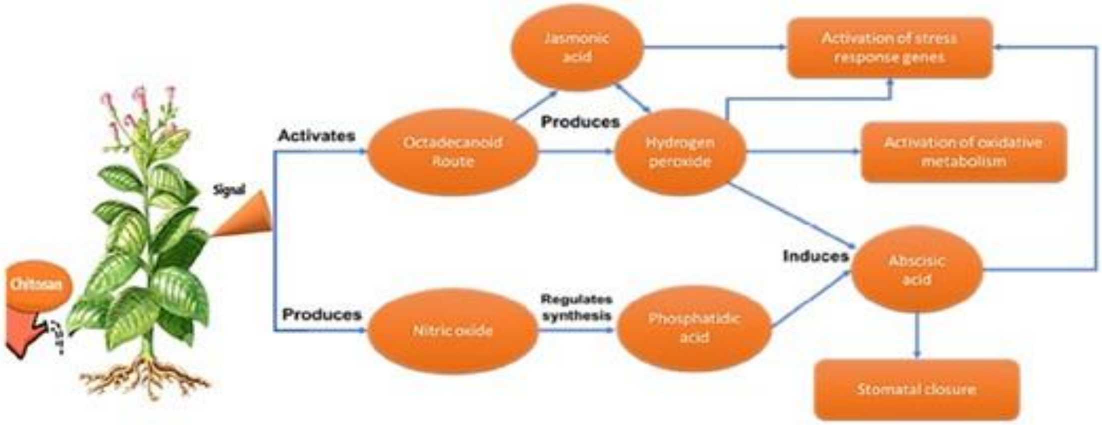

Figure 3. Processes involved in chitosan signaling

In the case of ROS, the plant treated with chitosan induces a greater activity of the enzymes related to oxidative metabolism such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) which, in addition to reducing the species reactive, they promote the production of malondialdehyde which in turn is responsible for reducing lipid peroxidation. Another enzyme that is affected by the application of chitosan is phenylalanine ammonium lyase (PAL), responsible for the biosynthesis of most bioactive compounds in plants; Likewise, various studies report an increase in the concentration of proline. These processes are positively related to the positive effects of chitosan in plants subjected to some type of abiotic stress, such as stress due to salinity and drought (Pichyangkura and Chadchawan; Hidangmayum *et al.*, 2019). While the Ca^{2+} content is responsible for regulating the activity of the enzyme callose synthase, which is responsible for producing callose, a substance responsible for repairing wounds in cells caused by mechanical damage or attack by pathogens (Malerba and Cerana, 2016; Hidangmayum *et al.*, 2019).

On the other hand, the accumulation of NO induces the production of phosphatidic acid, which is positively related to the production of abscisic acid (ABA), a phytohormone that can produce stomatal closure and thus reducing the rate of perspiration. altering the turgor of the occlusive cells and consequently causing their closure. In turn, the production of ABA also induces the synthesis of solutes such as proline, glycine, betaine, and trehalose; which decrease the water potential of the cell, allowing it to take in water that surrounds it or retain what it already has (Wilson *et al.*, 2014; Pichyangkura and Chadchawan, 2015). Another positive effect that chitosan produces in plants is the ability to activate plant defense genes through the octadecanoid pathway, increasing the synthesis of jasmonic acid (JA), a key compound for plants to protect themselves from attacks by pathogens, mainly insects. (Pichyangkura and Chadchawan, 2015; Hidangmayum *et al.*, 2019).

Chitosan as a biofertilizer

In recent years, the use of biostimulants as fertilizers in plants has generated many investigations, chitosan has stood out among this series of compounds due to its rapid degradation through enzymatic processes without affecting the beneficial rhizosphere microbiome of the soil and induces symbiotic exchange between the plant and microorganisms (Sharif *et al.*, 2018). The large number of articles published in the last year shows that chitosan is a unique product available in large quantities and at a very economical price and has a bright future in the development of sustainable agricultural practices, and its application as a biofertilizer has been shown to have positive effects on the absorption of other nutrients without negatively affecting the environment (Malerba and Cerana, 2016; Sharif *et al.*, 2018).

It is known that one of the main problems in agriculture is the low absorption in fertilizers, it is estimated that plants cannot absorb between 40% and 70% of nitrogen, 80-90% of phosphorus, and between 50% % and 70% of the potassium applied to the soil, this problem can be reduced by including nutrients in matrices based on chitosan; Chitosan nanoparticles combined with N, P and K were shown to delay the release, improving absorption and without negative effects on the soil (Malerba and Cerana, 2018). For their part, Ha *et al.* (2019) prepared chitosan nanoparticles by ionic gelling with NPK, generating a slow and controlled release nanofertilizer, resulting in a strong effect on nutrient absorption, chlorophyll content, photosynthetic activity, and growth of coffee seedlings. in a greenhouse.

Abdel-Aziz *et al.* (2016), used similar nanoparticles mixed with NPK, but their application was carried out in a foliar way, finding that chitosan facilitated absorption through the stomata and a mobility study using electron transmission microscopy showed that this type of fertilizer moved through the phloem from the source (leaves) to the sink organ (fruit, shoots or roots), in addition significant increases were found in the harvest index, crop index and index of the yield variables in wheat, shortening the life cycle 23.5% of the plants compared to normally fertilized plants, although this effect is related more to the application of nanoparticles than to the use of chitosan. In turn, Hussain et al. (2012), in their laboratory work, found that a combination of urea with chitosan microspheres delayed the release of the fertilizer, making this a promising mixture to analyze its effects in a field work.

Chitosan and its interaction with the use of iron and zinc

It is likely that the beneficial effects attributed to chitosan on plant development are due to a possible role as a carrier of nutrients and its metal chelating power. This phenomenon occurs because the structure of this compound facilitates the union of metal ions through ion exchange, complex formation and intra and intermolecular encapsulation (Liu *et al.*, 2014; Vasconcelos, 2014; Deshpande *et al.*, 2017).

This process has been presented in conjunction with the inclusion of metallic nanoparticles in agriculture and, as chitosan is the only natural polycationic polysaccharide, it allows an interaction with positively charged elements, which is why it has taken on great relevance within the scientific field. The implementation of these chitosan systems as nanoparticle carriers have proven to be an excellent alternative to the problem of low absorption of fertilizers and environmental pollution caused by the excessive use of agrochemicals (Velásquez, 2015).

It is known that transition metals, such as zinc (Zn), iron (Fe) and copper (Cu) are very important for the correct development of crops in general. Zn has a role as an enzymatic cofactor, participating in the reactions of key enzymes such as superoxide dismutase and alcohol dehydrogenase, it is also necessary to carry out the metabolism of nucleic acids, it has a central role in protein synthesis and is essential for photosynthesis to occur and carbohydrate metabolism in plants. For its part, Fe is a constituent of several enzymes, in addition to being a key element for the formation of chlorophyll, a fundamental compound to carry out photosynthesis. However, the absorption of these elements is limited by various factors, its application in the form of nanoparticles being a viable alternative and the chelating properties of chitosan emerge to maximize their potential (Marschner, 2011; Choudhary *et al.*, 2017b).

There are a large number of research works that relate the use of these two micronutrients and chitosan (Table 2), finding a greater emphasis on the use of Zn because its combination has shown positive effects on production, biomass and mechanisms of plant defense (Salimi *et al.*, 2019). In their study, Deshpande *et al.* (2017), showed that chitosan nanoparticles complexed with Zn, proved to be a good transporter for foliar application in wheat, in addition to that their release was slow and controlled, thus preventing the loss of nutrients. For their part, Mirbolook *et al.* (2020), found that when Zn chelated with chitosan and applied it both foliar and edaphic, the plants had better Zn absorption and greater root growth. Similarly, Choudhary *et al.* (2019), obtained a strong antifungal activity and an increase in antioxidant activity, in addition to a greater accumulation of Zn in corn plants, when applying this nutrient in a foliar way complexed with chitosan in the form of nanoparticles.

On the other hand, the combination of Fe with chitosan has not been widely studied, among the few studies where this combination was used, Salimi *et al.* (2019), found negative effects when they applied chitosan to the culture medium that contained Fe nanoparticles, however, when compared with the control, the results were higher in root and aerial growth.

Table 2. Effects of chitosan applied in combination with Zn and/or Fe

Nutrient	Application form	Crops	Results	References
Zn	Encapsulated in chitosan nanoparticles (priming and foliar)	Maize (*Zea mays* L.) Cv. Suria local	Antifungal effects, increased Zn content and increased plant growth	Choudhary *et al.*, 2019
	Chitosan Nanoparticles and TPP * (Foliar)	Wheat (*Triticum durum* L.) Cv. MACS 3125 and UC 1114	Increase in the concentration of Zn in grain	Deshpande *et al.*, 2017
	Foliar in combination with chitosan and/or humic acids	Bean (*Phaseolus vulgaris* L.) Cv. Nebraska	Inconsistent results on performance parameters under temperature stress	Ibrahim and Ramadan, 2015
	In the form of nanoparticles of ZnO plus chitosan	Chickpea (*Cicer arietinum L.*) Cv. GJ-62	Antifungal activity against *Fussarium oxysporum* and promotion of plant growth	Kaur *et al.*, 2018
	Directly to the ground in combination with chitosan	Sunflower (*Helianthus* L.) Cv. Pioneer Hybrid 6946	Reduced nickel levels in contaminated soil and increased antioxidant levels in plants	Turan *et al.*, 2018
	Foliar in combination with chitosan	Tomato (*Lycopersicon esculentum* L.)	Increase in biomass and enzymatic activities of SOD and PAL	Salimi *et al.*, 2019
	In combination with amino acids and chitosan	Bean (*Phaseolus vulgaris* L.)	Better Zn absorption and higher root growth	Mirbolook *et al.*, 2020
Fe and Zn	In culture medium plus chitosan	Pepper (*Capsicum annuum* L.) Cv. LJ-King	The application of chitosan favored growth when combined with Zn, but did not obtain positive results when combined with Fe	Zhao *et al.*, 2019
Fe	New fertilizer with chitosan and silica	N/A	A stable fertilizer with a slow release was obtained, but it is not yet tested on plants	Mangallo *et al.*, 2020

* Sodium tripolyphosphate

In general, these studies position chitosan as one of the most suitable compounds to be used together with macro and micronutrients, due to its wide range of characteristics that include biocompatibility, biodegradability, high permeability, cost-benefit ratio, non-toxicity, and excellent film-forming ability to be used as covers, plus their uses can be expanded with pesticides, herbicides, genetic material, and plant hormones. However, there are still many challenges to make this compound an accepted commercial product, due to the continuous rejection of new technologies by producers since the production process is not yet standardized, generating a material that has different characteristics and that in turn time causes variable results, so it is necessary to continue with these studies (Mujtaba *et al.*, 2020).

Conclusions

In the last 10 years. The advances in the use of chitosan are impressive, as it is a unique, inexpensive product that can be incorporated into sustainable production systems. Its physiological effects are proven and the benefits on growth, production and especially in the induction of defense of plants under stress conditions are promising for use in situations where production conditions are unfavorable, or to face the problems that the climatic situation presents us. Also, its use as a carrier of nutrients is an alternative that can be used to improve the efficiency of fertilization and achieve better absorption, especially with micronutrients that present difficulties for their absorption such as Zn, this would avoid excess fertilization that causes contamination of soils and aquifers. In addition, its effects as a fungicide, bactericide and antiviral can also be used to reduce the use of agrochemicals. However, despite these positive effects, many differences are still observed in terms of its production and the forms of application, which generates a discrepancy in the results, so it is necessary to expand the investigations to seek a homogenization in these aspects and reach the maximum potential that chitosan possesses.

Authors' Contributions

Conceptualization: A.P.-M., E.S., D.L.O.-B.; Data Curation: A.P.-M., C.A.R.-E.; Formal analysis: A.P.-M., E.S., D.L.O.-B.; Writing original draft: A.P.-M., C.A.R.-E.; Writing-review and editing: E.S., D.L.O.-B., C.C.-M., J.P.S.-A, P.P.-R.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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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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3. EFFICIENCY OF FOLIAR APPLICATION OF ZINC OXIDE NANOPARTICLES VERSUS ZINC NITRATE COMPLEXED WITH CHITOSAN ON NITROGEN ASSIMILATION, PHOTOSYNTHETIC ACTIVITY, AND PRODUCTION OF GREEN BEANS (*Phaseolus vulgaris* L.).

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Efficiency of foliar application of zinc oxide nanoparticles versus zinc nitrate complexed with chitosan on nitrogen assimilation, photosynthetic activity, and production of green beans (*Phaseolus vulgaris L.*)

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ABSTRACT

The individual application of ZnO nanoparticles and chitosan improve the growth and production of crops, compared to conventional fertilizers, their effectiveness is higher, and the doses can be reduced. However, there is little information regarding foliar application of ZnO nanoparticles complexed with chitosan and its effect on crop physiology. Therefore, the aim of the present study was to evaluate the efficiency of foliar application of zinc oxide nanoparticles versus zinc nitrate complexed with chitosan on the assimilation of nitrogen, photosynthetic activity, and production of green bean cv. Strike. Two sources of zinc were foliar-applied: zinc nanoparticles and zinc nitrate at doses of 0, 25, 50, and 100 ppm with and without chitosan. The results obtained indicate that the foliar application of Zn nanoparticles at 25 ppm and zinc nitrate at 50 ppm were the most efficient doses in favoring biomass accumulation and production. The addition of chitosan favored the biomass, production, and the parameters related to photosynthesis, especially when combined with zinc nitrate while the combination with ZnO nanoparticles presented a possible chelation of the nanoparticles, delaying the release of Zn. Finally, the best nanoparticle treatment was ZnO (25 ppm), which had similar results to the 50 ppm NZN treatment. This indicates that ZnO nanoparticles can reduce the amount of fertilizer to be used without affecting crop yield, so they could be used as nanofertilizers to maximize the productivity of agriculture crops. Finally, this indicates that more in-depth studies are required to know the physicochemical properties of ZnO nanoparticles complexed with chitosan and its effect on the physiology and biochemistry of plants.

1. Introduction

The common bean (*Phaseolus vulgaris L.*) is the legume with the highest direct consumption in the world since it is the main food source for more than 300 million people. In nutritional terms, it is the main source of protein of plant origin in addition to its high content of minerals, especially iron and zinc. In turn, consumption of beans in the form of green beans has anti-glycemic, antioxidant and lipid-lowering effects (Abu-Reidah et al., 2013; Blair, 2013). However, production of bean plants is limited due to various stress factors including micronutrient deficiency, especially zinc (Zn). Zinc is a necessary element to produce chlorophyll, protein synthesis and key in the production of biomass. It also plays an important role as a cofactor in the enzymes involved in the oxidative metabolism of plants; Zn deficiencies have also been reported to affect the photosynthetic rate, sugar accumulation, and the integrity of cell membranes. This deficiency is caused by the low availability of this element in the soil and the low efficiency in the use of fertilizers (Marschner, 2011; Cakmak and Kutman, 2018; Mahdieh et al., 2018).

Foliar fertilization has become an important tool to overcome micronutrient deficiencies in crops and has shown that it can improve crop yield and quality. However, the application of fertilizers in a foliar way can be limited by the formulation, the source and the particle size (Fernández et al., 2013). An advance in foliar fertilization is the use of nanotechnology, which emerges as an alternative to achieve the optimal development of plants in a sustainable and precise way. Because of the reduction in the size of the particles, the specific surface will increase and, as a consequence, the contact area with the plants will increase as well. With this, the plants can absorb them with a different dynamic,

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which has an additional advantage compared to the sources of traditional fertilization. All the particles smaller than 100 nm that provide some essential nutrient for plants are considered nanofertilizers (Subbaiah et al., 2016; Raliya et al., 2017; Sturikova et al., 2018).

The application of nanofertilizers in a foliar way, have positive effects on growth, development, and production in different crops. In addition to that, compared to conventional fertilizers, their effectiveness is higher, and the doses can be reduced, reducing the environmental impact (Davarpanah et al., 2016; El-Ramady et al., 2018). Applications of Zn nanoparticles, at low concentrations, have been shown to have positive effects on germination, vegetative growth, chlorophyll, carotene content, and significant increases in yield (Rossi et al., 2019; Siddiqui et al., 2019). On the other hand, applications in high concentrations have stressful effects that can inhibit root growth, delay crop development, and affect protein and DNA synthesis (Ma et al., 2015; Sturikova et al., 2018).

A viable alternative to mitigate stress situations is the foliar application of substances that have a biostimulant property, where the use of chitosan stands out, which is the deacetylated form of chitin, the second most abundant waste material and comes mainly from the exoskeleton of crustaceans and insects. Different authors have reported positive effects on vegetative growth, photosynthetic pigment concentration and yield in more than 20 plant species (Pichiyangkura and Chadchawan, 2015; Ibrahim and Ramadan, 2015; Orzali et al., 2017).

In general, there is little information on the use and application of nanoparticles along with organic substances such as chitosan. Therefore, the aim of the present study was to evaluate the impact of the foliar application of zinc nanoparticles plus urea and chitosan on the assimilation of nitrogen, photosynthetic activity, and production of green beans.

2. Materials and methods

2.1. Crop management

The experiment was carried out in a greenhouse covered with a shade house located at the CIAD facilities in Cd. Delicias, Chihuahua, Mexico, with an average temperature of 29.4 °C and an average relative humidity of 22.38 %. Bean plants cv. Strike were sown in polystyrene trays for germination, and 10 days later, they were transplanted into plastic pots with a diameter of 30.5 cm and a volume of 13.4 L, which were filled with a substrate composed of vermiculite and agricultural perlite in a proportion of 2:1. Two plants per pot were placed and watered with 500 mL of the following complete nutrient solution: 6 mM NH_4NO_3, 1.6 mM K_2HPO_4, 0.3 mM K_2SO_4, 4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5 μM Fe-EDDHA, 2 μM $MnSO_4$, 0.25 μM $CuSO_4$, 0.3 μM Na_2MoO_4, and 0.5 μM H_3BO_3. This solution was applied every third day during the first 30 days and daily the following 30 days. The treatments were applied as foliar spray. The foliar application was done every 10 days from the appearance of the first true leaves 15 days after transplantation (DDT) for a total of 4 applications.

2.2. Experimental design

A completely randomized design (DCA) with a 2*3*2 factorial arrangement plus an absolute control (control without application) and one with the application of chitosan (Quito) with four repetitions were used, factor A being the zinc sources: zinc nitrate of commercial use GoZinc® (NZN) and zinc nanoparticles (ZnO) added with urea to balance the nitrogen supply of zinc nitrate; factor B corresponded to the doses: 25, 50 and 100 ppm, and factor C was the addition of the bioregulator: with chitosan of commercial use Quitofyt® (Poly-D-glucosamine), which was selected for its anti-stress properties and its possible relationship as a carrier of metal particles at a dose of 50 ppm and without adding it; for a total of 14 treatments with four repetitions (Table 1).

Table 1
Treatments description

Zn Source	Dose (ppm)	Chitosan	Code
Control	0	No	Control
Control	0	Yes	Control+Q
ZnO	25	No	ZnO25
ZnO	50	No	ZnO50
ZnO	100	No	ZnO100
ZnO	25	Yes	ZnO25+Q
ZnO	50	Yes	ZnO50+Q
ZnO	100	Yes	ZnO100+Q
NZN	25	No	NZN25
NZN	50	No	NZN50
NZN	100	No	NZN100
NZN	25	Yes	NZN25+Q
NZN	50	Yes	NZN50+Q
NZN	100	Yes	NZN100+Q

2.3. Characterization of nanoparticles

Zinc oxide nanoparticles (ZnO) produced by the wet chemistry methodology in the form of Wurzite crystals were used, with a purity of 99.7% and an average size of 50 nm free of contaminants (Fig. 1). The morphology of the sample was obtained by means of scanning electron microscopy and transmission (Fig. 2). The material was provided by the company "Investigación y Desarrollo de Nanomateriales S.A. de C.V", located in San Luis Potosí, Mexico.

2.4. Plant sampling

After physiological maturity of the plants, 60 DDT, the samples were taken and separated into four parts: root, stem, leaf, and fruit and washed 3 times with distilled water and a 1% non-ionic detergent.

2.5. Plant analysis

2.5.1. Biomass

The dry weight of the leaf, stem, root and fruit was obtained with the help of an analytical balance (AND HR-120, San José, California, USA). Results will be expressed in grams per plant based on dry weight.

2.5.2. Production

The weight of the total fruit per plant was determined based on fresh matter and was expressed in grams per plant.

2.5.3. Nitrate reductase activity "in vivo"

In vivo nitrate reductase (NR) activity (EC 1.6.6.1) was determined using the method proposed by Sánchez et al. (2004). The leaf blades were cut into 7 mm sections and placed in 10 ml of incubation buffer (100 mM phosphate buffer K, pH 7.5 and 1% (v / v) propanol). The samples were infiltrated at a pressure of 0.8 bars. They were incubated at

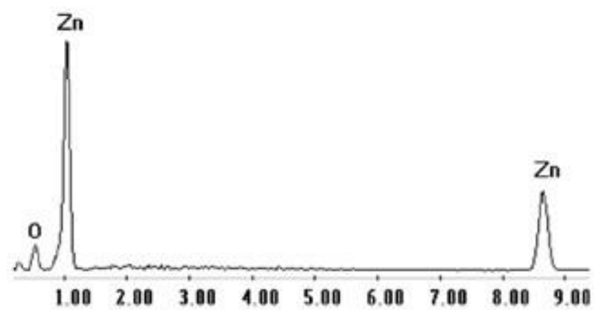

Fig. 1. Elemental analysis (chemical composition) of zinc oxide (ZnO) nanoparticles using X-ray scattering energy (EDX).

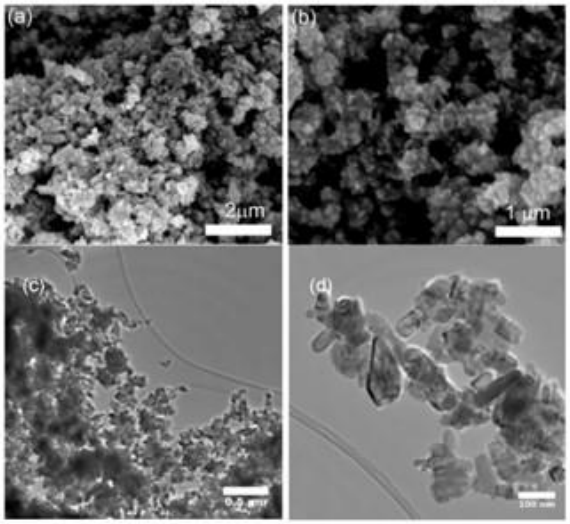

Fig. 2. (a, b) ZnO morphology using Scanning Electron Microcopy (SEM); (c, d) ZnO Morphology by Transmission Electron Microcopy (TEM).

30°C in the dark for 1 hr, and finally placed in a boiling water bath to stop NR activity. Then, 1 mL of enzyme extract was taken and 2 mL of 1% (w/v) sulfanilamide in 1.5 M HCl and 2 mL of 0.02% 1-naphthylethylenediamine N-dihydrochloride in 0.2 M HCl (w/v) were added. The resulting nitrite concentration was determined by spectrophotometry at 540 nm, against a standard curve of NO_2^-.

2.5.4. Photosynthetic activity and stomatal conductance

Photosynthetic activity and stomatal conductance were measured in leaves when the plant reached its physiological maturity (Kocal et al., 2008). A LI-COR 6400 portable meter (Lincoln, Nebraska, USA) was used, in each plant a healthy leaf of homogeneous color and free of damage was selected. A concentration of 400 μmol per mole of CO_2 was used in the reference cell, while the sample cell was kept at approximately 380 μmol per ml of CO_2. The vapor pressure deficit of the air in the sample chamber was less than 1.5 and the temperature of the block that housed the leaf was 25°C. Photosynthetic activity was expressed as μmol of $CO_2m^2{\bullet}s^{-1}$ and stomatal conductance was reported as mmol of $CO_2m^2{\bullet}s^{-1}$.

2.5.5. Chlorophyll index

The chlorophyll index was measured using a Minolta SPAD 502 chlorophyll reader (Konica Minolta Sensing, Inc., Osaka, Japan) for which five fully expanded leaves (without physical damage and rib-free parts) were used for each plant per treatment. The results obtained were expressed in SPAD units (Shrestha et al., 2012).

2.5.6. Photosynthetic pigments

Photosynthetic pigments were analyzed following the Wellburn (1994) methodology, for which 7 mm diameter leaf discs, free of ribs with an approximate weight of 0.125 g, were collected and placed in test tubes. Then 10 mL of methanol were added to each test tube and allowed to stand for 24 h in the dark. After this time, the reading was taken on a Genesis 10S UV-VIS spectrophotometer (Thermo Sientific, Waltham, Massachusetts, USA) at wavelengths of 666, 653 and 470 nm. The results were expressed in mg.g^{-1} of fresh weight and were calculated according to the following formulas:

$$Chla = [15.65(A666) - 7.34(A653)]$$

$$Chl\,b = [27.05(A653) - 11.21(A666)]$$

$$Carotenoids = [(1000 * A470) - 2.86(Chl\,a) - 129.2(Chlb)]/221$$

2.5.7. Amino acids and soluble proteins

For the quantification of amino acids and soluble proteins, 0.5 g of fresh material was weighed and homogenized in a 50 mM KH_2PO_4 buffer at pH 7 on a layer of ice to keep the sample cold. They were then centrifuged at 12,000 x g for 15 min. The supernatant obtained was used for the determination of total amino acids by the ninhydrin method with slight modifications (Sánchez et al., 2004); Total free amino acids were expressed as mg of glycine g^{-1} of fresh weight (FW). The soluble protein content was measured with the Bradford reagent (Kruger, 2009) and was expressed as mg.g^{-1} FW, using bovine serum albumin as standard.

2.6. Statistic analysis

An analysis of variance, a mean separation test using the LSD method, and a Pearson correlation analysis were performed using the SAS statistical package (SAS, 2004).

3. Results and Discussion

3.1. Biomass

Biomass accumulation is one of the most important variables to identify the correct functioning of plants and the efficiency of fertilization (Sánchez et al., 2016). In the present study, no statistical differences were found regarding the Zn source used (Fig. 3), placing nanoparticles as a viable alternative for biomass production, compared to a widely used traditional fertilizer. In addition, better performance was observed when the nanoparticle doses were lower, with ZnO25 being the treatment that presented the highest value, with an increase of 29.67% compared to the control without application. Various authors report similar effects when ZnO was used in a foliar way as a source of Zn. Mahdieh et al. (2018), found significant increases in relation to the control and traditional sources of zinc such as zinc sulfate and chelates, in the length of the shoots, the length of the internodes, the weight of the shoots and roots when they applied ZnO to bean plants. In turn, Burman et al. (2013) applied ZnO nanoparticles in chickpea and obtained an increase in biomass of 22.8% compared to their control when applying 1.5 ppm, but the increase in biomass was less when applying a higher dose. For their part, Raliya et al. (2015) found that doses above 250 ppm of ZnO reduced biomass in tomato and doses below had better results, which is adjusted with the results obtained in this study, although the results obtained are not significantly lower when increased the dose of ZnO, a downward trend can be observed. Previous studies mention that an excess of Zn can cause stress in plants, affecting their development and yield because they generate changes in the morphology and physiology of plants, (Sturikova et al., 2018; Balafrej et al., 2020).

Regarding the addition of chitosan, positive effects were found with respect to treatments without its application for the 50 and 100 ppm doses of ZnO and for the 25 and 50 ppm doses of NZN, the last two being the only ones that passed 26 g per plant dry weight. Previously, it was reported that foliar applications of chitosan improved vegetative growth under normal conditions and under stress conditions in crops such as beans, bell peppers and okra (Ghoname et al., 2010, Mondal et al., 2012; Abu-Muriefah, 2013). The increase in vegetative development can be related to studies where the stimulating action of chitosan against free oxygen radicals was demonstrated, mitigating possible stress situations, in addition to the fact that the use of chitosan as a biostimulant applied in a foliar way has had positive effects on the plant performance and growth (Pichyangkura and Chadchawan, 2015; Orzali et al., 2016; Morin-Crini et al., 2019).

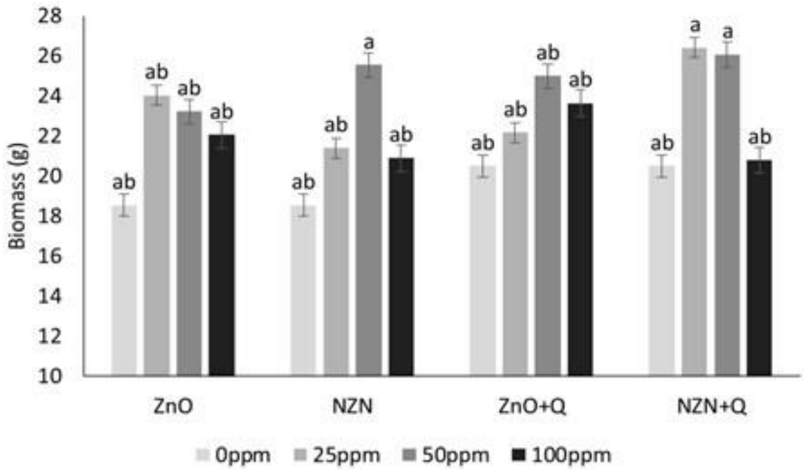

Fig. 3. Effect of the application of Nanoparticles and Zinc nitrate in combination with chitosan on the biomass (dry weight) of bean plants cv. Strike. The letters indicate significant differences.

3.2. Production

Studies related to the application of Zn nanoparticles are of great importance for modern agriculture, as they have shown that they can improve crop productivity and growth (Raliya et al., 2017). The production results showed significant differences (Fig. 4), highlighting the treatments of ZnO25, NZN50, NZN25+Q and NZN50+Q, which had increases of 18, 19.25, 30.75 and 29.5 g, respectively in relation to the control. In turn, these 4 treatments exceeded the average of 78 g per plant reported by Salinas-Ramírez et al. (2012). On the other hand, a positive correlation was found with biomass (Table 2) and likewise, the source factor did not obtain significant differences. However, the ZnO25 treatment obtained the same yield as NZN50. These results are consistent with the theory that the use of nanofertilizers can reduce the doses to be used without affecting the production of the crops (El-Ramady et al., 2018; Naresh et al., 2018). Previous results show that the application of ZnO nanoparticles increases the production of various crops, Mahdieh et al. (2018), found significant increases in yield in bean plants when ZnO nanoparticles are applied at a dose of 500, 1000 and 1500 ppm. Also, Genaidy et al. (2020), found increases of 9.08% in relation to their control in the yield of olive trees when 100 and 200 ppm of ZnO were applied via foliar application.

Foliar application of chitosan did not show significant differences. However, in the treatment of 100 ppm of nanoparticles, which was the one with the lowest production, an increase of 21.99% was observed when chitosan was applied. In the same way, the treatments of 25 and 50 ppm of NZN increased their production by 27.42 and 11.75%. Hassnain et al. (2020), found that the application of chitosan at doses of 50, 100 and 150 ppm increased the production of tomato plants, under normal conditions and under water stress. Similarly, Fahmy and Nosir (2021), obtained increases in the total yield of lavender plants when they added chitosan to a solution with micronutrients (Zn and Fe). The results obtained in the present study demonstrate positive effects of foliar application of chitosan and agree with previous studies where production was increased or maintained in plants such as pepper, beans and okra (Pichyangkura and Chadchawan, 2015).

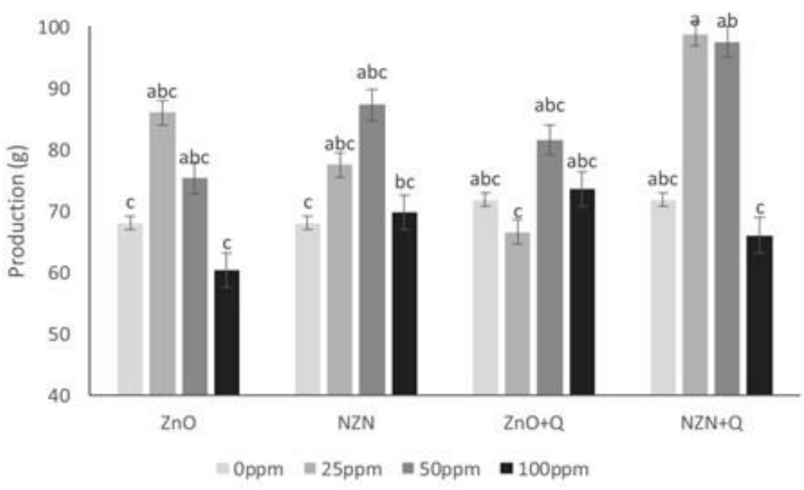

Fig. 4. Effect of the application of Nanoparticles and Zinc Nitrate in combination with Chitosan on the yield of bean plants cv. Strike. The letters indicate significant differences.

Table 2
Pearson correlation analysis for 14 green bean variables.

	SPAD1	SPAD2	SPAD3	Biomass	Production	NR	NR +NO_3	Dif NR	Chl	Car	PA	StCon	AA's	Protein
SPAD1	1	0.413**	0.418**	0.049	0.023	-0.065	0.024	0.067	0.247	0.246	0.112	-0.097	0.426	0.563*
		0.002	0.001	0.719	0.864	0.632	0.863	0.622	0.066	0.068	0.409	0.477	0.129	0.036
SPAD2	0.413**	1	0.555**	0.008	0.026	-0.301*	-0.299*	-0.175	0.204	0.145	-0.011	-0.156	0.326	0.444
	0.002		0.000	0.955	0.851	0.24	0.025	0.196	0.131	0.288	0.935	0.249	0.255	0.112
SPAD3	0.418**	0.555**	1	0.061	-0.003	-0.035	-0.135	-0.140	0.163	0.094	-0.002	-0.085	0.229	0.374
	0.001	0.000		0.656	0.983	0.796	0.319	0.303	0.230	0.489	0.987	0.531	0.430	0.188
Biomass	0.049	0.008	0.061	1	0.908**	0.074	-0.063	-0.119	0.004	-0.026	0.023	-0.063	-0.317	0.334
	0.719	0.955	0.656		0.000	0.589	0.644	0.381	0.978	0.851	0.865	0.644	0.27	0.242
Production	0.023	0.026	-0.003	0.908**	1	0.022	-0.200	-0.251	0.046	0.032	0.081	-0.050	0.31	0.326
	0.864	0.851	0.983	0.000		0.874	0.139	0.062	0.734	0.815	0.554	0.717	0.281	0.256
NR	-0.065	-0.301*	-0.035	0.074	0.022	1	0.544**	0.047	-0.172	0.008	-0.099	-0.211	-0.374	-0.09
	0.632	0.24	0.796	0.589	0.874		0.000	0.732	0.205	0.954	0.466	0.119	0.187	0.761
NR +NO3	0.024	-0.299*	-0.135	-0.063	-0.200	0.544**	1	0.864**	-0.174	0.114	0.053	0.072	-0.239	-0.307
	0.863	0.025	0.319	0.644	0.139	0.000		0.000	0.201	0.402	0.697	0.599	0.41	0.285
Dif NR	0.067	-0.175	-0.140	-0.119	-0.251	0.047	0.864**	1	-0.103	0.131	0.123	0.212	-0.035	-0.300
	0.622	0.196	0.303	0.381	0.062	0.732	0.000		0.499	0.336	0.366	0.117	0.907	0.298
Chl	0.247	0.204	0.163	0.004	0.046	-0.172	-0.174	-0.103	1	0.495**	0.340*	0.299*	0.587*	0.755**
	0.066	0.131	0.230	0.978	0.734	0.205	0.201	0.499		0.000	0.010	0.025	0.027	0.002
Car	0.246	0.145	0.094	-0.026	0.032	0.008	0.114	0.131	0.495**	1	0.228	0.073	0.256	0.249
	0.068	0.288	0.489	0.851	0.815	0.954	0.402	0.336	0.000		0.091	0.595	0.376	0.391
PA	0.112	-0.011	-0.002	0.023	0.081	-0.099	0.053	0.123	0.340*	0.228	1	0.580**	0.301	0.509
	0.409	0.935	0.987	0.865	0.554	0.466	0.697	0.366	0.010	0.091		0.000	0.296	0.063
StCon	-0.097	-0.156	-0.085	-0.063	-0.050	-0.211	0.072	0.212	0.299*	0.073	0.580**	1	0.22	-0.183
	0.477	0.249	0.531	0.644	0.717	0.119	0.599	0.117	0.025	0.595	0.000		0.449	0.53
AA's	0.426	0.326	0.229	-0.317	0.31	-0.374	-0.239	-0.035	0.587*	0.256	0.301	0.22	1	0.462
	0.129	0.255	0.430	0.27	0.281	0.187	0.41	0.907	0.027	0.376	0.296	0.449		0.096
Protein	0.563*	0.444	0.374	0.334	0.326	-0.09	-0.307	-0.300	0.755**	0.249	0.509	-0.183	0.462	1
	0.036	0.112	0.188	0.242	0.256	0.761	0.285	0.298	0.002	0.391	0.063	0.53	0.096	

. La correlación es significativa al nivel 0,01 (bilateral). *. La correlación es significante al nivel,05 (bilateral). **Abbreviations: Nitrate reductase activity (NR), induced nitrate reductase activity (NR+NO_3), nitrate reductase activity difference (Dif NR), clorofila (Chl), Carotenoids (Car), photosintetic activity (PA), stomatal conductivity (StCon), aminoacids (AA's).

3.3. Nitrate reductase activity "in vivo"

The enzyme Nitrate reductase (NR) oversees transforming the nitrate of the plants into nitrite for its subsequent assimilation and conversion to nitrogenous metabolites, so that its reaction is inducible with the application of nitrate (Maldonado, 2013). In the present work, significant differences were found in the activity of the NR enzyme (Table 3). All the treatments, except NZN100+Q, stimulated NR activity, with the ZnO100, NZN50, ZnO50+Q and NZN25+Q treatments being the most outstanding, obtaining an increase of more than 600% in relation to the control. Tapan-Adhikari et al. (2015) found similar increases when applying nanoparticles and Zn sulfate in corn plants, also mentioning that there are no outstanding differences between the two sources they used. In turn, Hemantaranjan and Trivedi (2015), found that the foliar application of Zinc increases NR activity. Furthermore, they mentioned that Zn acts indirectly on the assimilation of nitrogen due to the fundamental role it plays in the development and synthesis of proteins in plants. Regarding the application of chitosan, positive effects on the activity of this enzyme have been reported in crops such as corn, soybeans, and okra (Mondal et al., 2012).

When infiltrating the samples with NO_3^-, an increase in the NR activity was observed for all the treatments, highlighting the control which presented an increase 20 times greater than the sample without infiltration. These results indicate a deficiency of N for this treatment, which confirms the importance of Zn and the indirect participation in the assimilation of N (Hemantaranjan and Trivedi, 2015). Likewise, the NR activity of the treatments in which nanoparticles were applied obtained lower values than those of the treatment with conventional fertilizer, without affecting their production, consistent with the theory that the application of Zn in the form of nanoparticles shortened the time in which plants reached maturity, possibly associated with the phenomenon of nanoparticle penetration and their participation in enzymatic activities (Tapan-Adhikari et al., 2015).

3.4. Photosynthetic activity and stomatal conductance

Photosynthetic activity is a key process for the development of crops because it is the main source of energy for plants, growth, and production depends on it (Azcón-Bieto et al., 2008). In the present investigation, significant differences were obtained for photosynthetic activity (Fig. 5). In this case, the source factor was significant, finding that the application of NZN increased the values by 33.51% in relation to the ZnO treatments, highlighting the NZN25 and NZN50+Q treatments, which obtained increases of 48.39% and 30.18% respectively, in relation to control without application. Even though the ZnO treatments had less photosynthetic activity, the biomass and yield parameters were not affected, so it can be assumed that the plants treated with zinc nanoparticles reached maturity in a shorter time. On the other hand, Chávez-Simental and Álvarez-Reyna (2012) found that photosynthetic activity in beans tends to decrease as the harvest period approaches, finding its maximum point in the flowering stage. For their part, Rossi et al. (2019) reported higher values for photosynthetic activity in coffee plants during the first 45 days of development when applying 100 ppm of ZnO nanoparticles. While Wang et al. (2018) mentioned that the ZnO doses are very important to maintain a correct photosynthetic activity, since in their study said activity decreased as the dose increased, which supports the theory that nanoparticles work better at low doses.

Table 3
Effect of the application of Nanoparticles and Zinc nitrate in combination with chitosan on the activity of the enzyme nitrate reductase of bean plants Cv. Strike. The letters indicate significant differences.

Treatments	NRend	$NRNO_3$	Dif NR
Control	0.100 [cd]	2.113 [abc]	2.014 [a]
Control+Q	0.130 [cd]	0.964 [c]	0.834 [bc]
ZnO25	0.240 [cd]	1.533 [abc]	1.293 [abc]
ZnO50	0.442 [abcd]	1.473 [abc]	1.031 [abc]
ZnO100	0.622 [abcd]	1.104 [c]	0.482 [c]
NZN25	0.384 [bcd]	2.394 [ab]	2.010 [a]
NZN50	1.004 [a]	2.536 [a]	1.531 [ab]
NZN100	0.454 [abcd]	1.844 [abc]	1.390 [abc]
ZnO25+Q	0.148 [cd]	1.336 [bc]	1.188 [abc]
ZnO50+Q	0.811 [ab]	2.023 [abc]	1.212 [abc]
ZnO100+Q	0.128 [cd]	1.187 [c]	1.059 [abc]
NZN25+Q	0.626 [abc]	1.288 [bc]	0.663 [bc]
NZN50+Q	0.270 [bcd]	1.249 [bc]	0.979 [bc]
NZN100+Q	0.057 [d]	0.992 [c]	0.936 [bc]

Regarding stomatal conductance (Fig. 6), the results were similar, finding that the application of NZN increased the values by 21.27% more than the ZnO treatments; however, for this variable, both controls were in the outstanding group and it was found that the ZnO50 and ZnO100+Q treatments reduced stomatal conductivity by 31.4 and 30.6%, respectively. In the same way that, in photosynthetic activity, the application of ZnO obtained smaller values on average, which is possibly due to the correlation found between these two variables (Table 2). Various studies support the theory that the treated plants with nanoparticles they shortened their vegetative cycle (Chávez-Simental and Alvarez-Reyna, 2012; Rossi et al., 2019). In addition, Abdel-Aziz et al. (2016), applied nanoparticles of macronutrients in combination with chitosan showed significant increases in the harvest index, crop index and index of the yield variables in wheat, shortening the life cycle of the plants by 23.5% compared to normally fertilized plants, although this effect is related more to the application of nanoparticles than to the use of chitosan.

3.5. Chlorophyll index

The chlorophyll index is a fast and non-destructive way to detect chlorophyll levels in plants and thus form an idea of their nutritional situation (Shrestha et al., 2012). In the present research, significant differences were found for the chlorophyll index, in response to each application of the treatments carried out (Table 4). Regarding the first measurement of SPAD values, an increase of 12.9% in the treatment of NZN25+Q was obtained with respect to the control and 20.4% with respect to the control+Q. In the second SPAD measurement, the ZnO25, NZN25+Q and NZN100+Q treatments stood out, which obtained increases of 9.8, 11.2 and 9.3% respectively. Finally, the third SPAD measurement followed a similar trend, highlighting the same 3 treatments in addition to NZN50+Q. In this sampling, there was a decrease in chlorophyll levels. However, in the treatments where chitosan was applied, the decrease was less. The results obtained agree with those published by Medina-Pérez et al. (2018) which found a range of 35-50 SPAD units for Pinto beans applying 1.3 and 6 g of ZnO through fertigation; but, they found no significant difference from the control. Similar results were found by Medina-Velo et al. (2017) which applied different types of ZnO, without finding significant differences regarding their control. In relation to the application of chitosan, various studies reported that a foliar application increases the chlorophyll index despite stressful situations (Mondal et al., 2012; Abu-Muriefah, 2013; Behboudi et al., 2018).

3.6. Photosynthetic pigments

Photosynthetic pigments are very important for plants not only for their ability to capture light and convert it into energy, but also for the reducing power they possess, which allows them to face stressful situations (Jaleel et al., 2009). In relation to the present research, significant differences were obtained in the chlorophyll content (Fig. 7). The application of nanoparticles presented an increase of 15.45% compared to the control, while the use of NZN only increased by 4.67%. The results found were like those published by Ponce-García et al. (2019) who applied ZnO nanoparticles in an edaphic way in beans and found increases in chlorophyll levels of up to 83% in relation to a control without

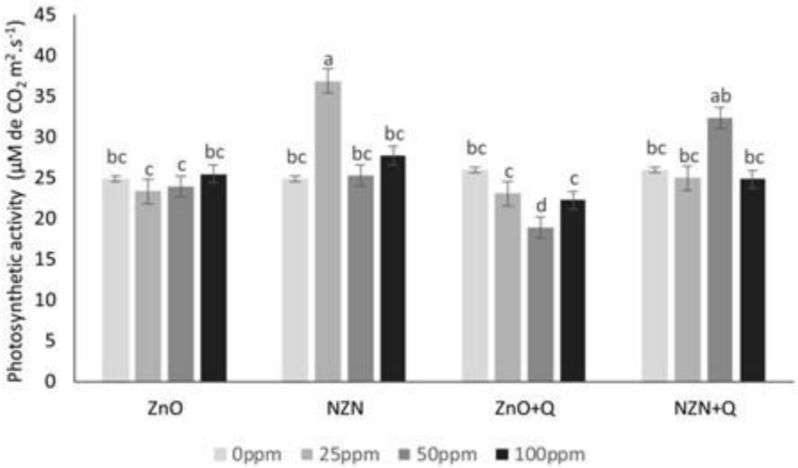

Fig. 5. Effect of the application of Nanoparticles and Zinc nitrate in combination with chitosan on the photosynthetic activity of bean plants Cv. Strike. The letters indicate significant differences.

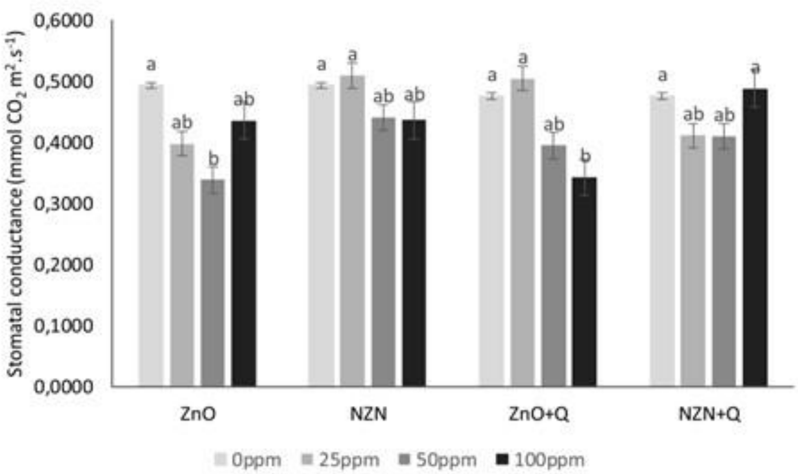

Fig. 6. Effect of the application of Nanoparticles and Zinc nitrate in combination with chitosan in the stomatal conductance of bean plants Cv. Strike. The letters indicate significant differences.

Table 4
Effect of the application of Nanoparticles (ZnO) and Zinc nitrate (NZN) in combination with chitosan on the chlorophyll index (SPAD units) of bean plants cv. Strike. The letters indicate significant differences.

Treatments	SPAD (30 DDT)	SPAD (40 DDT)	SPAD (50 DDT)
Control	37.0 ^{de}	37.89 ^{b}	36.61 ^{e}
Control+Q	34.64 ^{e}	37.765 ^{b}	36.98 ^{de}
ZnO25	40.08 ^{abc}	41.62 ^{a}	41.24 ^{ab}
ZnO50	40.45 ^{abc}	40.28 ^{ab}	38.06 ^{bcde}
ZnO100	39.5 ^{abcd}	39.52 ^{ab}	38.40 ^{abcde}
NZN25	40.44 ^{abc}	39.75 ^{ab}	37.72 ^{cde}
NZN50	38.75 ^{bcd}	38.13 ^{b}	39.48 ^{abcde}
NZN100	40.41 ^{abc}	39.66 ^{ab}	39.62 ^{abcde}
ZnO25+Q	40.13 ^{abc}	40.28 ^{ab}	40.21 ^{abc}
ZnO50+Q	38.30 ^{cd}	39.49 ^{ab}	39.22 ^{abcde}
ZnO100+Q	39.68 ^{abc}	40.21 ^{ab}	40.1 ^{abcd}
NZN25+Q	41.79 ^{a}	42.02 ^{a}	40.27 ^{abc}
NZN50+Q	40.22 ^{abc}	40.34 ^{ab}	41.34 ^{a}
NZN100+Q	41.15 ^{ab}	41.31 ^{a}	41.36 ^{a}

the application of zinc, while Torabian et al. (2016) reported increases of 14.7% in the total chlorophyll content applying foliar ZnO on sunflower plants. In turn, no significant differences were found for the chitosan factor. Despite this, the combination NZN+chitosan increased the chlorophyll content by 28.98% in relation to the plants to which only NZN was applied and in 34.99% with respect to the control; these results like those reported by Abu-Muriefah (2013), which increased chlorophyll levels in beans when applying chitosan. Various studies mention that the increase in chlorophyll levels is related to the effect of chitosan on cellular respiration and a greater efficiency to uptake nutrients (Ibrahim and Ramadan, 2015; Hidangmayum et al., 2019). In contrast, the combination of chitosan with ZnO, decreased chlorophyll levels by 3.5% compared to the control, this can be related to a possible chelation of ZnO by chitosan delaying its release. Vasconcelos (2014) mentions that chitosan has the property of chelating metal ions such as Zn, Cu, Ni, among others. For their part, Deshpande et al. (2017) mention that nanoparticles are slow release products and combining them with chitosan slow their release, having positive long-term results in the loss of nutrients and reduction of environmental contamination caused by

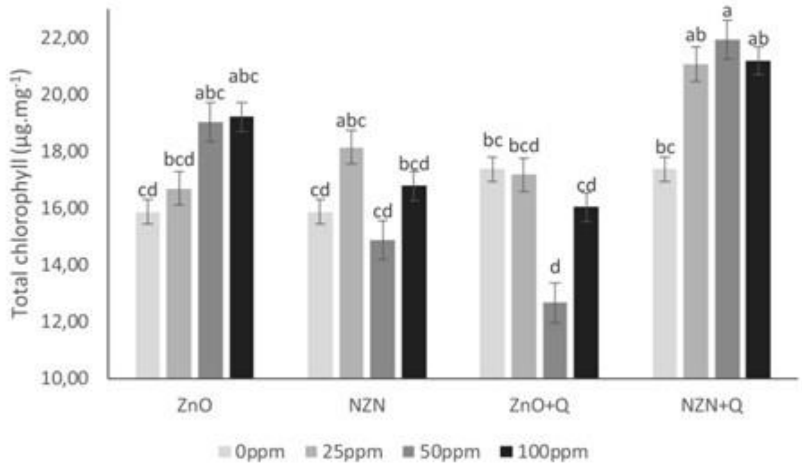

Fig. 7. Effect of the application of Nanoparticles and Zinc nitrate in combination with chitosan in the total chlorophyll of bean plants Cv. Strike. The letters indicate significant differences.

excess fertilizer applications.

Regarding the carotenoid content, an increase of 8.42% was obtained when ZnO was applied in relation to the control, while the application of NZN reduced the content by 4.21% (Fig. 8). Siddiqui et al. (2019) found increases in the carotenoid content when applying 0.010 $mg.ml^{-1}$ of ZnO in carrot plants. In the same way, Wang et al. (2016) obtained increases in the carotenoid contents when applying various doses of ZnO in Arabidopsis plants. These results indicate a possible acceleration in maturation in the plant when applying ZnO in the form of nanoparticles, since previous studies have related the increase in carotenoid content with the maturity state of a plant (Bramley, 2013; Llorente et al., 2016).

The application of chitosan + NZN found significant differences, following a trend like the chlorophyll content with which a positive correlation was found (Table 2), increasing carotenoid levels by 50.52% compared to the control and from 57.14% with the treatments without application of chitosan. On the other hand, the combination of ZnO + Chitosan did not obtain favorable results, this is possibly due to what was previously explained (Deshpande et al., 2017).

3.7. Amino acids and soluble proteins

The production of amino acids and proteins is the final step in the assimilation of nitrogen, which is why they become an indicator of good nutrition in plants (Sánchez et al., 2004). In the present investigation significant differences were found for both parameters. In the case of amino acids (Fig. 9), a positive correlation was found with the chlorophyll content and a similar trend with these results. The most outstanding treatments were ZnO100 and NZN100+Q, which had increases of 13 and 23% in relation to the control without application. Regarding the source used, the application of ZnO increased the amino acid content by 5.98% compared to the control without application, while the application of NZN reduced it by 7.8%. These results agree with those published by Patra et al. (2013), who found an increase in the amino acid content in Vigna radiata plants when they applied zinc nanoparticles but did not find significant differences between treatments. In the same way, the addition of chitosan showed a behavior similar to the chlorophyll content, finding an increase in the amino acid content compared to the control of 2.1% when combined with NZN and a reduction of 18.82% when ZnO was used. Previous studies reported

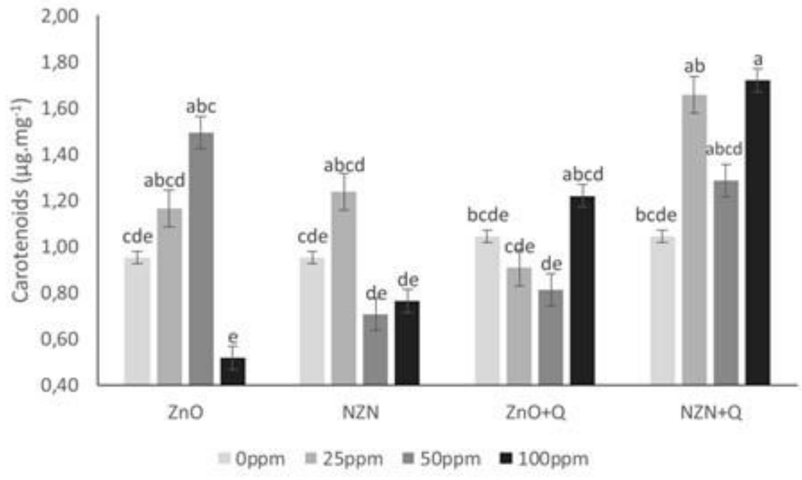

Fig. 8. Effect of the application of Nanoparticles and Zinc nitrate in combination with chitosan in the carotenoids of bean plants Cv. Strike. The letters indicate significant differences.

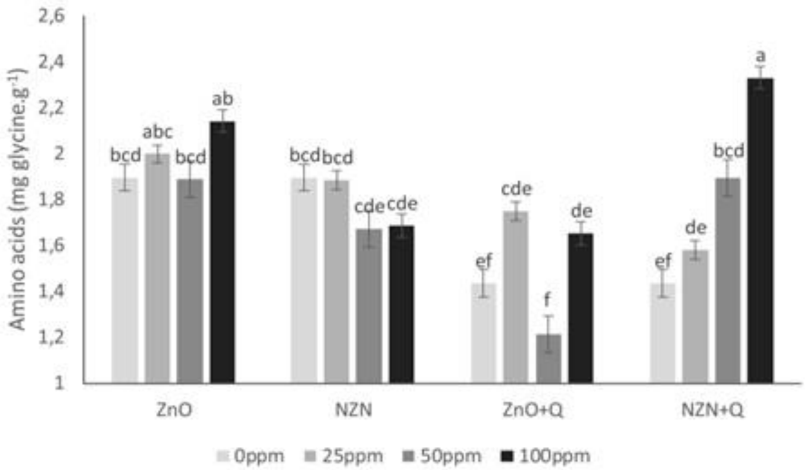

Fig. 9. Effect of the application of Nanoparticles and Zinc nitrate in combination with chitosan on the amino acid content of bean plants Cv. Strike. The letters indicate significant differences.

positive effects of foliar application of chitosan on parameters related to nitrogen growth and assimilation (Pichyangkura and Chadchawan, 2015). On the other hand, the results obtained from the combination with ZnO nanoparticles support the theory that chitosan had a chelating effect, delaying the release of zinc and, therefore, its absorption and use compared to the combination with a traditional source of zinc (Deshpande et al., 2017).

Similarly, the soluble protein content presented a positive correlation with the chlorophyll content (Table 2) and a similar trend to the amino acid content. Finding that the most outstanding treatments were NZN50+Q, NZN25+Q and ZnO100 (Fig. 10) with increases higher than 50% in relation to the control without application. In general, the foliar application of zinc increased the concentration of proteins, because zinc is a fundamental nutrient for the synthesis and structure of these, since it has a role in the translation and transcription of genetic material, it is believed that approximately 2,800 proteins depend on a correct supply of Zn (Marschner, 2011). In the present study, the use of nanoparticles stood out, which presented an increase of 45.13%, while the use of NZN only increased by 27.43% in relation to the control treatment. Similar results were published by Salama et al. (2019) which found increases in the total protein content as the dose of ZnO nanoparticles increased in common bean plants. For their part, Raliya and Tarafdar (2013) obtained an increase of 27.12% when applying ZnO nanoparticles in guar gum.

The addition of chitosan in combination with both sources had positive results compared to the control without application, increasing 2.45% when combined with ZnO and 56.04% in combination with NZN. This can be explained due to the positive effects of chitosan on the development of various crops, in addition to increases in the production of nitrogen compounds (Hidangmayum et al., 2019). Abu-Muriefah (2013) obtained an increase in the content of soluble proteins when applying chitosan in bean plants under water stress. However, as in previous variables, NZN+Q presented an increase in relation to treatments without chitosan application, while ZnO+Q had a lower concentration than the same treatment without chitosan application; therefore, in the same way as amino acids, it indicates the possible chelating effect of chitosan on ZnO nanoparticles (Deshpande et al., 2017).

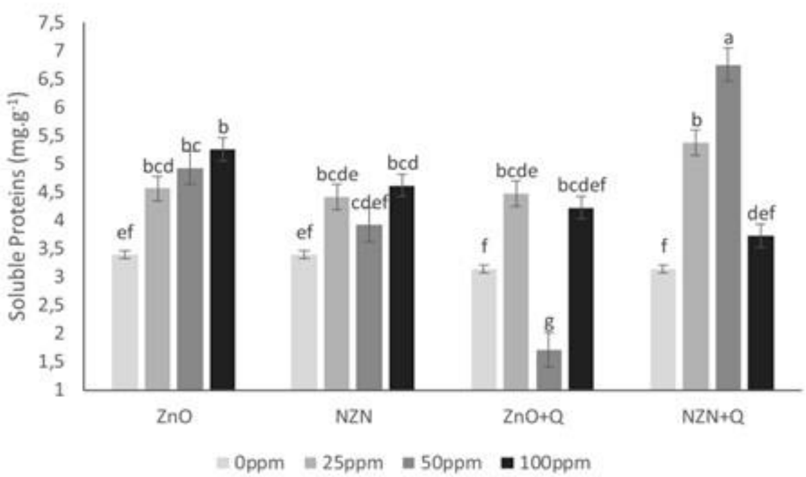

Fig. 10. Effect of the application of Nanoparticles and Zinc Nitrate in combination with Chitosan on the soluble protein content of bean plants Cv. Strike. The letters indicate significant differences.

4. Conclusions

The foliar application of nanoparticles of ZnO (25 ppm), NZN (50 ppm), NZN (25 ppm) +Chitosan and NZN (50 ppm) +Chitosan were the most efficient doses for favoring biomass accumulation and production in green beans. Likewise, foliar application of chitosan favored the biomass, production, and the parameters related to photosynthesis, especially when combined with NZN, while the combination with ZnO nanoparticles presented a possible chelation of the nanoparticles, delaying their release. The results obtained in the present study, particularly the carotenoid content and photosynthetic activity, suggest that the application of ZnO nanoparticles accelerated the maturation process of the plants, reaching their harvest time in a shorter time than those treated with the conventional fertilizer. The best nanoparticle treatment was ZnO (25 ppm), which had similar results to the 50-ppm treatment of NZN, which indicates that ZnO nanoparticles can reduce the amount of fertilizer to be used without affecting crop yields, for which could be used as nanofertilizers to maximize the productivity of agriculture crops. Finally, indicate that more in-depth studies are required to know the physicochemical properties of ZnO Nanoparticles complexed with chitosan and its effect on the physiology and biochemistry of plants.

Declaration of Competing Interest

None.

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5. BIOFORTIFICATION WITH NANOPARTICLES AND ZINC NITRATE PLUS CHITOSAN IN GREEN BEANS: EFFECTS ON YIELD AND MINERAL CONTENT.

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Biofortification with nanoparticles and zinc nitrate plus chitosan in green beans: effects on yield and mineral content

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Abstract

Approximately 33% of the world's population is affected by Zinc (Zn) deficiency, making it the fifth leading cause of human disease and mortality. An innovative strategy to this problem in the food diet is biofortification. Therefore, the use of nanotechnology emerges as a possible way to achieve the optimal development of plants in a sustainable and precise way. The objective of the present study was to increase the Zn content in bean plants cv. 'Strike', through the application of nanoparticles versus Zn nitrate plus chitosan. Two sources of Zn were applied via foliar: Zn nanoparticles and Zn nitrate at doses of 0, 25, 50 and 100 ppm with and without chitosan. The results indicate that the application of Zn favours the biofortification process, finding increases for all the treatments used. The treatments that stood out were Zn nitrate plus chitosan at 50 and 100 ppm, which increased the Zn content in fruits by more than 110%. The application of Zn nanoparticles at 25 ppm and Zn nitrate at 50 ppm favoured biomass accumulation and production. Furthermore, the addition of chitosan helped biomass and yield, especially when combined with Zn nitrate. Finally, indicate that a greater number of studies are required regarding the use of nanoparticles and chitosan in horticulture to determine with certainty their effect on the physiology and nutrition of plants.

Keywords: biofortification; bioregulators; nanofertilizers; *Phaseolus vulgaris*; zinc

Introduction

Globally, malnutrition affects more than 2 billion people around the world, causing problems such as heart disease, cancer, stroke, and diabetes. There are currently around 821 million people who suffer from hunger (WHO, 2018; FAO, 2019). Malnutrition has become a serious child health problem, being the cause of death of about 3.1 million children under 5 years of age, which represents 45% of all child deaths annually. It also contributes to increased infectious mortality, neurological disability, physical and mental growth

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retardation; In addition, early malnutrition increases the risk of obesity in adulthood. One of the causes of this problem is the deficiency of vitamins and nutrients such as iron (Fe) and zinc (Zn) (Bouis and Welch, 2010; Kane *et al.*, 2015; Bourke *et al.*, 2016; WHO, 2018).

Zn is an essential nutrient for almost all organisms and plays a key role as a metal cofactor in more than 300 proteins within the human body. In addition, it plays an essential role as a stabilizer and hormone receptor; It also acts as a regulator, being essential for the synthesis of biomolecules such as DNA and binds to nuclear proteins and forms complexes called "Zn fingers" (Caro *et al.*, 2016; Taboada-Lugo, 2017). The requirement for Zn in adults is 11 $mg.day^{-1}$ and it is present in an amount of 2 to 3 g, being the second most abundant micronutrient in the human body. Zn deficiency is very common in countries where the diet is unbalanced, mainly in places with cereal-based diets and low protein intake. Approximately 33% of the world population is affected by Zn deficiency, being the cause of 5% of deaths in children under 5 years of age; while, in developing countries, Zn deficiency is the fifth leading cause of human disease and mortality (Bilski *et al.*, 2012; Singh and Prasad, 2014; Reed *et al.*, 2015).

Currently, an innovative strategy to the problem of micronutrient malnutrition in the diet is "Biofortification". Which has been defined as the process of increasing the bioavailable concentrations of essential elements in edible portions of cultivated plants through agronomic management (fertilization) or genetic improvement (White and Broadley, 2005; Cakmak and Kutman, 2018). The common bean (*Phaseolus vulgaris* L.) is one of the five species that have been considered for biofortification programs because it is the legume with the highest direct consumption in the world, since it is the main food for more than 300 million people in regions of Africa and Latin America. In nutritional terms, beans are the main source of plant-based protein, in addition to their high content of minerals, especially Fe and Zn, and their great contribution of vitamins (Blair, 2013; Petry *et al.*, 2014).

To carry out the process of increasing the concentration of minerals in plant tissues, the exogenous application of these nutrients through fertilization is necessary. An important tool to combat micronutrient deficiencies in crops is foliar fertilization, which has been shown to improve crop yield and quality. However, the application of fertilizers in a foliar way can be limited by the formulation, the source, and the particle size (Fernández *et al.*, 2013; Blasco, 2015). Based on the above, the use of nanotechnology emerges as a possible way to achieve the optimal development of plants in a sustainable and precise way; given their size, plants can absorb them with different dynamics, which presents an additional advantage. However, the excessive use of these fertilizers can cause stress damage to crop, so the study of nanoparticles and their interaction with plants requires further studies to determine the extent of their benefits (Raliya *et al.*, 2017; Sturikova *et al.*, 2018). An alternative to mitigate stress situations caused using this new technology is the use of chitosan, which favors root growth and nutrient absorption, in addition to helping the plant to face stress situations and against pathogen attacks (Pichyangkura and Chadchawan, 2015; Saharan *et al.*, 2016; Deshpande *et al.*, 2017).

In general, there is little information on the use and application of nanoparticles together with organic substances such as chitosan in biofortification works. Based on the above, the objective of this research work was to increase the Zn content in bean plants (biofortification) through the application of nanoparticles versus Zn nitrate plus chitosan, as well as to evaluate the effect of these compounds on the mineral content in fruits of the bean.

Materials and Methods

Crop management

The experiment was carried out in a greenhouse covered with shade mesh located at the CIAD facilities in Cd. Delicias, Chihuahua, Mexico, with an average temperature of 29.4 °C and an average relative humidity of 22.38%. Green bean plants cv. 'Strike' which were transplanted 10 days after emergence in plastic pots with a diameter of 30.5 cm and a volume of 13.4 L, a substrate formed by vermiculite and agricultural perlite was used in a 2:1 ratio. Two plants were placed per pot and watered with the following nutrient solution: 6 mM NH_4NO_3, 1.6 mM K_2HPO_4, 0.3 mM K_2SO_4, 4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5 µM Fe-EDDHA, 2 µM $MnSO_4$, 0.25 µM $CuSO_4$, 0.3 µM Na_2MoO_4 and 0.5 µM H_3BO_3; which was applied every third day during the first 30 days and daily the following 30 days.

Experimental design

A completely randomized experimental design (DCA) was used with a 2*3*2 factorial arrangement plus an absolute control (control without application) and one with the application of chitosan with four repetitions, being factor A the Zn sources: Zn nitrate(7% N and 17% Zn) of commercial use brand GoZinc® (NZN) and nanoparticles of Zn oxide (ZnO) added with urea to equalize the nitrogen supply; factor B, corresponded to the doses: 0, 25, 50 and 100 ppm and factor C, was the addition of the bioregulator: with commercial use chitosan brand Quitofyt® (Poly-D-glucosamine) at a dose of 50 ppm and without the addition thereof; for a total of 14 treatments (Table 1) with four repetitions. The treatments were applied via foliar four times every 10 days from the appearance of the first true leaves.

Table 1. Description of the zinc plus chitosan treatments

Source of Zn	Dose (ppm)	Chitosan	Abbreviation
Control	0	No	Control
Control	0	Yes	Control + Q
ZnO	25	No	ZnO25
ZnO	50	No	ZnO50
ZnO	100	No	ZnO100
ZnO	25	Yes	ZnO25+Q
ZnO	50	Yes	ZnO50+Q
ZnO	100	Yes	ZnO100+Q
NZN	25	No	NZN25
NZN	50	No	NZN50
NZN	100	No	NZN100
NZN	25	Yes	NZN25+Q
NZN	50	Yes	NZN50+Q
NZN	100	Yes	NZN100+Q

Characterization of nanoparticles

Zinc oxide (ZnO) nanoparticles produced by wet chemistry methodology were used in the form of Wurzite crystals, with a purity of 99.7% and an average size of 50 nm free of contaminants (Figure 1). The morphology of the sample was obtained by scanning and transmission electron microscopy (Figure 2). The material was provided by the company "Investigación y Desarrollo de Nanomateriales S.A. de C.V", located in San Luis Potosí, Mexico.

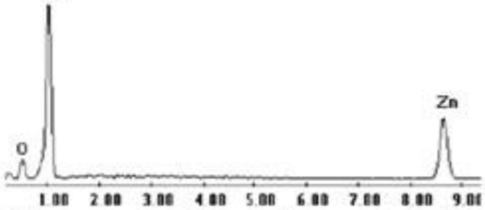

Figure 1. Elemental analysis Elemental analysis (chemical composition) of zinc oxide (ZnO) nanoparticles by energy dispersive X-rays (EDX)

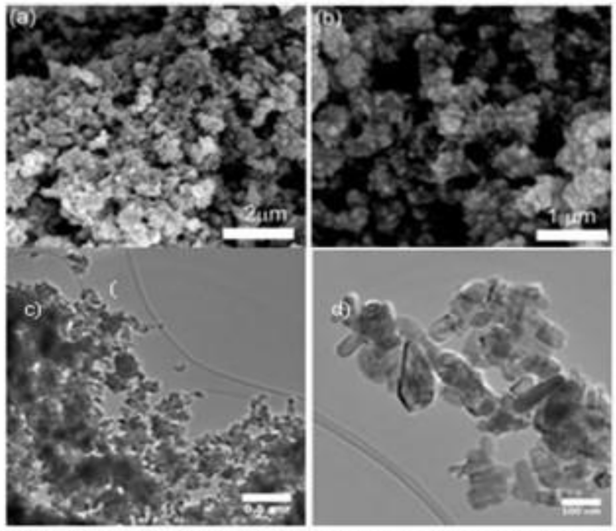

Figure 2. (a, b) ZnO morphology by Scanning Electron Microcopy (SEM); (c, d) ZnO morphology by Transmission Electron Microscopy (TEM)

Plant sampling

Once the plants reached maturity (60 days after sowing), the samples were taken and separated into 4 parts: root, stem, leaf, and fruit and washed 3 times with distilled water and a non-ionic detergent at 1%. The samples were dried in an oven at 70 °C for 48 h for biomass, mineral content, and protein analysis.

Variables evaluated

Biomass

The dry weight per plant was obtained with the help of an analytical balance (AND HR-120, San José, California, USA). The results were expressed in grams per plant on a dry weight basis.

Yield

The weight of the total fruits per plant was determined based on fresh matter and expressed in grams per plant.

Nitrogen (N), sulphur (S) and protein content

The content of N, S and protein was determined using a Flash 2000 unit (Thermo Scientific, Waltham, MA, USA), using the methodology proposed by Calvo *et al.* (2008). The results were expressed as a percentage for the three variables.

Mineral content

The mineral content was determined using the method proposed by Wolf (1982). In which, one gram of dry sample was weighed and 25 mL of triacid mixture (88.9% HNO_3, 8.9% HCl and 2.2% H_2SO_4) were added and placed in a digester oven at 300 °C. The resulting sample was made up to 50 ml with distilled water (main sample). The reading of potassium (K), calcium (Ca), magnesium (Mg), zinc (Zn), iron (Fe), manganese (Mn), copper (Cu) and nickel (Ni) was performed using an atomic absorption apparatus (AAS, iCE 3000 Series, Thermo Scientific, Waltham, MA, USA.). In the case of K, Ca, and Mg, a 1:100 dilution was made for reading.

For its part, the reading of phosphorus (P) was carried out following the ammonium metavanadate method (NH_4VO_3). 0.5 mL of the main sample was taken in a test tube to which 1 mL of phosphorus reagent plus 3.5 mL of distilled water was added, stirred, and left to stand for one hour for later reading. The reading was made in a spectrophotometer (Genesis 10s UV/Vis, Thermo Scientific, Waltham, MA, USA.) at 430nm against a K_2HPO_4 standard curve. The concentration of P was expressed as a percentage.

Degree of biofortification and distribution pattern

The degree of biofortification was expressed as a percentage and was determined according to the following formula:

$$GB = \left(\frac{ZnF * 100}{ZnC}\right) - 100$$

Where:

GB: degree of biofortification

ZnF: Zn content in the fruit of plants with foliar application of Zn

ZnC: Zn content in the fruit of the control treatment

While the distribution pattern consisted of the fraction of Zn that each organ of the plant contained based on the total Zn of the same and was expressed as a percentage.

Statistical analysis

An analysis of variance, a mean separation test using the LSD method, and a Pearson correlation analysis using the SAS statistical package (SAS, 2004) were performed on the data obtained.

Results and Discussion

Biomass

The food industry seeks sustainable solutions in modern agriculture for biomass production, due to the excess resources it consumes to maintain the required food production (Joshi *et al.*, 2018). In the present investigation, no changes were found regarding the Zn source used (Figure 3), placing nanoparticles as a viable alternative for biomass production, compared to a widely used traditional fertilizer. In addition, a better performance was observed when the doses of nanoparticles were lower, with ZnO25 being the treatment that presented the highest value with an increase of 29.67% compared to the control without application. Various authors report similar effects when ZnO was used in a foliar manner as a Zn source. Garcia-Lopez *et al.* (2019) applied ZnO nanoparticles in habanero pepper plants and obtained an increase in biomass of 10.21% compared to their control with their lowest dose and when their dose doubled, biomass was reduced. For their part, Elizabath *et al.* (2017) found greater growth in carrot plants when they applied 50 ppm of ZnO and when the dose was increased, this growth was reduced. Previous studies mention that Zn excess may cause stress in plants, affecting their development and yield (Sturikova *et al.*, 2018).

In relation to the addition of chitosan, positive effects were found with respect to the treatments without its application for the doses of 50 and 100 ppm of ZnO and for the doses of 25 and 50 ppm of NZN, the last two being the only ones that passed of 26 g per plant of dry weight. Previously, Salimi *et al.* (2019), reported that by combining chitosan with Zn and applying it foliarly, increases in the biomass of tomato plants were obtained. Furthermore, foliar applications of chitosan improved vegetative growth under stress conditions in bean plants (Abu-Muriefah, 2013). The increase in dry matter production can be related to studies where the stimulating action of chitosan against oxygen free radicals was demonstrated, mitigating possible stress situations generating greater vegetative growth (Pichyangkura and Chadchawan, 2015).

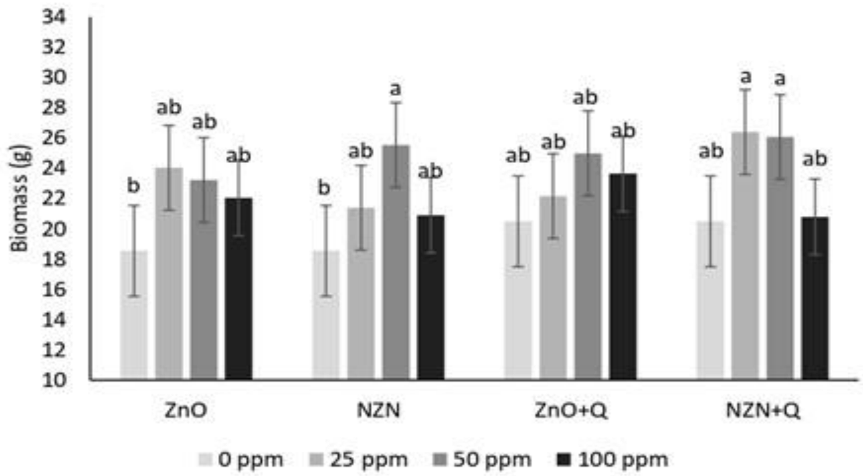

Figure 3. Effect of the application of nanoparticles and Zn nitrate in combination with chitosan on the biomass (dry weight) of bean plants cv. 'Strike'
Different letters indicate significant differences. Vertical bars indicate ±S.D.

Yield

Studies related to the application of nanoparticles and their effects on plants are of great importance to determine the exact amount of nutrients that should be applied and standardize the productivity and growth of crops (Feregrino-Pérez *et al.*, 2018). The production results showed significant differences (Figure 4), standing out the treatments of ZnO25, NZN50, NZN25+Q and NZN50+Q, which had increases of 18, 19.25, 30.75 and 29.5 g, respectively in relation to the control. In turn, these 4 treatments exceeded the average of 78 g per green bean plant reported by Salinas-Ramírez *et al.* (2012). In contrast, the source factor did not obtain significant differences, however, the ZnO25 treatment obtained the same performance as NZN50. These results support the theory that the application of nanofertilizers can reduce the amount of conventional fertilizer to be used, without affecting crop production (El-Ramady *et al.*, 2018).

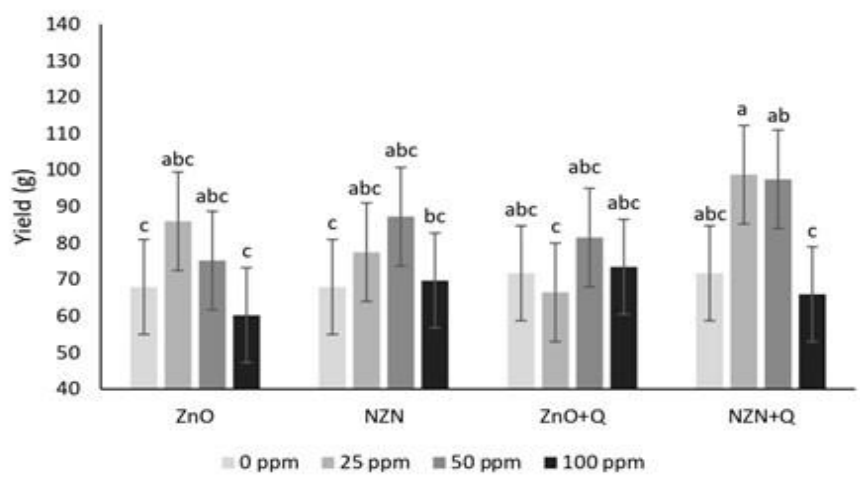

Figure 4. Effect of the application of nanoparticles and Zn nitrate in combination with chitosan on the yield of bean plants cv. 'Strike'

Different letters indicate significant differences. Vertical bars indicate ±S.D.

The foliar application of chitosan did not present significant differences, however, in the treatment of 100 ppm of nanoparticles, which was the one with the lowest production, an increase of 21.99% was observed when chitosan was applied. In the same way, the 25 and 50 ppm NZN treatments increased their production by 27.42 and 11.75%. The results obtained in the present study demonstrate positive effects of the foliar application of chitosan as well as those published by Shehata *et al.* (2012), who obtained increases in the yield of cucumber plants during 2 cycles when applying chitosan foliarly; likewise, Mahmood *et al.* (2017), through the application of chitosan increased the weight, diameter, and number of fruits in chili plants. Several studies show positive effects when applying chitosan in a foliar way in numerous crops, mainly when they are under stress situations (Pichyangkura and Chadchawan, 2015; Malerba and Cerana, 2018).

Zinc content

Zn fertilization has increased in recent years both in soil and foliar, with the objective of increasing production. However, its use as an alternative to increase the content of this nutrient in edible parts remains little studied (Cakmak and Kutman, 2018). The present research work presented significant differences for the Zn content in the different organs of bean plants (Figure 5), because all treatments obtained increases in relation to the control without application. While the source used did not present differences, placing nanoparticles as an equally effective alternative to correct Zn deficiencies; while the dose factor showed a linear trend in Zn content as the dose increased. These results agree with those published by Salama *et al.* (2019), who applied ZnO in increasing doses up to 40 ppm and obtained an increase in the Zn content in bean leaves as the dose increased. In the same way, Akanbi-Gada *et al.* (2019), mentions that when applying Zn nanoparticles in an edaphic way, the same result is obtained, that is, a linear increase in the Zn concentration. However, she mentions that the translocation of Zn is lower in this way, so this may explain why the control treatment did not present a high concentration of Zn in leaves. For their part, Broadley *et al.* (2012), mentions that plants can develop with a concentration of 20 $mg.kg^{-1}$ in mature leaves, presenting symptoms of deficiencies below 15 $mg.kg^{-1}$, while Mitra (2015), considers a range of 25 to 150 ppm of Zn as the optimal range for the correct development of plants in general.

On the other hand, the application of chitosan did not favour the total content of Zn in relation to its counterparts without application, but it presented a better result when combined with NZN, slightly increasing the content of Zn in fruit, root and stem, only decreasing in leaf, but without affecting the total Zn content (less than 1% difference), which indicates a greater translocation; these results differ from those published by Mirbolook *et al.* (2021), which indicate that chitosan favoured the absorption of Zn when applied to the roots, but the translocation factor was lower than when applying Zn only in the form of Zn sulphate ($ZnSO_4$).

Degree of biofortification and distribution pattern

The agronomic biofortification process can increase the content of a specific nutrient in the fruit of the plants without genetically affecting their composition (Sida-Arreola *et al.*, 2017). In the present study, significant differences were obtained between treatments, finding that the treatments that included Zn applied foliarly increased the Zn content in the green bean (Table 2), in a range of 32.96 to 129.05 % in relation to the control without application. Cambraia *et al.* (2019), indicate that the foliar application of Zn can increase the content in bean grains from 4 to 40%, while in other crops the increases can reach up to 420%; however, there is no previous reference of the increase that the bean in the form of green beans can reach.

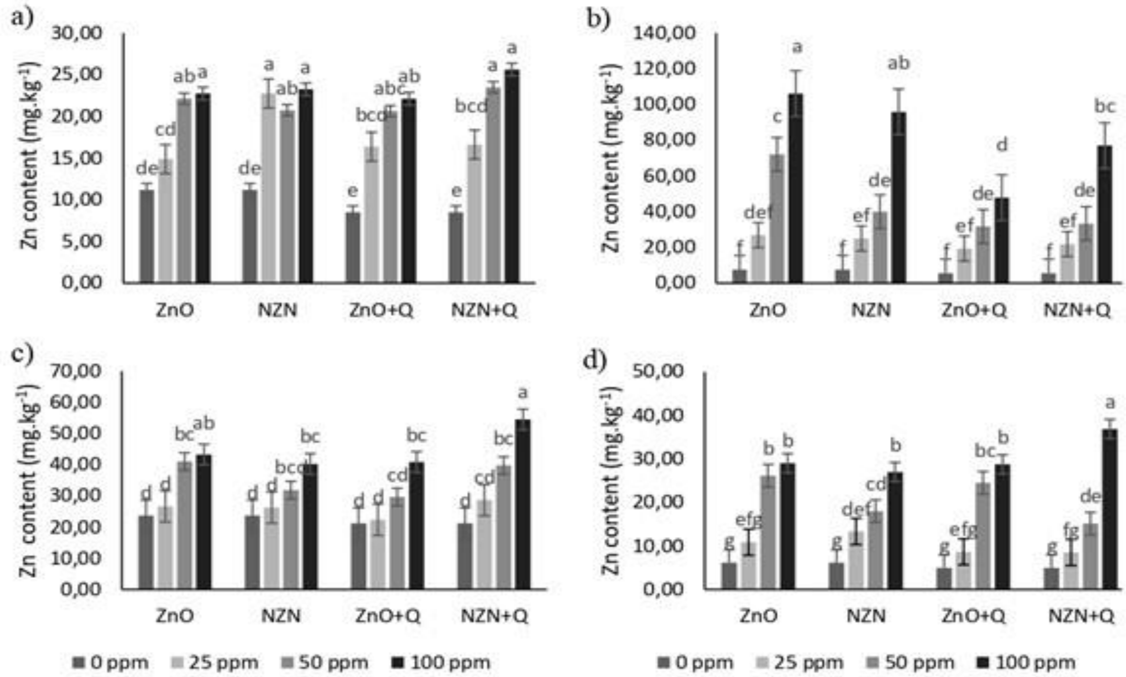

Figure 5. Effect of the application of nanoparticles and Zn nitrate in combination with chitosan on the zinc content in bean plants cv 'Strike'.
a) fruit, b) leaf, c) root, d) stem; Different letters indicate significant differences. Vertical bars indicate ±S.D.

Table 2. Degree of biofortification achieved by the application of nanoparticles and Zn nitrate in combination with chitosan in green bean cv. 'Strike'

Treatment	Degree of biofortification (%)
ZnO25	32.96 c
ZnO50	97.77 ab
ZnO100	103.35 a
NZN25	103.35 a
NZN50	85.47 abc
NZN100	107.82 a
ZnO25+Q	46.37 bc
ZnO50+Q	84.36 abc
ZnO100+Q	97.77 ab
NZN25+Q	48.60 bc
NZN50+Q	110.06 a
NZN100+Q	129.05 a

Different letters indicate significant differences.

For their part, Haider *et al.* (2021), report a range of 11.81% to 106% in various varieties of mung bean with the soil application of Zn. Alternatively, Poblaciones and Rengel (2016), report an increase in Zn content of more than 300% in pea fruits with their foliar application treatment in relation to the control without Zn application. In addition, they report that this increase was reduced when foliar application was combined with a soil application, in agreement with various studies that foliar Zn application may be a more effective way to achieve biofortification than soil application and priming in legume seeds (Jha and Warkentin, 2020). This could be explained by Fernández *et al*. (2013), which indicate that Zn tends to move from the leaves and stems to the fruits when the senescence stage of the plants begins.

Within the treatments, the one that obtained the best result was NZN100+Q, increasing the Zn content in the fruit by 129.05%. However, among the treatments that are in the same range of significance, the NZN50+Q treatment should be highlighted, which increased the Zn content in the fruit by 110.06% in relation to the control and stands out in the biomass and yield variables (Figures 3 and 4). In addition, when comparing it with products found on the market, it had an increase of 21.89% in relation to the values reported by Fernández-Valenciano and Sánchez-Chávez (2017), for green beans obtained in supermarkets in Mexico.

Regarding the distribution pattern, a marked difference is observed due to the foliar application of Zn (Figure 6), generating a higher concentration in the leaf in relation to the control treatments, which had a higher concentration in the root. These results were expected because Zn is considered a medium or conditionally mobile element, for which it is logical to find a higher concentration of Zn in the organs where it was applied, however, various studies indicate that despite its reduced mobility these applications have a significant benefit in the development of the plant (Broadley *et al*., 2012; Fernández *et al*., 2013). Concerning the source used, a slight change in the distribution pattern can be observed, especially in the concentration of Zn in the fruit, increasing by 3.84% when NZN was applied compared to the application of ZnO. Fernandez *et al.* (2013), mentions that the chemical form in which a product is applied can influence its foliar absorption, but it is not confirmed that it can influence its mobilization within the plant, so more in-depth studies are necessary to determine whether the chemical source can influence the distribution pattern of nutrients in the plant.

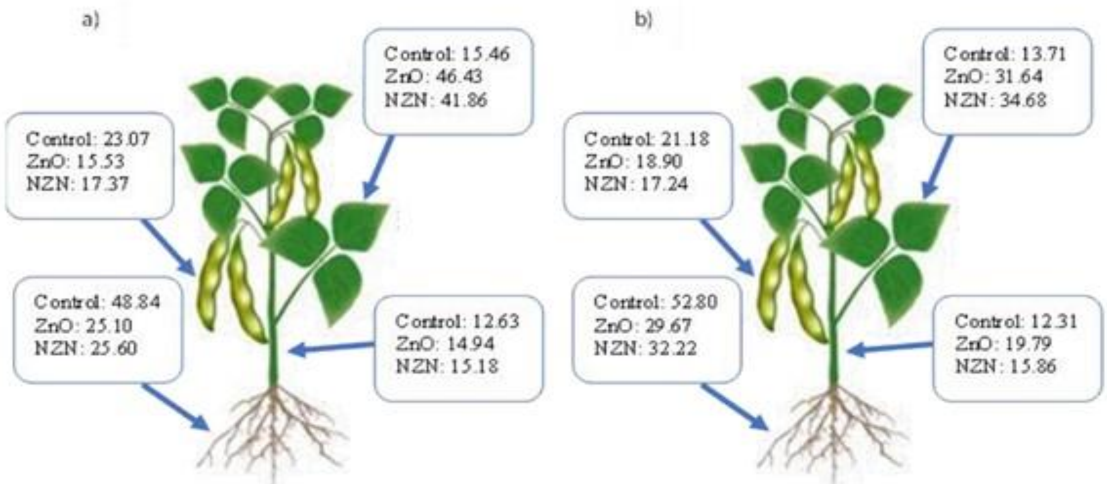

Figure 6. Zn distribution pattern in response to the application of nanoparticles and Zn nitrate in combination with chitosan in bean plants cv.

a) without chitosan. b) with chitosan; The results are expressed in percentage

The application of chitosan, when combined with Zn sources, reduced the leaf percentage for ZnO by 14.79% and 7.18% for NZN, indicating a possible increase in Zn mobility. In addition, in the case of the combination with nanoparticles, increases in the distribution pattern of 3.37 % in fruit, 4.57 % in root and 4.58 % in stem were obtained; results that indicate a possible effect of chitosan on nanoparticles that allows a greater mobility of Zn in the plant. This hypothesis had been reported by Choudhary et al. (2019), whose results suggests that the Zn released from a chitosan matrix applied to maize plants moved from the leaf to the grain during the filling stage, increasing the Zn concentration.

Mineral content

Mineral content is essential for the correct development of plants; N and P are considered the elements that most affect plant growth, however, the content of other nutrients is just as important to achieve a maximum physiological response in plants (Weih *et al.*, 2018). In the present study, significant differences were obtained for the mineral content of the elements analysed except for Cu (Table 3). In the same way as Zn, all the treatments increased the N content in the leaf, obtaining a positive correlation between these two elements (Table 4). These results could be explained due to the nitrogenous contribution included in the treatments; In addition, various studies suggest a beneficial relationship between N and Zn, finding that N deficiency does not allow the ideal absorption of Zn (Hafeez *et al.*, 2013; Montoya *et al.*, 2020). On the other hand, González *et al.* (2019), mention that when the Zn content is sufficient or high, there is a positive effect of N in the biofortification process.

Table 3. Effect of the application of Nanoparticles and Zn nitrate in combination with chitosan on the foliar mineral content in bean plants cv. 'Strike'

Treatment	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	S (%)	Fe (ppm)	Mn (ppm)	Cu (ppm)
Control	2.01 de	0.35 b	1.73 b	3.79 ab	2.39 abcd	0.12 c	144.31 ab	151.69 fg	18.94 a
Control + Q	1.58 e	0.39 a	2.10 a	2.16 ef	2.42 abc	0.10 c	151.94 a	166.13 def	15.63 a
ZnO25	3.18 bcd	0.19 cd	1.31 d	4.72 a	2.36 bcdef	0.17 c	127.50 bcd	142.88 fg	17.38 a
ZnO50	3.58 abc	0.20 cd	1.32 d	3.05 bcde	2.37 abcde	0.18 c	129.13 bc	156.13 efg	19.50 a
ZnO100	4.77 a	0.20 cd	1.25 d	3.76 abc	2.46 ab	0.32 ab	110.63 def	136.63 g	17.25 a
NZN25	4.58 ab	0.18 d	1.38 cd	1.63 f	2.38 abcde	0.15 c	105.00 f	184.00 cde	18.38 a
NZN50	4.13 abc	0.22 c	1.34 d	1.62 f	2.28 bcdefg	0.14 c	117.13 cdef	199.50 bc	20.38 a
NZN100	4.20 abc	0.19 cd	1.25 d	3.12 bcde	2.47 a	0.15 c	118.75 cdef	184.75 cde	18.63 a
ZnO25+Q	3.37 abcd	0.20 cd	1.50 bcd	3.43 bc	2.23 defg	0.17 c	107.88 ef	200.00 bc	16.63 a
ZnO50+Q	3.56 abc	0.22 c	1.35 d	2.80 bcde	2.18 fg	0.20 bc	130.13 bc	216.63 ab	17.88 a
ZnO100+Q	3.83 abc	0.22 c	1.35 d	3.26 bcd	2.38 abcd	0.35 a	109.63 ef	187.50 cd	15.63 a
NZN25+Q	2.81 cde	0.22 c	1.72 bc	2.76 cde	2.25 cdefg	0.11 c	122.88 cde	228.00 ab	17.38 a
NZN50+Q	3.58 abc	0.21 cd	1.57 bcd	2.26 cde	2.15 g	0.18 c	113.50 cdef	229.50 a	16.25 a
NZN100+Q	3.69 abc	0.22 c	1.50 bcd	3.36 bc	2.20 efg	0.19 c	119.38 cdef	208.25 abc	18.38 a

Different letters indicate significant differences.

Besides, the Zn application presented negative effects on the content of P, K and Fe, which had average decreases of 40, 19.08 and 18.49 %, respectively, in relation to the control without application. Various studies confirm an imbalance between Zn and P in the plant, explaining the negative correlation presented in this work; P being the main limiting factor for the absorption, translocation, and utilization of Zn in the aerial parts of the plant because high levels of P in the cells affect the specific Zn functions (Mousavi, 2011; Hafeez *et al.*, 2013). Similarly, the Zn-Fe and Zn-K ratio can be affected when the nutrients are in solution form and the way they are applied, especially the Fe content, which tends to decrease when the amount of Zn applied is older and vice versa (Hafeez *et al.*, 2013).

The application of chitosan individually presented significant differences (Table 3) for P, K and Ca; obtaining an increase of 11.43 and 21.39 % in the content of P and K respectively in relation to the control treatment. These results agree with those published by El-Miniawy *et al.* (2013), who obtained significant increases in the content of P and K when chitosan was foliarly applied to strawberry leaves; and as in the present work, they obtained slight decreases in the N content without obtaining significant differences. For his part, Vasconcelos (2014), mentions that, although promising results have been obtained with the application of chitosan on the mineral content, especially with metals, the results are still highly variable, so it is necessary to carry out more studies to validate the use of this biocompound in biofortification works or in cases of bioremediation.

Table 4. Pearson correlation analysis for 13 green bean variables

	Biomass	Produc.	N	P	K	Ca	Mg	S	Zn	Fe	Mn	Cu	Protein
Biomass	1	.719**	0.149	-0.16	-0.148	-0.061	-0.034	-0.002	-0.03	-0.096	.347**	-0.216	-0.243
		0	0.272	0.238	0.277	0.654	0.802	0.989	0.825	0.48	0.009	0.111	0.071
Produc.	.719**	1	0.064	-0.018	0.105	-0.128	-0.069	-0.171	-0.243	-0.028	.294*	-0.185	-0.162
	0		0.637	0.895	0.442	0.349	0.611	0.208	0.071	0.839	0.028	0.173	0.233
N	0.149	0.064	1	-.481**	-0.242	-0.03	0.026	.372**	.365**	-.396**	0.006	-0.046	0.12
	0.272	0.637		0	0.073	0.825	0.848	0.005	0.006	0.003	0.966	0.734	0.379
P	0.16	0.018	.481**	1	.706**	0.056	0.202	0.174	.452**	.621**	0.186	0.087	.271*
	0.238	0.895	0		0	0.683	0.135	0.199	0	0	0.171	0.524	0.043
K	-0.148	0.105	-0.242	.706**	1	-0.057	-0.126	-.319*	-.465**	.424**	0.018	-0.164	-0.11
	0.277	0.442	0.073	0		0.675	0.356	0.016	0	0.001	0.895	0.227	0.42
Ca	-0.061	-0.128	-0.03	-0.056	-0.057	1	0.212	0.211	0.135	0.09	-0.261	0.011	-0.175
	0.654	0.349	0.825	0.683	0.675		0.116	0.118	0.32	0.511	0.052	0.938	0.196
Mg	-0.034	-0.069	0.026	0.202	-0.126	0.212	1	0.178	0.188	0.103	-.376**	0.133	0.05
	0.802	0.611	0.848	0.135	0.356	0.116		0.188	0.165	0.45	0.004	0.328	0.713
S	-0.002	-0.171	.372**	-0.174	-.319*	0.211	0.178	1	0.21	-.470**	-0.082	-0.004	0.113
	0.989	0.208	0.005	0.199	0.016	0.118	0.188		0.12	0	0.548	0.977	0.409
Zn	-0.03	-0.243	.365**	-.452**	-.465**	0.135	0.188	0.21	1	-0.159	-0.149	0.149	0.061
	0.825	0.071	0.006	0	0	0.32	0.165	0.12		0.243	0.273	0.273	0.655
Fe	-0.096	-0.028	-.396**	.621**	.424**	0.09	0.103	-.470**	-0.159	1	-0.12	0.183	-.360**
	0.48	0.839	0.003	0	0.001	0.511	0.45	0	0.243		0.379	0.176	0.006
Mn	.347**	.294*	0.006	-0.186	0.018	-0.261	-.376**	-0.082	-0.149	-0.12	1	0.174	0.004
	0.009	0.028	0.966	0.171	0.895	0.052	0.004	0.548	0.273	0.379		0.2	0.979
Cu	0.216	0.185	0.046	0.087	0.164	0.011	0.133	0.004	0.149	0.183	0.174	1	0.211
	0.111	0.173	0.734	0.524	0.227	0.938	0.328	0.977	0.273	0.176	0.2		0.118
Protein	-0.243	-0.162	0.12	-.271*	-0.11	-0.175	0.05	0.113	0.061	-.360**	0.004	0.211	1
	0.071	0.233	0.379	0.043	0.42	0.196	0.713	0.409	0.655	0.006	0.979	0.118	

Produc. - production

Protein content

Zinc (Zn) is a fundamental nutrient for the synthesis and structure of proteins due to its role in the translation and transcription of genetic material, its correct contribution being of great importance (Broadley *et al.*, 2012). The results obtained in the present study indicate significant differences in protein content in response to foliar application of Zn plus chitosan (Figure 7). Among the treatments, those of NZN25, ZnO100 and ZnO25+Q, obtained values above 20 g per 100 g of dry weight. Similar results were published by Salinas-Ramírez *et al.* (2012), who obtained a range from 17.9 to 22.3 g per 100 g of dry weight in green beans grown in Mexico. In the same way, these 3 treatments were located within the range obtained by Suarez-Martínez et al. (2016), which indicate that 100 g of different bean varieties contain between 20 and 30% protein.

Regarding the source of Zn used, the results do not indicate significant differences, finding an average increase in relation to the control of 7.86 g when ZnO was used and 9.49 g when NZN was applied. The use of nanoparticles as well as the foliar Zn content (Figure 5) presented a linear trend when the dose was increased. These results are like those obtained by Salama *et al.* (2019) who report a similar increase in protein content as they increase the dose of nanoparticles in the form of ZnO in common bean plants.

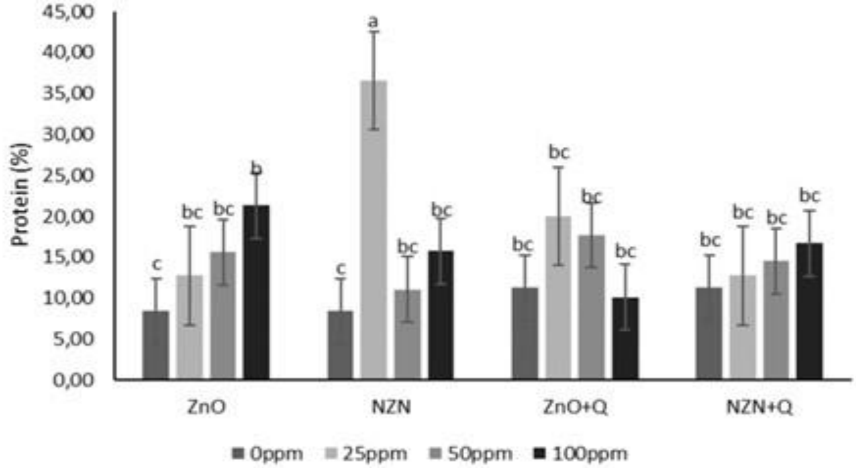

Figure 7. Effect of the application of nanoparticles and Zn nitrate in combination with chitosan on the protein content in bean plants cv. 'Strike'
Different letters indicate significant differences. Vertical bars indicate ±S.D.

While the application of chitosan individually favoured the protein content with an increase of 33.93% in relation to the control without application. Similar results were reported by Abu-Muriefah (2013) who obtained an increase in the content of soluble proteins when applying chitosan in short bean plants, however, the plants were under water stress. The positive effect of chitosan on protein content can be explained because various studies indicate an increase in the content of nitrogenous compounds because of chitosan application, generating greater development in the plant (Hidangmayum *et al.*, 2019).

Conclusions

The foliar application of Zn favours the biofortification process, finding increases in the content of Zn in the fruit for all the treatments used. The treatments that stood out were NZN50+Q and NZN100+Q, which increased the Zn content in fruits by more than 110%. In addition, the NZN50+Q treatment stood out in biomass production and exceeded the average yield reported for snap beans. In the present research work, no significant differences were found in production and biomass for the Zn source used, placing nanoparticles as a promising alternative for use in agriculture compared to a widely recognized fertilizer on the market. On the other hand, the application of chitosan plus nanoparticles did not present differences in the biofortification process, having similar results to the treatments without the application of this compound, however, the distribution pattern indicates a possible help in the translocation of Zn to the different plant organs. In the same way, the application of chitosan obtained favourable results when combined with NZN in biomass production, protein content, yield, and Zn content in fruit. Regarding the mineral content, a positive correlation between Zn and N was found, which indicates that, in biofortification works with Zn, a correct supply of N is essential. On the other hand, a negative correlation was obtained between P and Zn, and a similar trend between Zn and Fe, so it is important to be careful in the levels of these nutrients in future biofortification works. Finally, indicate that a greater number of studies are required regarding the use of nanoparticles and chitosan in horticulture to determine with certainty their effect on the physiology and nutrition of plants.

Authors' Contributions

ES and APM designed the study. APM and CRE analyzed the data. ES and APM. prepared the manuscript, while APM, CRE, CChM and DOB conducted the experiments. APM and ES organized the data and performed the statistical analysis. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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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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6. CONCLUSIONES

- La aplicación foliar de zinc favorece el proceso de biofortificación encontrando aumentos en el contenido de zinc en fruto para todos los tratamientos utilizados.
- Los tratamientos que presentaron un mayor grado de biofortificación fueron NZN 50 ppm + quitosano y NZN 100 ppm + quitosano, los cuales incrementaron más de 110 % el contenido de zinc en frutos. Además, el tratamiento NZN 50 ppm + quitosano destaco en la acumulación de biomasa y superó el promedio de producción reportado para frijol ejotero.
- La aplicación de ZnO 25 ppm, NZN 50 ppm, NZN 25 ppm + quitosano y NZN 50 ppm + quitosano fueron los tratamientos más eficientes en favorecer la acumulación de biomasa y la producción en frijol ejotero.
- La aplicación foliar de quitosano favoreció la biomasa, producción, los parámetros relacionados con la fotosíntesis, el contenido de proteína y el contenido de Zn en fruto, especialmente cuando se combinó con NZN.
- El tratamiento de nanopartículas de ZnO a 25 ppm, obtuvo resultados sobresalientes para las variables de biomasa y producción, sus resultados fueron similares estadísticamente al tratamiento de 50 ppm del NZN. Esto sugiere que las nanopartículas de ZnO pueden reducir la cantidad de fertilizante a emplearse sin afectar los rendimientos en los cultivos, por lo que podrían ser utilizadas para solucionar la problemática ambiental ocasionada por el uso excesivo de fertilizantes.
- El patrón de distribución del Zn dentro de la planta sugiere una mayor translocación de Zn a los diferentes órganos de esta cuando se aplicó quitosano, tanto para nanopartículas como para el fertilizante convencional.
- En cuanto al contenido mineral, se encontró una correlación positiva entre Zn y N lo que nos indica que, en trabajos de biofortificación con Zn, es fundamental un correcto suministro de N. En contra parte, se obtuvo una correlación negativa entre P y Zn, y una tendencia similar entre Zn y Fe, por lo que es importante ser cuidadoso en los niveles de estos nutrientes en futuros trabajos de biofortificación.

7. RECOMENDACIONES

- Los resultados obtenidos en el presente estudio, particularmente el contenido de carotenoides y la actividad fotosintética, sugieren que la aplicación de nanopartículas de ZnO aceleró el proceso de maduración de las plantas, alcanzando su tiempo de cosecha en un tiempo más corto que las tratadas con el fertilizante convencional. Sin embargo, son necesarios trabajos enfocados en el estudio de las etapas fenológicas y mediciones de las variables fotosintéticas a través del tiempo de desarrollo del cultivo para comprobar esta teoría.
- La combinación de quitosano con nanopartículas de ZnO, obtuvo resultados más bajos para el contenido de Zn y los parámetros relacionados con el crecimiento, pero, en el patrón de distribución presentó una mayor movilidad del elemento que la aplicación de nanopartículas de ZnO de manera individual, por lo que nos surge la teoría de que la adición de quitosano con nanopartículas de ZnO presentó una posible quelación de las nanopartículas retardando su liberación. Por lo que son necesarios estudios sobre la interacción de estas dos moléculas
- Potencialmente, el consumo de frijol ejotero biofortificado con los tratamientos utilizados aportaría entre 1.49 y 2.56 mg de Zn por cada 100 g de fruto fresco, lo que contribuiría con un 15 a 27% del requerimiento diario recomendado para este micronutriente. Aunque es necesario un análisis a fondo de la disponibilidad del Zn tanto después de la cocción como después del consumo para determinar exactamente la cantidad de nutriente que es aprovechado.
- Finalmente, indicar que se requieren más estudios para conocer a fondo los efectos de las nanopartículas de ZnO de manera individual y acomplejadas con quitosano sobre la fisiología y bioquímica de las plantas.

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