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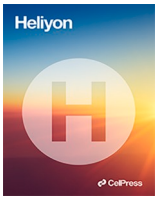

Research article

Foliar applications of zinc oxide nanoparticles and boric acid affect leaf oxidative metabolism and productivity in young pecan trees

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A R T I C L E I N F O

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A B S T R A C T

Zinc and boron are nutrients that often suffer low bioavailability to pecan trees grown in calcareous soils whereas adequate supplies of these two elements is essential for commercial pecan production. Working with young pecan trees, we evaluated changes in oxidative metabolism, levels of bioactive compounds, yield components and foliar nutrient concentrations in response to foliar sprays (50 or 100 mg L^{-1}) of zinc oxide nanoparticles (ZnO NPs) and boron (H_3BO_3). Four different treatment solutions were applied in a completely randomised design with six replications per treatment (24 trees in total). Zinc and B treatments were applied before pistil receptivity (3 weeks before anthesis) and at stem elongation stage 31, 39/60; flowering stage 69; fruit stages 7–75 and continued for a total of five applications at 14-day intervals. We evaluated enzyme activities (SOD, H_2O_2, CAT and GPx), AC, phenols, flavonoids, leaf area, chlorophyll, total anthocyanins and nut yield and quality (nut weight and % kernel). The mineral concentrations in the leaflets were also determined. The mineral concentrations (N, P, K, Ca, Mg, Fe, Cu, Mn, Ni, Zn and B) in the leaflets were also determined. Spraying ZnO NPs and B increased SOD activity, CA, chlorophyll concentration, mineral nutrients (N, K, Ca, Zn and B) and yield. However, reductions were observed for CAT activity, nut quality and concentrations of phenol, flavonoid, anthocyanin and Fe. Boron increased GPx activity and P concentration. These results demonstrate that spraying low doses (50 mg L^{-1}) of ZnO NPs and B can help reduce oxidative stress and increase yield, nut quality and leaf concentrations of Zn and B in young cv. Wichita pecan trees established on a calcareous soil.

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1. Introduction

On the calcareous and alkaline (pH ≥ 8.0) soils of many arid/semi-arid areas of the southwestern United States and northern Mexico the pecan [*Carya illinoinensis* (Wangenh.) K. Koch] is an important deciduous fruit tree species [1]. Under these soil and climate conditions, the nutrient uptake and productivity of established pecan trees can be significantly compromised [2,3] due to edaphic accumulations of salts such as Na^+, Mg^{2+}, Ca^{2+}, Cl^- and SO_4^{2-} (salinity values between 2.0 and 2.6 dS m^{-1}), carbonates, bicarbonates and boron (B) [4,5]. These conditions contrast with this species' native conditions of deep, sandy, alluvial (riverbank) soils with lower pH values - between 6.0 and 7.0 [6].

Commercial pecan production requires nitrogen (N) application as the nutrient of highest demand. However, it is necessary also to include supplements of Zn^{2+}, Ni^{2+} and B, which are involved in a range of metabolic processes associated with various enzyme groups, including oxidoreductases, lyases, isomerases, transferases, ligases, urease and carbonic anhydrase. These are associated with the metabolism of nitrogen, phenols, starch, proteins and with the synthesis of a number of key secondary metabolites [7–9]. Commercial pecan trees are often Zn-deficient due to limitations in the uptake, mobility and bioavailability of Zn in alkaline soils [10]. The uptake of B and Zn is also limited by the anatomy and physiology of pecan's root system which comprises a tap root with multiple fine, branching, lateral roots which lack root hairs [11,12]. These roots show obligate symbiotic associations with ectomycorrhizal fungi of different genera - mostly *Astraeus, Gyrodon, Pisolithus, Russula, Tuber and Tylopilus* [13].

The soils of many pecan growing regions are also very saline and with high B concentrations, with foliar analyses indicating B levels of around 500 mg kg^{-1}. Under these conditions the leaves often show symptoms of excess B – leaf margin chlorosis and necrosis [7]. However, the foliar B values that such trigger phytotoxicity symptoms have not been properly defined for pecan, so that young leaves and roots, even of the more B-tolerant genotypes, may suffer a degree of metabolic dysfunction prior to the appearance of visible toxicity symptoms. It is thought that such asymptomatic levels of B may well affect growth, yield and nut quality [14]. Over-exploitation of aquifers, and possibly the consequences of climate change, seem in recent years to have increased the edaphic accumulations of B via environmental temperature fluctuations and poor irrigation water quality (≥3 dS m^{-1}; C3–S1), [15–17]. These conditions have a direct impact on cropping by increasing premature flower drop and so decreasing fruit set. A strategy to minimise these problems has been to promote hormone flow through foliar applications of B so as to favour pollen tube growth [5].

Both the alkaline soils and the high salt concentrations of the irrigation water may be factors limiting the loading of B into the xylem of the roots. Within the pecan tree, B is known to be transported via the xylem, so its distribution is also affected by transpirational flow [18]. Boron's phloem mobility is limited, due to limited exchange between xylem and phloem, with the result that B availability is often low in the ovary tissues. Previous studies have demonstrated that foliar B sprays can be effective in increasing foliar B availability at critical stages, such as at flower set and fruit set, due to the absence or malfunction of xylem connections in the developing fruit [4, 19]. Achieving high B availability can help minimise certain physiological disorders associated with cracking of the fruits underlying testa (kernel shell) and sometimes of the involucre (husk) [20].

The application of foliar micronutrients to fruit trees can come at quite a high economic and environmental cost because it requires specialised equipment, its application causes significant soil compaction as it requires multiple application runs (often more than six) each season. It also requires the use of adjuvants and B products of high water-solubility [21]. Compared with soil applications of B, foliar applications are relatively fast-acting and efficient in terms of the mass of material required to optimise the nutrient levels in the plant tissues. Tree crop growing, including of pecan, requires the efficient delivery of plant nutrients to the crop so as to minimise the negative environmental effects of excessive fertiliser use, also of excessive cost which affects the economic viability and profitability of pecan production [22]. In recent years, the development of nanotechnology has opened up a wide range of new opportunities in industry and agriculture [23]. Nowadays, the commercial availability of nanofertilisers (N, Ca, Cu, Mn, Fe, Ni, Mo, Zn, Se and Ti) with particle sizes ≤100 nm creates a more environmentally and economically sustainable alternative for the supply of essential nutrients to increase fruit tree productivity, including that of Western Schley pecan [11,24].

Foliar sprays of individual mineral nutrients (or combinations of nutrients) formulated as nanoparticles has been proven more efficient than foliar applications of conventional mineral sources. In this way, both crop yield (t/ha) and crop quality (fruit size, colour, concentration of bioactive compounds etc) have been increased. These fruit tree species in which such benefits have been demonstrated include olive (*Olea europaea* L.) [25], apple (*Malus domestica* L.) [26], pomegranate (*Punica granatum* L.) [27,28], mango (*Mangifera indica* L.) [29], avocado (*Persea americana* L.) [30] and pecan (*Carya illinoinensis*) 'Western Schley' [11,24]. However, at this stage the results for pecan remain rather limited, and in some cases somewhat contradictory, perhaps due to variance associated with differences in cultivar, tree age, dosage, the number and timings of the spray applications, as well as the confounding effects of different soils and microclimates. Our study sets out to extend and diversify the body of information currently available for the use of foliar ZnO NP sprays in pecan and for B (as boric acid, H_3BO_3). The objective of this study was to evaluate changes in oxidative metabolism, bioactive compounds, yield components and foliar nutrients in response to foliar spray of ZnO NPs (50 and 100 mg L^{-1}) and B (H_3BO_3) (50 and 100 mg L^{-1}) on young cv 'Wichita' pecan trees established in calcareous soils.

2. Materials and methods

2.1. Study area, plant material and orchard management

This trial was conducted in the 2020 and 2021 growing seasons using cv. 'Wichita' pecan [*Carya illinoinensis* (Wangenh.) K. Koch] trees established in 2011 on 'Riverside' rootstocks in a 10 × 10 m planting grid (100 trees ha^{-1}). The orchard is located in Delicias, Chihuahua, Mexico (28°20′46.4''N, 105°34′03.3''W) at an altitude of 1162 m, which has a mean annual temperature of about 27 °C

and a mean annual precipitation about 300 mm. The physicochemical properties of the soil (Xerollic Calciorthid) over an arable depth (0–30 cm) were: soil texture, a crumbly light sand composed of sand 65.21 %; silt 17.08 %; clay 17.71 %; $CaCO_3$ 27 %; active $CaCO_3$ 12.1 %; pH 8.1; organic matter 0.6 %; EC 2.6 dS m^{-1}; NO_3^- 8.8 mg kg^{-1}; Ca 3912.5 mg kg^{-1} (moderately high); Zn 1.34 mg kg^{-1} (moderately low) and B 0.5 mg kg^{-1} (low). The trees used in the study had not previously received any Zn or B treatments. Nutrient supply was by a surface application of dry fertiliser (140 N: 80 P_2O_5: 100 K_2O). Standard commercial practices for weed control and irrigation scheduling were followed throughout.

Four different treatments were applied in a completely randomised design with six replications per treatment (24 trees in total). Each tree was an experimental unit. Tree height was 9 ± 0.3 m and trunk girth 55 ± 5 cm at 0.5 m above soil level. The solutions were 50 and 100 mg L^{-1} Zn (1.53 and 0.76 mM) as Zn NPs. These were of zinc oxide obtained by wet chemistry in the form of wurtzite crystals with an average size of 50 nm with no contaminants, a purity level of 99.7 %, and a density of 5.61 g cm^{-3} [(Fig. 1 (a, b, c and d) and Fig. 2 (A and B)]. Boron was supplied as doses of 50 and 100 mg L^{-1} B (0.80 and 1.61 mM) as H_3BO_3 in water through a hand sprayer. Zinc and B treatments were applied: (1) before pistil receptivity (3 weeks before anthesis) [stem elongation stage 31, 39/60, (2) flowering stage 69; (3) fruit stages 7–75 [31] and continued every 14 d thereafter to a total of five applications. In each application, the solution was sprayed to full foliage (17 L per tree in 2020 and 20 L per tree in 2021) between 05:00 and 08:30 h. In all formulations, 0.1 % urea was added as a carrier ion and 100 mg L^{-1} Tween® 20 was used as a non-ionic surfactant (Thermo Fisher Scientific™, USA). The pH of the solutions was adjusted to 6.5 with HCl to facilitate foliar uptake in the formulations with metallic nutrients.

2.2. Sampling of leaflets

Approximately 60 pairs of leaflets were collected during the growing season on July 26, 2020 and 2021. This timing was about 221 days after bud break in the water stage of the nut. Leaflets were taken from the mid canopy of each tree. Leaflets were collected by pooling samples from the four cardinal directions and from both vegetative and fruiting shoots. Samples were selected to be free of obvious mechanical damage from pests or diseases.

2.3. Oxidative metabolism and antioxidant capacity

Superoxide dismutase (SOD EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), according to the method described by Ref. [32] and modified by Ref. [33]. Enzyme activity is reported as units/min/g, where one unit of SOD activity corresponds to the amount of enzyme required to cause a 50 % inhibition of NBT reduction

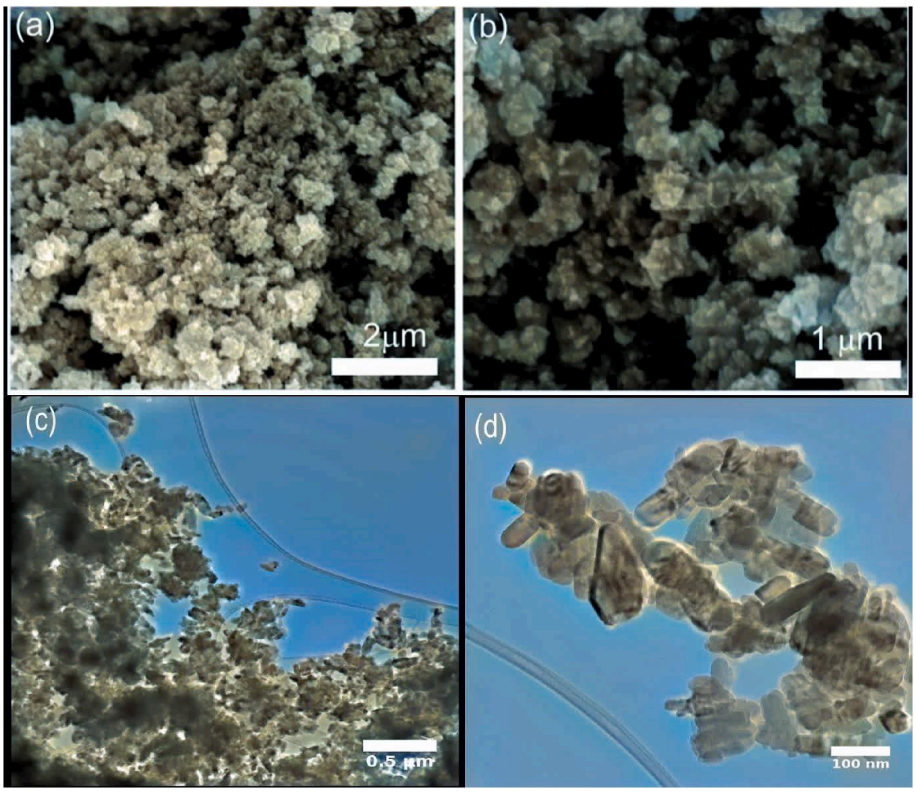

Fig. 1. Morphology of the ZnO NPs sample by scanning electron microscopy (a and b) and transmission electron microscopy (c and d).

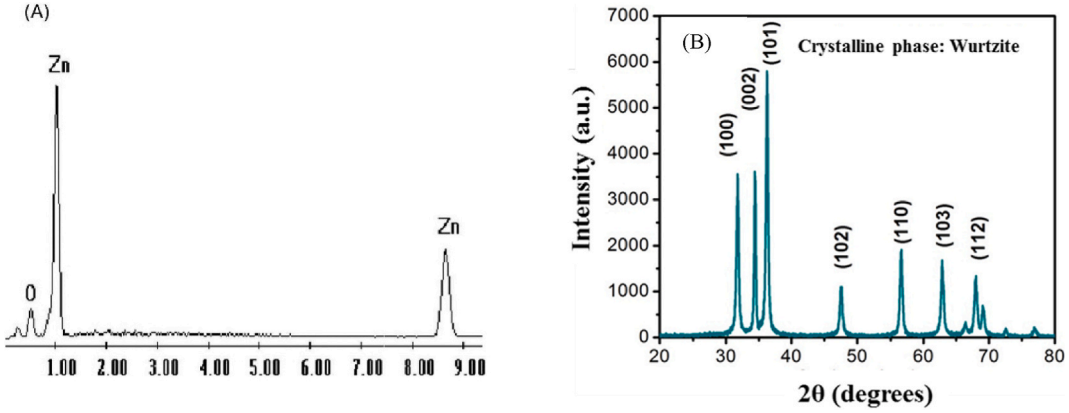

Fig. 2. Elemental analysis (chemical composition) by energy dispersive X-ray scattering (A) and crystalline structure by X-ray diffraction (B) of the sample ZnO NPs (zinc oxide nanoparticles).

evaluated at 560 nm. The extraction and analysis of total hydrogen peroxide (H_2O_2) employed a colorimetric method [33]. The results are reported in μmol H_2O_2 min^{-1} g^{-1} (total peroxides). Last, the extraction and determination of enzymatic activity of catalase and guaicol peroxidase used the method described by Ref. [33]. The results are expressed as mol H_2O_2 min^{-1} g^{-1} and nmol GSH min^{-1} g^{-1}, respectively.

Antioxidant capacity (AC) was assessed by two methods. In the first, the ABTS assay was carried out as described by Ref. [34] for which the ABTS solution was prepared by mixing equal volumes of 7 mM of the ABTS reagent [(2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid)] and potassium persulfate ($K_2S_2O_8$) at 2.45 mM (w:v), which was left to stand for 116 h at room temperature. Subsequently, 20 % ethanol was used to dilute the mixture to obtain an absorbance value between 0.24 and 0.67 at 734 nm and then 10 μL of the sample diluted in ethanol +90 μL of distilled water +3 mL of ABTS applied every 30 s were added and left to react for 15 min. The absorbance at 734 nm was obtained and the results are expressed as μmol TE (Trolox Equivalents) g^{-1}. Last, the DPPH• (2,2-diphenyl-1-picrylhydraill (DPPH•)) assay was carried out in accordance with [35]. Aqueous ethanol (80 %) was used for sample preparation. Briefly, 0.3 mL of extract and 5.7 mL of the compound DPPH (2,2-diphenyl-1-picrylhydrazyl) were mixed at a concentration of 0.0375 g L^{-1}. The mixture was kept in the dark for 30 min. The decrease in the DPPH radical was measured at 515 nm. The results are expressed as % inhibition of DPPH.

2.4. Bioactive compounds, leaf area and photosynthetic pigments

The total phenol (TP) content was determined by the Folin-Cilocalteu method [36]. Briefly, 0.5 g of sample was taken and homogenised with 5 mL of 70 % absolute ethyl alcohol (v:v). From this solution, 50 μL were taken to which 7.950 mL of distilled water and 500 μL of Folin-Ciocalteu reagent (2 N) were added. This mixture was stirred for 1 min with a digital vortex (Thermo Scientific®, USA) and allowed to stand for 8 min. Subsequently, 1.5 mL of Na_2CO_3 20 % (w:v) was added, stirred and allowed to stand for 2 h in total darkness at room temperature (23±1 °C). The absorbance values were recorded at 760 nm with a Lambda 25® UV–visible spectrometer (PerkinElmer, Waltham, USA). Concentrations of 0, 20, 40, 60, 80 and 100 mg L^{-1} were used for the construction of a gallic acid standard curve (Sigma-Aldrich, USA). The results are expressed in mg GAE g^{-1} FW. The total flavonoid content (TFL) was quantified according to the method published by Ref. [37]. Briefly, 1 g of tissue was added to 5 mL of methanol and then homogenised and kept at room temperature (22 ± 1 °C) until evaporation of the methanol and drying of the extract. Subsequently, 5 mL of distilled water was added to 0.01 g of the dried extract and shaken for 20 min with a digital vortex (Thermo Scientific®, USA). To 650 μL of this solution, 75 μL of $NaNO_2$ 5 % was added, shaken and left to stand for 6 min. After this time, 150 μL of $AlCl_3$ 10 % was added and then shaken and left to stand for 4 min. Finally, 500 μL of NaOH (1 M) and 1150 μL of distilled water were added. The absorbance value was recorded at 510 nm. The total flavonoid content was calculated by constructing a catechin standard curve (0, 20, 40, 60, 60, 80 and 100 mg L^{-1}) (Sigma-Aldrich, USA). The results are expressed in mg QE g^{-1} FW.

Leaflet area was measured using CI-202® leaf area meter equipment (Cid Bio-Science., Washington, USA). Extraction and quantification of the photosynthetic pigments was done using the method described by Ref. [38]. Briefly, the samples were placed in vials containing 100 mL of 80 % (v:v) acetone. Absorbance values were measured at 665, 653 and 470 nm using a Lambda 25® UV visible spectrophotometer (PerkinElmer, Waltham, USA). Results are expressed as mg kg^{-1} FW.

2.5. Yield, nut quality and leaf mineral nutrients

Nuts collection was carried out in the first week of November using mechanical vibration (1000-R, Casan®, México). The yield components were determined according to the Mexican Standard NMX–FF–084-SCFI-2009. The weight of the harvested nuts (fruits) was obtained using a Combo-Rhino-122 scale (Rhino®, Mexico) with a sensitivity of 0.1 g. Yield data is expressed as kg nuts•$tree^{-1}$. For the number of nuts per kg, 1 kg of nuts was randomly selected and counted. Next, 400 g of nuts were selected as a sub-sample and

the shells were removed and discarded. The kernels (the edible part) were weighed. Kernel percentage was calculated as the ratio of kernel weight divided by the nut sub-sample weight x 100.

Extraction and quantification of nutrients was carried out using the method of [39]. For analysis the leaflets were transported to the Plant Physiology Laboratory at the Universidad Autonoma of Chihuahua, Mexico. A triple wash was carried out with tap water, a 4 N HCl solution and deionized water. Surface moisture was completely removed from the leaflets at room temperature, which were then dried at 75 °C for 24 h in a Heratherm VCA 230® oven (Thermo Scientific, Waltham, USA). Each sample was homogenised in a Willey R-TE-650/1 mill with 1 mm mesh (Tecnal, São Paulo, Brazil). The extraction of total-N and P was done by the Kjeldhal method (Novatech®, USA and the Micro Kjeldahl Labconco®, USA and quantification by the ammonium metavanadate method (NH_4VO_3) (Thermo Scientific™, USA). The extraction of K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+} was determined by triacid digestion (HNO_3, $HClO_4$ and H_2SO_4) (25 mL of mixture in a 10:10:25, v:v:v) using 25 mL of the acid mixture on a hot plate under a fume-hood. Analyte quantification was carried out using an Analyst 100® atomic absorption spectrophotometer (PerkinElmer®, USA). Macronutrient concentrations are reported as g kg^{-1} and micronutrients as mg kg^{-1}. The B concentration was determined by a colorimetric method using the azomethine H reagent for a plant ash extract [40].

2.6. Statistical analyses

Data analysis was performed with information from the two years of the study. Prior to statistical analysis, a normal distribution was confirmed by the Shapiro-Wilk test ($p \leq 0.05$) [41]. Statistical analysis consisted of a general linear model with year and treatment effects. The comparison of means was carried out with a multiple comparison of means with Tukey's test ($p \leq 0.05$). The data was analysed using the statistical package Statistical Analysis Software, SAS/STAT|SAS, version 9.3.

3. Results

No significant effect was found for year ($p \geq 0.05$), which enabled the two-year trial to be analysed simply by treatment.

3.1. Oxidative metabolism and antioxidant capacity

Data on oxidative metabolism (SOD, H_2O_2, CAT, and GPx) and antioxidant capacity are shown in Table 1. Foliar spraying of ZnO NPs and 50 mg L^{-1} of H_3BO_3 significantly increased SOD activity in 40, 34, 32 % with respect to the control. Similar results were obtained for the generation of H_2O_2, a product of oxidative metabolism that is very damaging to plant cells. Catalase (CAT) is a key enzyme in the catalysis of H_2O_2 to water and oxygen, so in regulating H_2O_2 accumulation [42,43]. In general, Zn and B sprays caused significant reductions in CAT enzyme activity with values ranging from 2.53 ± 0.05 to 3.01 ± 0.04 nmol GSH min^{-1} g^{-1}, where the highest value corresponds to the control. Likewise, ascorbate also functions as a substrate for guaiacol peroxidase, an enzyme involved in a number of processes related to ROS-induced stress found in the cytoplasm, vacuole, cell wall and apoplast [44]. GPx increased ascorbate activity in trees sprayed with 50 or 100 mg L^{-1} B, with values of 5.93 ± 0.81 and 5.73 ± 0.87 nmol GSH min^{-1} g^{-1}, respectively.

The effect of the treatments on AC evaluated using the ABTS method was clear, where values fluctuated between 65.89 ± 1.1 and 75.01 ± 1.75 μmol TE (Trolox equivalents/g). However, when this parameter was determined by the DPPH method, no significant variation was found.

3.2. Bioactive compounds, leaf area and photosynthetic pigments

The pecan leaflets showed variation between treatments with respect to the concentration of total phenols (37.77 ± 0.62 and 52.55 ± 1.77 mg GAE g^{-1}) and total flavonoids (56.09 ± 2.51 and 69.92 ± 1.63 mg QE g^{-1}), where the application of 100 mg L^{-1} of ZnO and H_3BO_3 NPs showed significant reductions in both bioactive compounds (Table 2). On the other hand, the TChl values fluctuated

Table 1
Foliar application of zinc oxide nanoparticles or boric acid to young cv. Wichita pecan trees and their effect on oxidative metabolism and antioxidant capacity.

Treatments (mg L^{-1})	SOD	H_2O_2	CAT	GPx	AC	
					ABTS	DPPH
Control	1.35 ± 0.03 b	0.51 ± 0.01 a	3.01 ± 0.04 a	4.91 ± 0.09 b	65.89 ± 1.1 b	85.98 ± 0.39 a
^{50}NPs ZnO	1.89 ± 0.05 a	0.39 ± 0.01 b	2.81 ± 0.01 b	4.83 ± 1.1 b	71.80 ± 1.9 a	84.90 ± 0.05 a
^{100}NPs ZnO	1.78 ± 0.1 a	0.32 ± 0.01 c	2.53 ± 0.05 b	3.52 ± 0.85 c	73.76 ± 2.0 a	81.11 ± 2.67 a
$^{50}H_3BO_3$	1.69 ± 0.03 a	0.42 ± 0.02 b	2.69 ± 0.08 b	5.93 ± 0.81 a	70.09 ± 1.5 a	82.97 ± 0.47 a
$^{100}H_3BO_3$	1.31 ± 0.04 b	0.29 ± 0.03 c	2.97 ± 0.12 ab	5.73 ± 0.87 a	75.01 ± 1.75 a	83.07 ± 0.60 a

Means with the same letter within a column are not significantly different (Tukey's test, $p \leq 0.05$). Data are the averages between the 2020 and 2021 seasons. SOD - Superoxide dismutase (units min^{-1} g^{-1}); H_2O_2 - Hydrogen peroxide (μmol g^{-1}); CAT – Catalase (nmol GSH min^{-1} g^{-1}); GPx - Guaiacol peroxidase (nmol GSH min^{-1} g^{-1}); AC – Antioxidant capacity (BTS - 2,2′-azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (μmol TE (Trolox equivalents)/g); % of DPPH inhibition). Treatments: NPs ZnO, zinc oxide nanoparticles (50 and 100 mg L^{-1}); H_3BO_3, boric acid (50 and 100 mg L^{-1}).

Table 2
Bioactive compounds, leaf area and photosynthetic pigments in young cv. Wichita pecan trees sprayed with zinc oxide nanoparticles and boric acid.

Treatments (mg L^{-1})	TP	TFl	LA	μg g^{-1}	
				TChl	TA
Control	52.55 ± 1.77 a	69.92 ± 1.63 a	46.4 ± 1.6 a	37.50 ± 0.51 c	1.68 ± 0.2 a
^{50}NPs ZnO	50.93 ± 1.38 a	65.74 ± 1.10 a	34.8 ± 2.6 a	44.43 ± 0.85 a	1.35 ± 0.1 b
^{100}NPs ZnO	43.87 ± 1.71 b	56.09 ± 2.51 b	36.3 ± 2.2 a	41.01 ± 0.74 b	1.41 ± 0.1 b
$^{50}H_3BO_3$	37.77 ± 0.62 b	58.17 ± 2.29 b	33.2 ± 1.2 a	39.70 ± 0.85 bc	1.79 ± 0.1 a
$^{100}H_3BO_3$	42.17 ± 1.71 b	61.23 ± 1.57 ab	35.1 ± 2.1 a	42.24 ± 1.07 ab	1.49 ± 0.2 b

Means with the same letter within a column are not significantly different (Tukey's test, $p \leq 0.05$). Data are the averages between the 2020 and 2021 seasons. TP – Total phenols (mg GAE g^{-1}); TFl (mg QE g^{-1}); LA - leaf area (cm^2); TChl – Total chlorophyll (μg g^{-1}); TA: total anthocyanin's (μg g^{-1}). Treatments: NPs ZnO, zinc oxide nanoparticles (50 and 100 mg L^{-1}); H_3BO_3, boric acid (50 and 100 mg L^{-1}).

between 37.50 ± 0.51 and 44.43 ± 0.85 μg g^{-1} for the 50 and 100 mg L^{-1} ZnO NPs and H_3BO_3 sprays, increased the concentration of TChl, with leaflet area being unchanged. Likewise, anthocyanin concentration fluctuated between 1.35 ± 0.1 and 1.79 ± 0.1 μg g^{-1}, with a similar behaviour to chlorophyll, being a secondary pigment in the photosynthetic process in the leaflets.

3.3. Yield, nut quality and leaf mineral nutrients

Spraying leaflets with ZnO NPs and B significantly increased fruit yield with values between 16.02 ± 0.2 and 17.76 ± 0.6 (kg $tree^{-1}$), where the 100 mg L^{-1} dose of B showed a similar result to the control with 16.98 ± 0.5 kg $tree^{-1}$ (Table 3).

This trial was carried out over two years. It was found that nut weight (g kg^{-1}) fluctuated between 113.06 ± 4.6 and 128.01 ± 5.1, where the treatments with ZnO NPs and 50 mg L^{-1} of B did not show significant differences from the control. However, the 100 mg L^{-1} B spray showed a significant reduction in nut quality, i.e., nut weight and kernel percentage had values of 113.06 ± 4.6 g kg^{-1} and 53.49 ± 1.1 %, respectively (Table 3).

The total-N concentration was increased in the application of ZnO NPs and H_3BO_3 with values of 22.1 ± 0.3 and 24.9 ± 0.6 g kg^{-1}, respectively. However, the dose of 100 mg L^{-1} of ZnO was similar to the control (Table 4). Meanwhile, levels of P and K^+ were highly significant in leaflets sprayed with 100 mg L^{-1} of B and 50 mg L^{-1} of ZnO, respectively. It is well known that Ca^{2+} is a structural element of the cell wall of plants, including the leaflets, and in our work it showed wide variations in concentration between treatments, with values between 11.0 ± 0.06 and 13.0 ± 0.2 g kg^{-1}, where ZnO spraying favoured a significant increase in Ca^{2+} (13.0 ± 0.2 and 12.6 ± 0.2 g kg^{-1}). 0 ± 0.2 g kg^{-1}, where ZnO spraying favoured a significant increase in Ca^{2+} (13.0 ± 0.2 and 12.6 ± 0.2 g kg^{-1}) and showed no change in the concentration of Mg^{2+}, a structural element of the chlorophyll molecule, a key component of the photosynthetic process.

The foliar concentration of Fe^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} and B are presented in Table 4, where significant differences ($p \leq 0.05$) appear only for Fe^{2+}, Zn^{2+} and B. The values for Fe^{2+} fluctuated between 77.3 ± 0.7 and 87.6 ± 3.5 mg kg^{-1}, with all treatments showing a reduction compared with the control. Likewise, the Zn^{2+} concentrations ranged from 38.1 ± 0.5 to 39.5 ± 0.2 mg L^{-1} increased improved significantly with ZnO nanoparticle sprays for both concentrations.

The leaflet B concentration showed significant increases with each concentration of H_3BO_3 but the B concentration increase with 100 mg L^{-1} of ZnO NPs was similar.

4. Discussion

4.1. Oxidative metabolism and antioxidant capacity

Large areas of northern Mexico and southwestern United States are arid or semi-arid. In these regions pecan is important economically because it is planted over large areas. Pecan is also important as a food source because it is of high nutritional value [8]. The adverse climate and soil conditions of these regions - low rainfall, nutrient leaching, high soil solutes (salts and carbonates) can induce significant alterations in oxidative metabolism in the leaves (photosynthesis and cellular respiration). As a consequence, both

Table 3
Yield and quality nut in young cv. Wichita pecan trees sprayed with zinc oxide nanoparticles and boric acid.

Treatments (mg L^{-1})	Yield (kg $tree^{-1}$)	Nut quality	
		Nut weight (g kg^{-1})	Kernel (%)
Control	16.02 ± 0.2 b	120.06 ± 1.8 a	55.46 ± 0.8 a
^{50}NPs ZnO	17.76 ± 0.6 a	120.60 ± 3.5 a	55.35 ± 0.9 a
^{100}NPs ZnO	17.35 ± 0.3 a	121.02 ± 3.7 a	56.55 ± 0.8 a
$^{50}H_3BO_3$	17.68 ± 0.3 a	128.01 ± 5.1 a	57.04 ± 1.7 a
$^{100}H_3BO_3$	16.97 ± 0.5 ab	113.06 ± 4.6 b	53.49 ± 1.1 b

Means with the same letter within a column are not significantly different (Tukey's test, $p \leq 0.05$). Data are the averages between the 2020 and 2021 seasons. Treatments: NPs ZnO, zinc oxide nanoparticles (50 and 100 mg L^{-1}); H_3BO_3, boric acid (50 and 100 mg L^{-1}).

Table 4
Nutrient concentrations in leaflets of young cv. Wichita pecan trees sprayed with zinc oxide nanoparticles or boric acid.

Treatments (mg L^{-1})	N-total	P	K^{+}	Ca^{2+}	Mg^{2+}
Control	22.1 ± 0.3 b	1.9 ± 0.01 c	13.0 ± 0.2 b	11.5 ± 0.1 c	4.4 ± 0.2 a
^{50}NPs ZnO	24.8 ± 0.4 a	2.1 ± 0.05 bc	14.8 ± 0.2 a	12.6 ± 0.2 ab	4.1 ± 0.1 a
^{100}NPs ZnO	23.2 ± 0.5 ab	2.1 ± 0.04 bc	13.1 ± 0.5 b	13.0 ± 0.2 a	4.1 ± 0.17 a
$^{50}H_3BO_3$	24.2 ± 0.2 a	2.2 ± 0.04 b	13.1 ± 0.2 b	12.5 ± 0.1 b	4.0 ± 0.06 a
$^{100}H_3BO_3$	24.9 ± 0.6 a	2.5 ± 0. 09 a	12.5 ± 0.3 b	11.0 ± 0. 06 d	4.2 ± 0. 07 a
	Fe^{2+}	Cu^{2+}	Mn^{2+}	Ni^{2+}	Zn^{2+}	B
Control	87.6 ± 3.5 a	4.1 ± 0.2 a	70.3 ± 1.6 a	6.5 ± 0.2 a	24.3 ± 0.5 c	42.8 ± 0.9 c
^{50}NPs ZnO	83.3 ± 3.3 b	4.2 ± 0.3 a	62.6 ± 4.1 a	7.5 ± 0.3 a	38.1 ± 0.5 a	47.0 ± 0.4 b
^{100}NPs ZnO	77.3 ± 0.7 c	4.1 ± 0.2 a	70.8 ± 2.2 a	6.9 ± 0.3 a	39.5 ± 0.2 a	58.95 ± 1.1 ab
$^{50}H_3BO_3$	82.3 ± 2.8 b	4.1 ± 0.2 a	69.9 ± 2.8 a	7.3 ± 0.5 a	29.1 ± 0.2 b	60.8 ± 2.4 a
$^{100}H_3BO_3$	79.8 ± 2.4 bc	3.9 ± 0.2 a	70.7 ± 3.2 a	7.2 ± 0.3 a	27.7 ± 0.2 b	62.8 ± 3.1 a

Means with the same letter within a column are not significantly different (Tukey's test, $p \leq 0.05$). Data are the averages between the 2020 and 2021 seasons. Treatments: NPs ZnO, zinc oxide nanoparticles (50 and 100 mg L^{-1}); H_3BO_3, boric acid (50 and 100 mg L^{-1}).

tree development and pecan nut production are adversely affected [45,46] by accumulations of low molecular weight metabolic by-products and ROS that harm cells and the structural components of their organelles [47]. Plants normally possess specific mechanisms to deal with these. These detoxification mechanisms include the activation of antioxidant enzymes such as SOD, peroxidase (POX), CAT, ascorbate peroxidase (APX), glutathione (GR) and dehydroascorbate reductase (DHAR) [48,49]. It has been shown that Zn^{2+} is among the micronutrients that promote the reduction and stabilisation of the effects of oxidative and peroxidative stress on cell membranes by participating as a catalyst in the synthesis of several antioxidant enzymes and as a structural element of enzymes involved in the metabolism of lipids, carbohydrates and proteins [47,50]. It has also been shown that metal NPs (including of Cu, Fe, Ni and Zn) generate a certain level of toxicity and when applied as a foliar spray and can induce a controlled oxidative stress with the generation of ROS, a by-product of aerobic metabolism in all organisms including pecan [51,52].

Soil B toxicity significantly limits crop yield and is a common problem in many arid and semi-arid areas of the world, especially in those with calcareous soils. It is associated with low leaching and high salinity [53]. In our study, the pecan trees showed no visual symptoms of B toxicity in the leaves - viz. distal burning, marginal and interveinal necrosis of older leaves [53]. In spite of its economic importance, information on B deficiency and toxicity in pecan is limited and/or contradictory, especially for foliar applications. Our results are interesting in that many pecan orchards in northern Mexico and southwestern United States are established in areas with low rainfall, calcareous soils (pH between 7.5 and 8.5) and excessive B accumulation; a combination of abiotic factors that often limits tree development and production, due to induction and accumulation of ROS [45,55].

It is well known that plants possess a range of mechanisms (antioxidant enzymes and antioxidant metabolites) to minimise the impacts of oxidative stress caused by free radicals (superoxide anion, hydroxyl radical) or non-radical molecules (e.g., hydrogen peroxide, singlet oxygen) [56]. These have been extensively researched for their roles in the oxidation of membrane lipids, cellular proteins, carbohydrates, DNA and enzymes [57]. However, they are also recognised as secondary messengers in several biological processes, especially under stress conditions such as in calcareous soils [58], where AC is an indirect parameter that can help to monitoring the concentration levels of molecules with oxidative properties and the level of control associated with the joint biological activity between the antioxidant and oxidative system present in plants.

4.2. Bioactive compounds, leaf area and photosynthetic pigments

Foliar spraying of metal NPs, including of Zn, can provide a controlled stimulation of free radical formation in the leaflets of pecan. This activates the antioxidant system to counteract the effect, triggering the production of low molecular weight compounds such as anthocyanins, polyphenolic compounds, vitamins, dietary fibre, bioactive peptides, biogenic amines, among others [51,59]. Previous studies have conclusively demonstrated the role of these molecules in ameliorating the negative effects of numerous abiotic and biotic stresses including lack of water, pathogen attack, excessive radiation, and unfavourable soil conditions - pH, excess salts, sulphates and carbonates [60,61]. Results similar to those in our study have been reported by Ref. [26] in apple (*Malus x domestica* Borkh) cv. 'Golden Delicious' with foliar sprays of 250 mg L^{-1} of Zn nanoparticles. Likewise [62], in 'Grany Smith' apple trees sprayed with 0.2 % Zn ($ZnSO_4.7H_2O$) one month after full bloom showed a significant reduction in the concentration of total phenols in the fruit. This behaviour can be explained by the role played by phenolic compounds (e.g., phenols, total flavonoids) as a defence mechanism against abiotic stress, including conditions of high soil carbonate levels. On the other hand, in pomegranate (*Punica granatum*) cv. Ardestani with combined applications of different doses of nanochelates of Zn (0, 60 or 120 mg L^{-1}) and B (0, 3.25 or 6.5 mg L^{-1}) [27], report that only the highest concentrations of each micronutrient significantly modified the concentration ($\leq$1 %) of phenolic compounds. In this connection, B has been proposed as a micronutrient involved in the synthesis and metabolism for several secondary compounds, including antioxidant polyphenols [5].

By applying 118, 236 or 354 kg ha^{-1} of N to adult trees (≈35 years old) of cv. Western Schley pecan trees [63], showed that net photosynthesis, biomass accumulation, N and micronutrient concentration are broadly correlated with chlorophyll concentration in leaflets. Also, several researchers have indicated a role for Zn in net photosynthesis by catalysing the enzyme carbonic anhydrase, an important factor for CO_2 fixation in the Calvin cycle in the chloroplast stroma [3,64]. In other crops such as mulberry (*Morus alba* L.)

cv. Khalili B deficiency (0 and 0.02 mM) and B toxicity (0.5 and 1 mM) significantly reduced net photosynthesis and leaf chlorophyll content. There are few or no reports on the response of pecan to B sprays and its role in photosynthesis. However, the key roles of B in cell membrane integrity and stability, in photo-assimilate transport and photosynthesis have been determined. These all minimise the impacts of physiological stress associated with abiotic factors, including the presence of calcareous soils [65].

4.3. Yield, nut quality and leaf mineral nutrients

Among the most important and sensitive responses to calcareous soils (pH ≈ 8.0) is the linked to nut yield and quality parameters which are of fundamental interest to producers, technical advisors and marketers for this type of nut [9]. A previous study on ≈7-year-old trees of pecan cv. Western Schley by Ref. [24] with foliar sprays of ZnO NPs (200 mg L^{-1}) reported a significant effect on yield, with an average value over two production cycles of 12.2 kg $tree^{-1}$, with variation attributable to tree age, cultivar and degree of alternate bearing [66]. In contrast [11], after applying 2 or 4 g L^{-1} of ZnO NPs to ≈23-year-old cv. Western pecan trees report yield values between 48.62 ± 8.19 and 57.62 ± 8.78 kg $tree^{-1}$. However, this study was with adult trees in full production and the results correspond to only one year of evaluation, and with consideration given to the predilection of pecan to alternate bearing. Yield of adult pecan trees is strongly related to the levels of mineral nutrient reserves and non-structural carbohydrates held over from previous production cycles. However, the nutritional supply of the current year is also considered a determining factor in fruit growth [67]. On the other hand, some authors such as [54] suggest that B is available in sufficient quantities in the majority of orchards in southwest United States and northern Mexico, this suggests producers should be able to obtain acceptable yields and quality without foliar B applications, though the levels of the other nutrients and micronutrients should still be considered and if necessary amended.

The weight of nuts and the percentage kernel together define yield for pecan so are the key parameters for producers because they relate directly to their financial return. In contrast to the results of our study, a combined foliar spray containing 0.5 % urea +0.1 % boric acid +0.5 % zinc sulphate +5 ml L^{-1} of 'supramino' significantly increased nut and kernel weights in cv. Western Schley pecan trees as reported by Ref. [68]. In other fruit tree species such as almond (*Prunus dulcis*) cv. Butte [69], applied B in September, December and February in doses that fluctuated between 49 and 205 mg L^{-1} of B, they observed significant increases in fruit set and in yield. The best treatment was the one applied early, soon after the previous harvest. Some of the inconsistencies observed for foliar applications of B and Zn are undoubtedly due to the weather conditions at the time of application as these differ site, with season, and with canopy. Our data for nut quality with B sprays show effects on the percentage kernel which should fluctuate between 50 and 54 % according to the NMX–FF–084- SCFI-2009, 2009. Previous studies [27] indicate that the distribution of B in the plant involves the formation of a B-polyol complex in the mature leaves. Only in this complexed form can B be transported in the phloem to key sinks, including to swelling buds and flowers, where it affects both pollen tube growth, and thus fertilisation, and thus fruit set.

Nitrogen is an essential element that plays important roles in the fruit trees, including pecan, where the its interannual availability helps maintain productivity [66]. Our results for N lie within the sufficiency range (20.5 and 29.5 mg kg^{-1}) reported for pecan [70]. However, our results for N following with the application of ZnO NPs are low when compared to the 24.8 mg kg^{-1} reported by Ref. [71] for the soil conditions in the Sonora region, Mexico. These variations can be attributed to the different pecan cultivar, tree age, sampling date, management and the site's edaphoclimatic characteristics. It is well known that N is extremely mobile that is rapidly translocated to developing fruits that are sinks for many mineral nutrients – especially for K (highly phloem mobile) but not for Ca (not phloem mobile) [45,55] (Naira et al., 2013). There are few studies that deal with the simultaneous foliar spraying of ZnO NPs and B on pecan, and which measure aspects of yield, nut quality and the levels of key bioactive compounds. A study by Ojeda-Barrios et al. (2023) of the application of foliar sprays of 200 mg L^{-1} ZnO nanoparticles over two years on seven-year-old pecan trees (cv. Western Schley) showed slight increases in the interannual concentration of total-N from 24.3 to 25.9 g kg^{-1}, however, this was not significant over the two years evaluated. A similar behaviour was reported by Ref. [68], when 0.1 % B and 0.5 % $ZnSO_4$ were sprayed on pecan (cv. Western Schley), with leaf N values of 22.4 and 22.7 mg kg^{-1} with respect to the control. Zinc participates in numerous enzyme reactions, it is a structural component of various proteins and it plays critical roles in DNA synthesis through the tryptophan pathway, being an essential component of tryptophan [72]. In other deciduous fruit trees such as pomegranate, cv. Ardestani, foliar sprays of nano-Zn chelate fertiliser (0, 60 or 120 mg Zn L^{-1}) and nano-B chelate fertiliser (0, 3.25 or 6.5 mg B L^{-1}) [27] found significant increases in N in the leaf tissues and in fruit set. This response is likely linked to the participation of B in both the (early) cell-division and (later) cell-expansion phases of fruit development.

Unlike N, P is relatively immobile in the soil, so band application is used to improve its availability [73]. The profitability of pecan orchards is also strongly related to the management of K^+. Pecan is a strongly biennial bearing species so K^+ management is especially important in the 'on' years when crop loads are high [74]. Our K^+ values were within the sufficiency range reported for pecan of 10.0–15.8 mg kg^{-1} [70] but our P values were high where values between 1.4 and 3.0 g kg^{-1}.

A previous study by Ref. [24] reported P values between 1.7 and 2.7 g kg^{-1} and between 17.0 and 22. 2 g kg^{-1} for K with the spraying of nitra-zinc (NZN), $ZnSO_4$, Zn-EDTA and ZnO NPs at concentrations of 200 mg L^{-1}, where the zinc oxide nanoparticles significantly reduced P concentration between years (2.5 and 1.9 g kg^{-1}) while keeping K^+ values unchanged. Zinc sulphate sprays (50, 100 or 200 mg L^{-1} of $ZnSO_4 \bullet 7H_2O$) on pecan cv. Western Schley did not affect the trees' K^+, Ca^{2+}, Mg^{2+}, Cu^{2+} or Mn^{2+} levels [75].

The B/P ratio is of significance in plant nutrition but the reasons for this are not well understood. Research in tomato and maize indicates an antagonistic or competitive relationship between these B and P because they share the same uptake and transport system [76]. The mechanism of the B/K interaction is unknown, but it has been observed in crops such as tomato and tobacco, that B deficiency or toxicity are aggravated by increases in K^+. In tobacco, high levels of B induce accumulations of K^+ in the roots and leaves, also reducing the accumulation of Mg^{2+} [77]. The synergistic relationship between Ca^{2+} and B is better known, where it influences the

formation and stability of cell walls, where a B deficiency can hinder Ca transport and functionality, while an excess of Ca can accentuate the symptoms of B deficiency [76].

Our Fe and Zn concentration results are in the normal range for pecan: Fe, 50–250 mg kg^{-1} and Zn, 50–118 mg kg^{-1} [2]. In general plants, including pecan take up Fe^{2+} and Zn^{2+} through similar pathways, therefore these ions tend to show antagonistic behaviour towards one another, with a Zn^{2+} deficiency aggravating a Fe^{2+} deficiency, and with a high Fe^{2+} level limiting Zn^{2+} uptake [78]. This interactive behaviour requires optimal supplies of these micronutrients to avoid nutritional imbalance. Soil physical properties such as drainage and texture can also determine their availability. Our sprayed trees showed no visual symptoms of either Fe or Zn deficiency - green veins, chlorotic leaf blades, chlorosis of young leaves [3].

The nutritional management of pecan includes the supply of Zn^{2+} as the second most important nutrient after N to maximise production. Technical advisors usually recommend fertilizers such as NZN, $ZnSO_4$ or $ZnNO_3$, due to their high market availability and relative efficiency in maintaining levels of foliar Zn [75,79]. Till now, work on the spraying Zn^{2+} as ZnO NPs has been limited to only a few fruit tree species such as mango, olive, apple, pomegranate, banana and avocado [26–30]. For pecan, the reports on ZnO NPs sprays are limited to cv. Western Schley [11,24]. Hence our results are fairly novel.

A previous study by Ref. [24] when applying 200 mg L^{-1} of ZnO NPs and ZN-EDTA in pecan cv. Western Schley reported a significant interannual Zn reduction (39.3–35.8, 35.8 and 38.0 mg kg^{-1}, respectively). However, other sources ($ZnSO_4$ and NZN) maintained initial foliar Zn^{2+} concentrations of 39.6–40.4 mg kg^{-1}, and 48.5 and 45.3 mg kg^{-1}, respectively. The concentration values reported here for Zn are similar to both these reports and also in the range of sufficiency reported for pecan [70]. The leaflets of our trees over two seasons were not Zn deficient, which may explain the final leaf Zn concentrations, but the effects of dose, time of application, cultivar and soil type could also be involved.

The importance of B in pecan production lies principally in its roles in flowering and fruit set, especially as this species is considered to be biennial bearing. Applications of B also seek to minimise the incidence of the physiological disorder 'water-stage fruit-split' in the thin-shelled pecan cvs. including Wichita [19]. Therefore, the focus of research has been in this direction, which explains the observation that there are few studies that seek to increase or maintain foliar B concentrations using boric acid as the source of B. Our results are comparable to sufficiency reports for pecan in a wide range of regions and are similar to the 40 mg kg^{-1} reported for Georgia, USA [80] but they can be considered low compared with results for New Mexico (137 mg kg^{-1}, [80]) and Arizona, USA (111 mg kg^{-1}, [70]). A previous study in which pecan trees of cv. Mahan were sprayed with 3 g L^{-1} H_3BO_3 or 5 g L^{-1} of $ZnSO_4$ reported increases in foliar of B and Zn concentrations of 48.7 and 50.0 mg kg^{-1}, respectively [81]. The doses evaluated by these authors could have caused toxicity problems if sprayed prior to flower bud opening, especially if the initial status of the trees was not evaluated. Nevertheless, their results are similar to ours and also within the range of sufficiency for pecan. In other deciduous fruit tree species, such as in cv. Rubinola apple, when B was applied in foliar sprays at 0.018 kg ha^{-1} and Zn at 0.300 kg ha^{-1} in two soil conditions, significant increases in both B and Zn were reported in both leaves and fruits [82].

The biannual sprays of ZnO NPs and boric acid evaluated in our study were a determining factor in correcting possible deficiencies in these micronutrients exacerbated by the calcareous soil. Under the conditions of this study, foliar sprays of 50 mg L^{-1} of ZnO NPs and H_3BO_3 increased enzyme activity (SOD and GPx), yield (16.02 ± 0.2 and 17.76 ± 0.6 kg $tree^{-1}$), and nut quality and nutrient levels (N-total, P, K^+, Ca^{2+}, Fe^{2+}, Zn^{2+} and B). However, significant reductions were observed in CAT activity, in the concentrations of bioactive compounds such as TP and TFl and AC. On the other hand, low dose sprays of ZnO NPs and B could be an alternative way of increasing yield and nut quality, as well as of foliar concentrations of Zn and B in young cv. Wichita pecan trees in calcareous soils.

5. Conclusions

One of the main challenges to improve the quality and productivity in pecan orchard is the optimal supply of Zn and B. Under the conditions of this study, foliar sprays of Zn (ZnO NPs) and B (H_3BO_3) (50 mg L^{-1}) helped to increase SOD activity and yield (kg $tree^{-1}$), but only B affected GPx activity. Likewise, CA was significant for all treatments. A reduction in TP and TFl content of leaflets treated with Zn and B was observed, where doses of 50 mg L^{-1} ZnO NPs and 100 mg L_{-1} H_3BO_3 increased chlorophyll content. Nut quality expressed as number of nuts and kernel percentage were negatively affected with the application of 100 mg L^{-1} of B. The use of Zn as ZnO NPs and B (boric acid) helped to improve the foliar concentration of N-total, K^+, Ca^{2+}, Zn^{2+} and B while a significant reduction was observed for Fe^{2+}. These results demonstrate that spraying low doses (50 mg L^{-1}) of ZnO NPs and B can help reduce oxidative stress and increase yield, nut quality and leaf concentrations of Zn and B in young cv. Wichita pecan trees established on a calcareous soil. However, it is necessary to conduct further evaluations (different doses, time of application, stress factors, cultivars, among others) to improve the comprehension of the effect caused by foliar supply of Zn as ZnO NPs and B (boric acid) on the physiology and biochemistry of this temperate fruit tree.

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Data availability

Data will be made available on request.

CRediT authorship contribution statement

O. Cruz-Álvarez: Writing – review & editing, Writing – original draft. **E. Sánchez-Chávez:** Software, Methodology, Conceptualization. **A. Benavides-Mendoza:** Writing – original draft. **O.A. Hernández-Rodríguez:** Validation. **R.A. Parra-Quezada:** Methodology. **J.P. Ciscomani-Larios:** Conceptualization. **M.T. Martínez-Damián:** Writing – review & editing, Supervision, Data curation, Conceptualization. **D.L. Ojeda-Barrios:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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