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Full Length Article

Potential of Zinc Application in Improving the Growth, Mineral Elements and Antioxidative Response of Pecan (*Carya illinoinensis*) Seedlings

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Abstract

Zinc (Zn) is an essential micronutrient for plants; and its deficiency and excess are both detrimental, so optimum Zn level plays a vital role in plant growth. The effect of Zn was explored on the growth and physiology of pecan seedlings. The pecan seeds were treated with 0.0, 0.1, 1.0, and 10.0 mg/L Zn ($ZnSO_4 \cdot 7H_2O$, 22.6%) for 30 days. The biomass; shoot and root length and diameter; Zn, nitrogen (N), phosphorus (P), potassium (K) levels; activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and malondialdehyde (MDA) content were investigated under Zn application. The results showed that: at a low Zn level (0.1 mg/L Zn), the maximum seedling shoot length (146.07 mm), shoot diameter (2.53 mm), root length (228.44 mm), and root diameter (4.06 mm) were observed. With increasing Zn levels up to 10 mg/L, the Zn, N and K levels increased significantly, and the P content decreased significantly, compared to the controls ($P \leq 0.05$). Similarly, the activities of SOD, POD, and CAT and the MDA content showed a slight increasing trend. However, these trends were not significant. Excess Zn caused a change in mineral nutrients and did not result in very high antioxidant enzyme activities. In conclusion, application of 0.1 mg/L Zn seemed beneficial to improve germination and early seedling growth of pecan due to elevated nutrients uptake. © 2020 Friends Science Publishers

Keywords: Pecan; Seedlings growth; Zinc; Catalase; Phosphorus contents

Introduction

Zinc (Zn) is an essential micronutrient for plant growth and metabolism. It functions as a cofactor to many enzymes; and therefore plays a key role in various biochemical processes in plants, such as the metabolism of carbohydrates, lipids, auxins, and nucleic acids (Wood *et al.* 2004; Zhang *et al.* 2016). It performs important functions in many physiological reactions, such as enzyme activation, protein synthesis, gene expression and regulation, and reproductive development (Ojeda-Barrios *et al.* 2014; Mattiello *et al.* 2015). Zn deficiency severely affects the productivity and quality of plants (Hafeez *et al.* 2013). It causes chlorotic stripes and purple shading on the edges and sheath of maize (*Zea mays* L.) seedling leaves (Mattiello *et al.* 2015), decreases the leaf area of apple (*Malus domestica* L.) (Fu *et al.* 2015) and causes biomass reduction in red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra*) and chickpea (*Cicer arietinum* L.) (Hajiboland and Amirazad 2010; Ullah *et al.* 2019, 2020). In pecan trees, Zn-deficiency-related phenotypic traits are observed as follows: dwarfing of tree organs, curling of leaves, yellowing of leaves between veins, shortening of shoot sections, loss of apical dominance, failure to germinate, delayed bud breaking, resetting and poor development of the root system (Wood *et al.* 2004). Further studies show that Zn deficiency could lead to nutrient element imbalance, decreased leaf chlorophyll content, reduced palisade parenchyma thickness, and increased stomatal density and pore size in the foliage of pecan trees (Ojeda-Barrios *et al.* 2012). In summary, Zn deficiency is a typical nutritional disorder in pecan trees (Peng *et al.* 2012; Smith *et al.* 2012).

Zn fertilization could significantly improve the agronomic traits and yield of food crops, such as mungbean (*Vigna radiata* L.), pea (*Pisum sativum* L.), olive (*Olea europea* L.), apples, and wheat (*Triticum aestivum* L.) (Saadati *et al.* 2013; Rafique *et al.* 2015; Gomez-Coronado *et al.* 2016; Zhang *et al.* 2016; Haider *et al*. 2019, 2020). The symptoms of Zn deficiency in young or mature pecan trees could be alleviated by applying Zn fertilizers within a short period of time (Heerema *et al.* 2017; Haider *et al*. 2018). In pecan trees, Zn fertilization methods include drip

irrigation, soil banding in autumn, and foliar spraying in spring. Foliar spraying is considered to be superior to the other two strategies (Walworth 2013). The compounds in Zn fertilizers include Zn sulphate ($ZnSO_4$), Zn oxide (ZnO), Zn nitrate ($ZnNO_3$), chelated Zn ethylenediaminetetraacetic acid (Zn-EDTA), and Zn trisodium diethylenetriaminepentaacetate (Zn-DTPA) (Smith *et al.* 2012). Among these, Zn sulphate is most effective for young pecan trees when considering economic and practical factors (Núñez-Moreno *et al*. 2015).

Despite its importance as an essential element, excess Zn can have toxic effect on plants by disturbing the balance of nutrients and inducing oxidative stress in plants (Wang *et al*. 2009). Excess Zn decreases the net photosynthetic rate transpiration rate, stomatal conductance, and levels of chlorophylls a and b in tea (*Camellia sinensis* (L.) O. Kuntze) plants and young bean (*Phaseolus vulgaris* L. cv. Lodi) plants (Vassilev *et al.* 2011; Mukhopadhyay *et al.* 2012). In addition, Zn toxicity led to decreased catalase activity and reduced antioxidative substance activity in young bean plants and pigeon pea (*Cajanus cajan* (L.) Millspaugh) (Rao and Sresty 2000). Therefore, it is important to apply an appropriate concentration of Zn fertilizer to prevent Zn toxicity in plants.

A number of previous studies have mainly focused on Zn deficiency or Zn toxicity in plants and on methods to improve the production of food crops using Zn fertilizers during the reproductive period. Moreover, the types of Zn fertilizers and methods of application have received much attention (Ashraf *et al*. 2013). However, there is limited information on Zn application for pecan seedlings during the vegetative growth. To explore the optimum Zn levels, this pot study was conducted to evaluate the effects of different levels of Zn application on germination, growth, mineral elements contents, and antioxidant enzyme activity of pecan seedlings with the hypothesis that Zn application can enhance pecan growth.

Materials and Methods

Seed collection and storage

Healthy pecan seeds, which were collected from 'Pawnee' trees in early October 2017 from Nanjing Green Universe Pecan Science & Technology Co., Ltd., in Shanbei Village, Luhe District, Nanjing, Jiangsu Province, China (lat. 31º52'45"N, long. 119º9'6"E, altitude 170 m asl.). All seeds were peeled, dried in a cool and ventilated room and then stored in refrigerator at 4℃ for cold stratification and simulating natural dormancy.

Zn treatment and seed germination

The germination study began on November 18, 2017. A total of 360 pecan seeds were selected and divided into four treatments with three replicates. Each treatment consisted of a particular concentration of Zn in the form of $ZnSO_4 \cdot 7H_2O$ (Zn content, 22.57%). The seeds were soaked in solutions containing 10 L of purified water (Biosafer-30TBA; Soffice (China) Co. Ltd.), 30 mg/L gibberellin (GA, BR, Q/CYDZ 1315-2008, Sinopharm Chemical Reagent Co., Ltd.), and different concentrations of $ZnSO_4$ solutions (CK: 0.0 mg/L Zn; Z_1: 0.1 mg/L Zn; Z_2: 1.0 mg/L Zn; Z_3: 10.0 mg/L Zn). The soaking solutions were placed in plastic buckets for 10 days. While soaking, the seeds were pressed under the solution's surface constantly using a sealed heavy water bag. The soaking solutions were replaced every five days.

The soaked seeds were removed, air dried, and stored in wet sand at 0℃ in darkness in an artificial climate incubator for 30 days to disrupt seed dormancy. The seeds and wet sand were placed in hollowed-out plastic crates (length × width × height = 45.5×31×24 cm) with one layer of seeds covered with one layer of wet sand. Each layer of wet sand was 10 cm thick. Each seed layer contained 30 tiled seeds. During the storage period, the moisture of the wet sand remained at 60~80% and was checked every day. Following this standard, purified water was used to spray the seeds in a timely manner.

After the storage period, all the seeds were removed and rinsed with tap water followed by purified water. After air drying, the seeds were transferred into square plastic flowerpots with round holes at the bottom (length × width × height = 49×20×14 cm). Each pot contained a 10 cm thick vermiculite layer to maintain humectation, and then, 30 seeds were buried in the pot, which was then sealed with plastic wrap to prevent water loss. Each treatment was carried out with three replicates. All the pots were placed in an artificial climate incubator for 30 days. The culture conditions were as follows: light 16 h/d, light level 400~500 $\mu mol \cdot m^{-2}$ s^{-1}, temperature 25/22℃, relative humidity 65/40%.

Measured parameters

After culturing for 30 days, the germination number of the pecan seeds was determined. Ten young seedlings were randomly selected from each treatment for analysis of biomass, growth, mineral elements, and antioxidant enzyme activity. The seedlings were then rinsed with tap water followed by purified water, and dried in air. After removing the endosperm, growth parameters were measured for pecan seedlings, which were then stored at -80℃ until further use.

Germination rate: Seed germination rate (%) = (germinated seeds / total seeds sown) × 100%; total seeds sown = 30; three replicates each treatment.

Biomass weight: Fresh weight per seedling (g) = total fresh weight/total number of seedlings per treatment; dry weight per seedling (g) = total dry weight/total number of seedlings per treatment. Total number of seedlings per treatment = 10, with three replicates per treatment.

Growth parameters: Growth parameters (shoot and root length and diameter) were measured with steel tape (5 × 16 mm, Deli, Deli Stationery Co., Ltd.) and Vernier calipers (150 ± 0.01 mm, Master Proof, Germany), respectively. Each growth was determined as the mean value per treatment, and each treatment contained 10 pecan seedlings, with three replicates each treatment.

Mineral elements: To measure the seedling mineral concentrations, five dry seedlings per treatment were sampled and mixed, immediately blotted and oven-dried at 105℃ for 20 min and then kept at 80℃ for 72 h, with three replicates per treatment. The dried material was ground. To measure the nitrogen (N), 0.5 g of powdered sample was digested with 10 mL of a mixed acid solution (v/v, 10:1) consisting of concentrated sulfuric acid (density 1.84 g/mL, AR) and perchloric acid (70%, AR), and heated at 100~200℃ until boiling to yield a colorless solution. The boiled solution was dissolved in 5 mL of 5% sulfuric acid and ultrapure water was added up to a final volume of 25 mL. The total N content was determined by the indophenol blue colorimetric method in the boiled solution (Piper 1945). To measure the phosphorus (P), potassium (K) and zinc (Zn) content, 0.5 g of powdered sample was digested with 10 mL of a mixed acid solution (v/v, 5:1) consisting of concentrated nitric acid (68%, AR) and perchloric acid (70%, AR), and heated at 100~200℃ until near dryness. The boiled solution was dissolved in 5 mL of 5% dilute nitric acid, and distilled water was added up to a final volume of 25 mL. The total P content was measured with ultraviolet absorption spectrophotometry (UV-mini1240, Island Ferry, Japan) by the molybdenum-antimony-scandium colorimetric method. The total K and Zn content was measured by a flame atomic absorption spectrometer (PE900T, U.S.A.) (Wallace 1951).

Antioxidant enzyme activity: For estimation of the superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) activities and malondialdehyde (MDA) content, 0.5 g of frozen sample was ground in liquid N_2 and homogenized with 1 mL of 50 m*M* potassium phosphate buffer (PBS, pH 7.8). The homogenate in 10 mL of ice-cold PBS was centrifuged at 4000 rpm for 20 min at 4°C (Wang *et al.* 2009). The supernatant obtained was used as the enzyme extract and stored at 4°C. All operations were carried out at 4°C and completed in one week.

To initiate SOD activity, an aliquot of 0.01 mL of enzyme extract was mixed with 3 mL of reaction solution consisting of 50 m*M* phosphate buffer (pH 7.8), 13 m*M* L-methionine, 75 μM nitroblue tebrazolium (NBT) for photochemical reduction, 10 μM EDTA-Na_2, 2 μM riboflavin, and distilled water (15:3:3:3:3:2.5, v/v). The reaction solution was placed in a test tube without enzyme extract as the control group. The tubes were cultured for 30 min at 4000 Lux light in an illuminated incubator, and the reaction was stopped by covering with a black cloth. The absorbance of the reaction mixture tube was measured at 560 nm (Liu *et al.* 2018). SOD activity was calculated as $U \cdot g^{-1}$ FW (unit of SOD activity per gram of fresh weight).

POD activity was determined following the method described by (Heath and Packer 1968). The mixture for the POD activity assay comprised 50 mL of 0.1 *M* phosphate buffer (pH 6.0), 28 μL of guaiacol and 19 μL of 30% H_2O_2. An aliquot of 20 μL of enzyme extract was reacted with the assay mixture for 3 min and then, the absorbance was measured at 470 nm. The recorded activity was calculated as $U \cdot g^{-1} \cdot FW\ min^{-1}$ (unit of POD activity per gram of fresh weight per minute).

CAT activity was estimated by monitoring the decrease in absorbance due to decomposition of H_2O_2 at 240 nm. For this assay, 0.1 mL of enzyme extract was mixed with 3 mL of reaction mixture containing 0.6 mL of 0.1 *M* phosphate buffer (pH 7.0). A blank was run simultaneously as described above, substituting the 0.1 mL of enzyme extract with 0.1 mL of phosphate buffer. CAT activity was expressed as the change in absorbance per min and calculated as $U \cdot g^{-1} \cdot FW\ min^{-1}$ (unit of CAT activity per gram of fresh weight per minute).

The MDA content was determined as the lipid peroxidation product levels in the fresh frozen samples via the method described by (Chen *et al.* 2003) using a spectrophotometer (UVmini-1240 ultraviolet spectrophotometer, Shimadzu Corporation, Suzhou, China). For this assay, 0.5 g of frozen sample was ground in 5 mL of 10% trichloroacetic acid (TCA, w/v) using a mortar and pestle, and then centrifuged at 4000 rpm for 20 min. The supernatant (1 mL) was mixed with 3 mL of 6% thiobarbituric acid (TBA, w/v). After boiling at 100°C for 15 min, the mixture was cooled rapidly in an ice bath. The absorbance values (OD values) were recorded at 450 nm, 532 nm and 600 nm. The MDA content was calculated using the following formula:

$$C\ (\mu mol/L) = 6.45(OD_{532} - OD_{600}) - 0.56OD_{450}$$

$$\text{MDA content}\ (\mu mol/g) = (C \times V_t)/W \times V_i$$

V_t is the total volume of the supernatant, V_i is the volume of supernatant used for analysis, and W is the weight of the sample.

Data analysis

The data were processed using Excel 2016 (Microsoft, Redmond, WA) and analysed using one-way analysis of variance (ANOVA) technique. In case of significant effect, means were separated using Tukey's test at $P \leqslant 0.05$. All the figures were plotted by Origin Pro, version 9.1, and all the tables were prepared in Microsoft Office Excel 2016.

Results

Effects of Zn on pecan seedling growth parameters

Analysed data indicated significant improvement in biomass

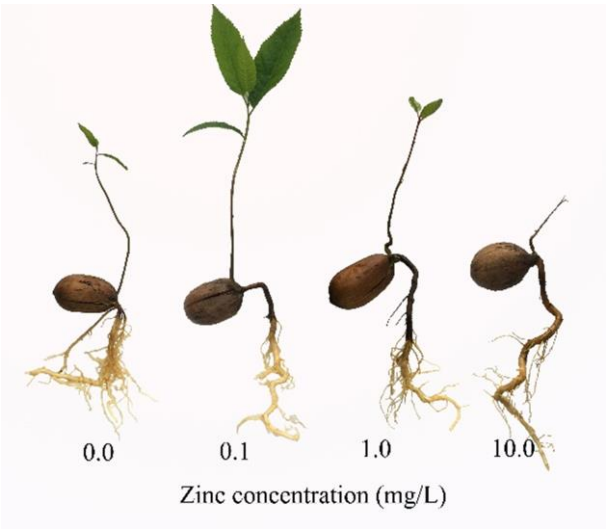

Fig. 1: Effect of zinc application on growth of pecan seedling

and shoot and root length and diameter at 0.1 mg/L Zn ($P \leqslant 0.05$). At 0.1 mg/L Zn, the maximum seedling fresh weight (3.98 g), dry weight (2.54 g), shoot length (146.07 mm), shoot diameter (2.53 mm), root length (228.44 mm), and root diameter (4.06 mm) were recorded, despite the minimum germination rate (87.33%) being observed at this concentration. At high Zn concentrations ($\geqslant$1 mg/L Zn), slight inhibition was observed. High concentrations significantly improved the germination rate ($P \leqslant 0.05$). The maximum germination rate was 93.33%, obtained with 1.0 mg/L Zn (Table 1 and Fig. 1).

Effects of Zn on pecan seedling mineral elements

Upon Zn application, Zn, N and K accumulation in pecan seedlings significantly increased, while the P content decreased (Fig. 2, $P \leqslant 0.05$). At 10 mg/L Zn, the highest Zn and K levels were 112.57 mg/kg and 1560.89 mg/kg, respectively (Fig. 2A, D), while the highest N content was 9835.14 mg/kg at 0.1 mg/L Zn (Fig. 2B). Application of Zn decreased the seedling P content. The lowest P content was 6201.73 mg/kg, observed upon application of 1.0 mg/L Zn (Fig. 2C).

Effects of Zn on pecan seedling antioxidant enzyme activity

Compared to the controls, the SOD, POD, and CAT activities and MDA content of pecan seedlings exhibited an increase with increasing Zn concentration. However, these increasing trends were not significant (Fig. 3). At 10.0 mg/L Zn, the maximum POD activity and MDA content were 3235.83 μmol g^{-1} min^{-1} and 8.91 μmol g^{-1}, respectively; and were significantly higher than those at 0.1 and 1.0 mg/L Zn (Fig. 3A, D; $P \leqslant 0.05$). At a Zn concentration of 1.0 mg/L,

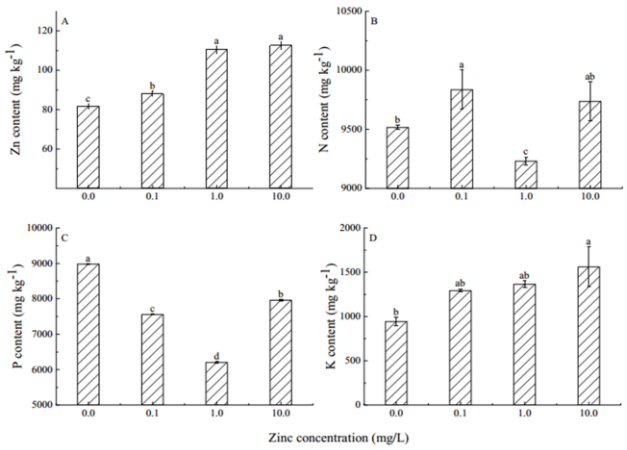

Fig. 2: Effect of zinc application on Zn (**A**), N (**B**), P (**C**) and K (**D**) contents in pecan seedlings
Means ± S.E. with different letters are significantly different from each other at $P \leq 0.05$ according to Tukey's test
Here Zn= Zinc; N= Nitrogen; P= Phosphorus; K= Potassium

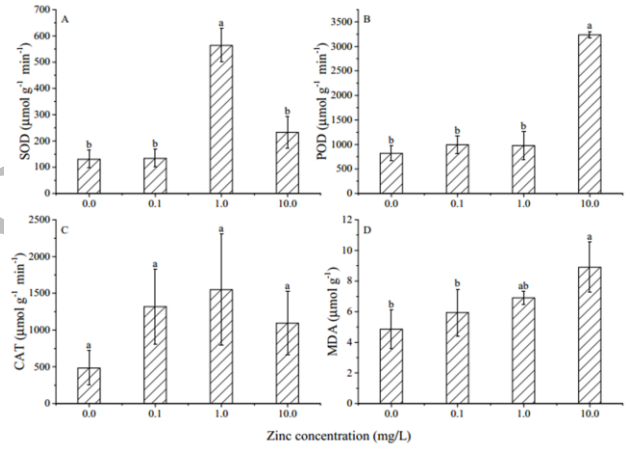

Fig. 3: Effect of Zn application on activities POD (**A**), SOD (**B**), CAT (**C**) and MDA (**D**) contents of pecan seedlings
Means ± S.E. with different letters are significantly different from each other at $P \leq 0.05$ according to Tukey's test
Here SOD= Superoxide dismutase; POD= Peroxidase; CAT= Catalase; MDA= Malondialdehyde

the SOD and CAT activities gradually reached their highest values of 563.49 μmol g^{-1} min^{-1} and 1550 μmol g^{-1} respectively (Fig. 3B, C).

Discussion

In this study, the seedling biomass increased with 0.1 mg/L Zn application. At this concentration, the shoot and root length and diameter also improved. No toxicity symptoms were observed. Studies on woody as well as herbaceous plants have demonstrated that application of Zn promotes plant growth (Hafeez *et al*. 2013). In a pot study conducted by (Prom-u-thai *et al.* 2012) on seedling vigour and viability using rice (*Oryza sativa* L.) as the test crop, Zn priming

Table 1: Effect of zinc application on growth traits of pecan seedlings

Zinc level (mg/L)	Biomass weight		Germination (%)	Shoot length (mm)	Shoot diameter (mm)	Root length (mm)	Root diameter (mm)
	Fresh weight (g)	Dry weight (g)					
0.0	3.56 ± 0.16b	2.25 ± 0.03b	90.33 ± 1.53ab	119.95 ± 33.74b	2.49 ± 0.40a	148.77 ± 38.79bc	3.73 ± 0.74ab
0.1	3.98 ± 0.20a	2.54 ± 0.23a	87.33 ± 2.08b	146.07 ± 28.17a	2.53 ± 0.53a	228.44 ± 179.35a	4.06 ± 0.73a
1.0	3.79 ± 0.22ab	2.29 ± 0.05b	93.33 ± 1.53a	51.89 ± 10.49c	2.51 ± 0.42a	138.61 ± 50.64c	3.93 ± 0.60a
10.0	3.69 ± 0.11ab	2.22 ± 0.04b	90.33 ± 1.53ab	35.06 ± 8.15c	2.13 ± 0.38b	172.44 ± 44.81b	3.49 ± 0.63b

Means ± SD in the same column with different letters are statistically different from each other at $P \leq 0.05$ according to Tukey's test

significantly enhanced the germination rate, root number and dry weight at up to 5 m*M* $ZnSO_4$. Based on a 2-year field study, accumulation of Zn has been reported to increase seed yield significantly for pea (Rafique *et al.* 2015). The Zn concentration range for 95% maximum pea yield was 42~53 mg kg^{-1} in leaves and 45~60 mg kg^{-1} in seeds. Our findings were consistent with these results. There were obvious symptoms of Zn application in pecan indicating that Zn may have a function in pecan seedlings.

As a response to Zn application, nutrient absorption may enhance or reduce in plant growth. The pecan seedlings exhibited significantly increased Zn, N and K levels under a high Zn nutrient level. The results were similar to those of (Ojeda-Barrios *et al.* 2014). Ojeda-Barrios reported that the accumulation of Zn was related to other mineral nutrients. Based on a 3-year study, it was demonstrated that foliar Zn compounds ($ZnNO_3$, Zn-EDTA, Zn-DTPA) significantly increased the N, K, Ca, Mg, Fe, Cu, Mn levels in the leaves of eight-year-old 'Western Schley' pecan trees. Usually, Zn fertilizers promote N absorption in plants (Lošák 2007). For the same reason, during the rapid growth period of pecan, increased amounts of N and Zn fertilizers are required (Smith *et al.* 2012). During the growing season, combining zinc sulphate with urea in foliar applications increased the concentration of Zn from 0.7 to 1.5 mg per kg of apple tissue (Amiri *et al.* 2008). There was a slight increase in the N and K levels in apple leaves between June and July in all the Zn fertilizer treatments.

As the Zn levels improved, the P content in pecan seedlings decreased. It was suggested that there was P-Zn antagonism regulation in pecan seedlings. Wang reported that trees are considered healthy when the *P*/Zn value is ≤ 100 in the leaves of apple trees (Wang *et al.* 2010). This regulation may result from the interaction between P and Zn termed 'P-induced Zn deficiency' (Yan *et al.* 2010). The nutritional disorder could be alleviated by reducing the P content and increasing the Zn content (Fu *et al.* 2015). This regulation also occurs in pecan trees. In orchard nutrient management, commercial pecan growers routinely apply supplemental nitrogen and zinc. Only 40% routinely apply phosphorus, and fewer use boron, iron, and copper (Walworth 2013). Eight-year-old 'Western Schley' pecan trees with the best appearance were treated with $ZnNO_3$ (100 mg/L Zn) and Zn-DTPA (100 mg/L Zn), which led to increases of 73 and 69%, respectively, in leaf Zn concentration (Ojeda-Barrios *et al.* 2014). Therefore, it was indicated that $ZnNO_3$ and Zn-DTPA are good options for foliar Zn fertilization in pecan trees.

The results showed that the SOD, POD, CAT activities and MDA content in the seedlings increased concomitantly with increasing Zn concentrations up to 10 mg/L, with all of these parameters showing a slight increase. This increase could eliminate the production of reactive oxygen species (ROS). ROS induce lipid peroxidation and are harmful to plant growth (Candan *et al.* 2018). Heavy metals usually cause lipid peroxidation in a concentration-dependent manner (Wójcik *et al.* 2006). Madhava reported that 6-day-old seedlings of two pigeon pea (*Cajanus cajan* (L.) Millspaugh) cultivars, namely, LRG30 and ICPL87, were studied under excess Zn and Ni, and the activities of antioxidative enzymes such as SOD, POD and glutathione reductase (GSHR) registered higher values than the activity of CAT and antioxidative substances such as ascorbic acid (Vc) and total glutathione (Rao and Sresty 2000). Under external Zn stress with 0.07~1.12 m*M* Zn, the NADH oxidase and POD activity increased in the leaves and roots of repeseed (*Brassica napus*) seedlings, but the SOD, CAT and POD activities decreased (Wang *et al.* 2009). Tavallali reported that salt stress significantly increased the activity of antioxidant enzymes, but Zn supplementation efficiently reduced the adverse effects of salt stress, such as inducing high oxidative stress and increasing lipid peroxidation, electrolyte leakage, and lipoxygenase activity to high levels in pistachio (*Pistacia vera* L. '*Badami*') seedlings (Tavallali *et al.* 2010).

Conclusion

The growth of pecan seedlings increased with low Zn levels (0.1 mg/L Zn), and was inhibited under high Zn levels. In this study, pecan seedlings exhibited significantly higher Zn, N and K levels under increased Zn nutrition than control, while P content decreased. The SOD, POD, and CAT activities and MDA content in the seedlings showed a slight increase with increasing Zn levels up to 10 mg/L. We inferred that the increase in antioxidant enzymes activities alleviated the effects of Zn application. Hence, it could be concluded that Zn supplementation was beneficial to pecan seedlings.

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Author Contributions

This work was carried out in collaboration between all authors. YS collected the literature along with references pertaining to zinc and wrote the first draft of the manuscript. PT, XZ, ZL and FC conducted the experiment, collected and analyzed the data while FP and YL gave the suggestions of the experiment process and improved the English. All authors read and approved the final manuscript.

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