Original Article

Zinc Nutritional Status of Pecan Trees Influences Physiological and Nutritional Indicators, the Metabolism of Oxidative Stress, and Yield and Fruit Quality

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Abstract

In the United States of America and in Mexico, zinc deficiency is a common nutritional disorder in pecan trees [*Carya illinoinensis* (Wangenh.) C. Koch], especially in calcareous soils. This study in Chihuahua, northern Mexico, analyses the effects of zinc nutritional status on various physiological and nutritional indicators, on the metabolism of oxidative stress, and on the yield and fruit quality of pecan. The aim was to identify possible bioindicators of soil zinc deficiency. The experimental design was completely randomized with four nutritional conditions with respect to zinc: a control and three levels of zinc deficiency - slight, moderate and severe. Zinc deficiency is characterised by small leaves with interveinal necrosis and rippled leaf margins. The lowest values of leaf area, SPAD values, total N and NO_3 concentration were observed under conditions of severe zinc deficiency. With worsening zinc deficiency, results indicate an increased enzymatic activity of superoxide dismutase, catalase and glutathione peroxidase. Interestingly, under severe zinc deficiency there are decreases in trunk cross-sectional area growth, in yield and in percentage kernel. Increased activity of superoxide dismutase, catalase and peroxidase enzymes is associated with detoxification of reactive oxygen species. The activity of enzymes detoxifying reactive oxygen species lessens the negative effects of zinc deficiency stress, and may be good bioindicators of zinc deficiency and its visual symptoms on pecan trees.

Keywords: *Carya illinoensis*; catalase; glutathione peroxidase; superoxide dismutase; zinc deficiency

Introduction

The pecan tree (*Carya illinoensis* [Wangenh.] K. Koch) is native to northern Mexico and the southern United States. After the United States, Mexico is the second largest producer of pecans in the world, with a planted area of 113,000 ha, yielding some 40% of world production and 56% of the internationally-traded volume (SIAP, 2016).

The planted area in the state of Chihuahua (northern Mexico) is about 70% of the national total and this produces some 80,000 tons of pecans. A factor limiting pecan production in this region is the low availability of zinc (Zn) in the alkaline (calcareous) soils which are also low in organic matter. Zinc deficiency symptoms are common here (Ojeda-Barrios *et al.*, 2012, 2014).

In pecan trees Zn deficiency presents as 'pecan rosette', a characteristic visual symptom in which leaf size and internode length are reduced. Severe symptoms include interveinal chlorosis, eventually developing to necrosis. Terminal growth dieback can also occur. Zinc deficiency can also reduce catkin length, fruit number per shoot, fruit development and, when severe, it can inhibit production of inflorescences (Walworth *et al.*, 2017).

Zinc is an essential micronutrient playing important roles in the function of several enzymes. In plants, enzymes either containing Zn, or activated by it, are involved in carbohydrate metabolism, protein synthesis, maintenance of the integrity of cell membranes, regulation of auxin synthesis and pollen formation (Hafeez *et al.*, 2013).

The micronutrient Zn plays a major role in cell defence against reactive oxygen species (ROS) and as a protective factor for several targets of oxidation, such as membrane lipids, proteins, chlorophyll and enzymes containing SH and DNA (Cakmak, 2000). Zinc plays multiple roles in plant metabolism, including the maintenance of cell membrane structure and the synthesis of hormone precursors and chlorophylls, as well as in the constitutive enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), which are involved in the detoxification of ROS (Hacisalihoglu *et al.*, 2003). Plants also require Zn for tryptophan synthesis, a key amino acid in the production of the auxin indole-3-acetic acid. Zinc operates in the control of plant development through its indirect action on the auxins (Ojeda-Barrios *et al.*, 2012). Zinc deficiency affects the catalytic activity of enzymes and metabolic pathways (Cakmak, 2000; Hafeez *et al.*, 2013). Zinc deficiency is also reflected in change in the levels of amino acids (Acevedo-Barrera *et al.*, 2017).

Plant cell metabolism produces ROS naturally through respiration and, because these products are toxic to cells, enzymatic and non-enzymatic systems have been developed to neutralise and prevent oxidation of cellular components. Zinc is present in the activation of several antioxidant enzymes involved in the scavenging for superoxide anion radical (O_2^-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyle radical (*OH) (Hafeez *et al.*, 2013). Plants have evolved mechanisms to reduce damage to cellular components including proteins, membrane lipids and nucleic acids, by the free radical oxygen molecules (Cakmak, 2008; Lopez-Millan *et al.*, 2005; Sida-Arreola *et al.*, 2015).

Working with pecan trees, the objective of this study was to record the effects of Zn nutritional status on various physiological and nutritional indicators, on the metabolism of oxidative stress and on yield and fruit quality (percent kernel and nut weight), as possible bioindicators of Zn deficiency and the visual symptoms.

Materials and Methods

The study area

The study was carried out in 2014, near Aldama, Chihuahua, Mexico, located at 28° 50' N, 105° 53' W, at 1262 masl. The climate and soil conditions are similar to those in other regions of northern Mexico where pecan trees are grown (Ojeda-Barrios *et al.*, 2014). Average annual temperatures are 22 to 24 °C and average annual rainfall is in the range of 200 to 300 mm. Precipitation normally occurs from May to October. The prevailing wind is from the south and of 27 to 44 km h^{-1}. Frost frequency is from 0 to 20 days per year and hail from 0 to 1 day per year (SIAP, 2016).

Orchard management

The pecan orchard was established twelve years previously. Western Schley scion was grafted onto native pecan rootstock. The planting scheme was 12 × 12 m, equivalent to 70 trees ha^{-1}. This study used 24 trees, selected according to trunk diameter and the concentration of foliar Zn. Average leaf Zn concentration was 7.5 mg kg^{-1}

determined in July 2013, indicating the occurrence of Zn deficiency (Ojeda-Barrios *et al.*, 2012). The trunk diameter was measured 30 cm above soil level on May 20, 2014 and this was used to estimate initial trunk cross-sectional area (TCSA). Trunk diameter was re-measured on Dec 20, 2014 (dormant) and the diameter change used to assess TCSA change. The orchard was on a calcareous soil, having an arable layer of 0 to 35 cm with a pH of 7.2 in 1:1 soil:water, 1.1% organic matter, 30.0% total $CaCO_3$, 10% active $CaCO_3$ by the Droineau method (Duchaufour, 1987), 8.8 mg·kg^{-1} NO_3^- and 0.44 mg·kg^{-1} DTPA-extractable Zn (Rivera-Ortiz et al., 2003). The trees were surface fertilised in the first week of April each year with a granular fertiliser (120 N, 183 P_2O_5, 96 K_2O). After fertilisation, the soil was immediately tilled and the trees irrigated. The irrigation system was gravity-fed at 20-day intervals, resulting in a cumulative application of 120 to 140 mm of water from late March to the end of October. There was a low occurrence of pests and diseases.

Experiment design

A fully randomized experimental design was used with six repetitions of four Zn nutrition conditions: control, slight, moderate and severe Zn deficiency (Fig. 1). Zinc nutrition conditions were in accordance with visual symptoms and Zn nutritional status. Monitoring was carried out to measure possible disease symptoms. The 24 selected trees had similar initial trunk diameter measurements.

Plant material

Leaflets were selected 95 days after flowering (DAF) based on their appearance in terms of colour, shape and branch size due to Zn nutrition status. Four Zn deficiency status were selected: control (> 20-11 mg Zn kg^{-1}), slight (20 mg Zn kg^{-1}), moderate (11 to 9 mg Zn kg^{-1}) and severe (≤ 9 mg Zn kg^{-1}) (Fig. 1). Each Zn nutritional condition and its replicate leaflets were sampled during the growing season on July 25, 2014 (fruit in early cotyledon stage). Following the method of Ojeda-Barrios *et al.* (2014), 40 leaflets were taken from the mid-canopy of the crown. These were of current year's growth, and taken from north, south, east and west orientations (so they included sun-exposed and shaded leaflets from vegetative and from fruiting shoots). The collected material was placed in transparent plastic bags and frozen at -80 °C pending enzymatic analysis. Leaflet area and SPAD chlorophyll readings were determined in all 40 leaflets per tree. SPAD was measured using a SPAD 502 meter (Konica Minolta Sensing America Inc., Ramsey, NJ) in the middle part of each leaflet and avoiding major veins at 95 DAF.

Plant analysis

The leaflets were selected 95 days after flowering and then transferred to the laboratory of Plant Physiology of the University of Chihuahua. Leaflets were washed in dilute detergent solution (0.1% Mistol, Henkel, Barcelona, Spain), tap water, 1% HCl and three separate 7-L demineralised water baths, following the protocol of Smith & Storey (1979) (Ojeda-Barrios *et al.*, 2014). Leaves were oven-dried at 55 °C to constant weight, ground and stored in airtight containers pending analysis. Samples (500 mg) were washed

at 50 °C and dissolved in HNO₃ and HCl following the procedure of the Association of Official Analytical Chemists (Latimer, 2012). Zinc was determined by atomic absorption spectrometry. Nitrogen concentration was determined by the micro-Kjeldahl method in separate samples, and NO₃ as in Bremner (1965).

Leaflets collected for enzymatic analysis were washed in deionised water and stored at -80°C pending analysis. The enzymatic determination was carried out at 4 °C. The leaflets were homogenised in a mortar with 100 mM of potassium buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM dithiotreitol and 5% (W/V) PVPP at a ratio of 2:1 (volume of the pellet / fresh weight). The samples were centrifuged for 30 min at 10,000 × *g* and the supernatant were stored in separate aliquots at -80 °C (Azevedo *et al.*, 1998).

Yield and nut quality sample

At harvest, yield was estimated by shaking trees and measuring the weight of nuts in a wedge-shaped grid enclosing 1/100 of the area beneath the tree. Four sample grids were used per tree, and the nut weights were summed and multiplied by 25 to estimate total nut yield per tree. A 50-nut sample was collected from each tree for analysis of individual nut weight (Wells, 2012). To determine edible kernel weight, a 300 g sample was selected per replicate, the husk was separated from the edible kernel, and the two fractions weighed separately. The percentage of kernel was calculated following the Mexican Standard NMX-FF-084-SCFI-2009.

Antioxidant systems

At 95 DAF (i.e. early cotyledon stage) leaflet samples were selected at random, harvested and transferred to the laboratory. Forty leaflets were harvested per tree for each analysis of the antioxidant system.

Extraction and assay of peroxidase and catalase

The methods used for extraction and assay of peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6) were modified versions of those proposed by Sanchez *et al.* (2000). Fresh plant material was ground with 50 mM Tris-acetate buffer, pH 7.5, 5 mM 2-mercaptoethanol, 2 mM DTT, 2 mM ethylene-diaminetetraacetic acid (EDTA), 0.5 mM PMSF, and 1% (w/v) PVP. The homogenate was filtered through two layers of Miracloth and centrifuged for 30 min at 37 000 *g*. The pellet was discarded and the supernatant used for POD and CAT assays, and to measure the protein concentration by the method of Bradford (1976), using BSA as standard. All these procedures were carried out at 0-4 °C.

POD activity was determined by following the change in A₄₈₅ due to guaiacol oxidation. CAT activity was determined by following the consumption of H₂O₂ at A₂₄₀ for 5 min in 3 mL of reaction mixture, due to H₂O₂ oxidation (Sanchez *et al.*, 2000). To determine whether the reactions were enzymatic, control extracts were boiled and then assayed.

Extraction and assay of SOD

SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), according to the methods of Giannopolitis and Ries (1977) and Beyer and Fridovich (1987), with some modifications (Wilde & Yu *et al.*, 1998). Frozen leaf samples were weighed and homogenised on ice in a mortar and pestle for 2 min with 1.5 g of quartz sand and 10 mL of homogenising solution containing 50 mM HEPES buffer and 0.1 mM Na₂EDTA (pH 7.6). The homogenate was centrifuged at 4 °C for 15 min at 15 000 *g*, then filtered through qualitative filter paper (MN 617, Macherey -Nagel, Germany) to produce the crude extract. It was then used for SOD assay and to measure the protein concentration by the method of Bradford (1976), using
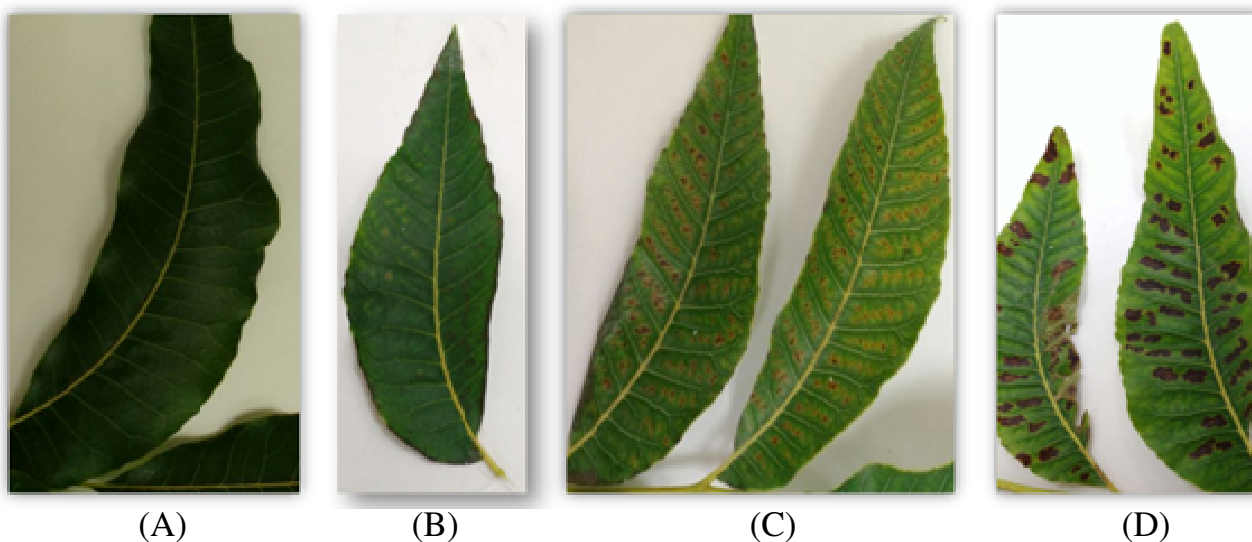

(A)   (B)   (C)   (D)

Fig. 1. Zn nutrition status in pecan tree leaves in accordance with its symptomatology. (A) Control: even green leaf (≥20 mg kg⁻¹). (B) Slight: leaves with early stage Zn deficiency symptomatology showing spotted regions, general internode chlorosis (> 20-11 mg kg⁻¹)(C) Moderate: leaves with advanced stage Zn deficiency (11 a 9 mg kg⁻¹) (D) Severe: leaves with serious Zn deficiency symptoms showing chlorosis, necrosis and leaves with undulate margins (< 9 mg kg⁻¹)

BSA as standard. All these procedures were carried out at 0-4 °C (Sanchez *et al.,* 2000).

For total SOD assay, a 5-mL reaction mixture was used, containing 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na_2CO_3 (pH 10.0), 13 mM methionine, 0.25% (w/v) Triton X-100, 63 μM NBT, 1.3 μM riboflavin and an appropiate aliquot of enzyme extract. The reaction mixtures were illuminated for 15 min; PPFD was 380 μmol $m^{-2}s^{-1}$. Identical reaction mixtures that were not illuminated were used to correct for background absorbance. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm (Sanchez *et al.,* 2000).

Extraction and assay of total H_2O_2

The methods used were those proposed by Brennan and Frenkel (1977). Hydroperoxides form a specific complex with titanium (Ti^{4+}), which can be measured by colorimetry at 415 nm. The concentration of the peroxide in the extracts was determined by comparing the absorbance against a standard curve representing a titanium-H_2O_2 complex from 0.1 to 1 mM. The hydroperoxides represent the total peroxides. Concentrations of total soluble proteins in the leaf extracts used for enzyme assays were determined with the Bradford protein assay kit (BioRad, California, USA) according to the manufacturer's protocol.

Statistical analyses

Data were subjected to ANOVA at 95% confidence. Means were compared by Tukey test (P ≤ 0.05). The data shown are mean values ± standard error (SE). Levels of significance are represented by * at P < 0.05 and NS: not significant.

Results and Discussion

Leaf pigments

The assessment was based on the characteristic visual symptoms of Zn deficiency, which include interveinal necrosis, chlorosis and the formation of leaf rosette. Sampled trees were classified into three levels of Zn deficiency, on the basis of the degree of visible foliar damage (slight, moderate and severe) when compared to a Zn-sufficient control without visible indications of Zn deficiency in Fig. 1.

The relationship between SPAD and leaflet area is shown in Fig. 2. The SPAD values observed were 45.3, 35.0, 33.2 and 27.3 for the control, and the slight, moderate and severe Zn deficiencies, respectively. Severe deficiency samples showed a 40% reduction in SPAD, as well as 68% decrease in leaflet surface area compared with the controls. These results are in line with previous studies (Ojeda-Barrios *et al.,* 2012), who observed that applications of foliar Zn fertiliser led to increases in leaf surface area and leaf chlorophyll.

The decrease in leaf surface area with increasing Zn deficiency is in line with the observation that Zn deficiency affects the biosynthesis of tryptophan, a precursor of the auxin IAA responsible for cell division (Ojeda-Barrios *et al.,* 2012). Zinc deficiency generally causes a decrease in leaf size, shoot length and cell division (Hafeez *et al.,* 2013). Studies showed that the growth of tea leaves (*Camellia sinensis*) decreased with increasing Zn deficiency. Similar results were obtained with mulberry trees [*Morus alba* (L)] affected by Zn deficiency (Tewari *et al.,* 2008). Mukhopadhyay *et al.* (2013) attributed decreased foliar surface area to damage during cell division. Zinc deficiency affects chlorophyll synthesis (Hafeez *et al.,* 2013) and chloroplast membranes (Lin *et al.,* 2005). Zinc is involved in both the development and the functioning of chloroplasts, since Zn activates the Zn-dependent SPP peptidase, and reconstitutes photo damaged D1 protein to repair photosystem II. In the case of Zn deficiency, the D1 protein is not reconstituted and chloroplast membranes are easily damaged by ROS (Hansch and Mendel, 2009). A significant reduction in the concentration of chlorophylls *a* and *b* in pistachio seedlings grown in soil with 0 and 5 mg Zn kg^{-1} as compared to those grown in soil with 10 mg Zn kg^{-1} has also been reported (Tavallali *et al.,* 2010).

Yield and nut quality

There was a significant decrease in yield (kg/tree), with a 57% yield reduction in the severe Zn deficiency trees (Table 1). The differences in TCSA revealed a similar response, with a 55% decrease. The percent of the kernel and of the fruit were similarly affected showing decreases of 86 and 66%, respectively.

Also, under severe Zn deficiency, there was a decrease in both nut production and nut quality. Increased activities of
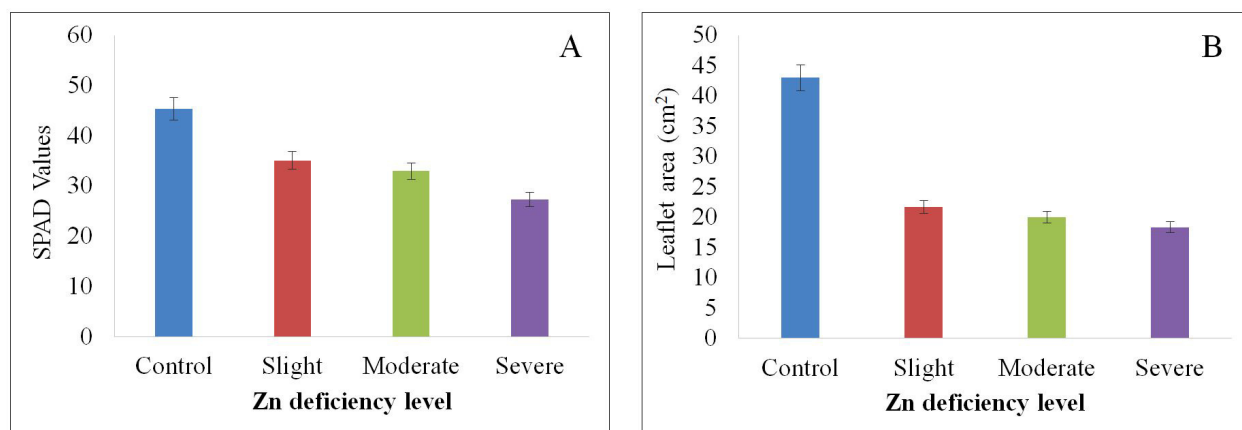

Fig. 2. Effects of slight, moderate and severe Zn deficiency on (A) pecan tree leaf chlorophyll concentration and (B) leaflet area. Data are means ± SE (n = 6). Means were compared using the Tukey's test (p ≤ 0.05)

Table 1. Yield, percent kernel, nut weight and trunk cross-sectional area in pecan trees exposed to control, and to slight, moderate or severe zinc deficiencies

Characteristic	Yield (kg per tree)	Percent kernel (%)	Nut weight (g)	Difference in TCSA (cm^2)
Control	15.67 ± 1.96 a	56.67 ± 1.37 a	10.75 ± 0.52 a	3.33 ± 0.67 a
Slight	13.50 ± 1.64 ab	54.33 ± 1.21 a	8.33 ± 0.75 b	2.50 ± 0.68 ab
Moderate	12.50 ± 1.64 b	51.33 ± 2.25 b	7.75 ± 0.52 bc	2.33 ± 0.56 ab
Severe	9.00 ±1.26 c	49.00 ± 1.26 b	7.08 ± 0.37 c	1.83 ± 0.20 b

Values shown are mean ± SE (n=6). Means were compared by the Tukey's test (p ≤ 0.05). TCSA, trunk cross-sectional area.

SOD, CAT and POD enzymes are directly linked to ROS detoxification. Zinc sufficiency is required for normal shoot elongation and leaf expansion. Also, because Zn deficiency results in incomplete shoot development, pecan yield is drastically reduced under these conditions (Sparks, 1993; Wells *et al.*, 2012).

Antioxidant systems

In moderate and severe Zn deficiencies, compared to the control, we observed increases up to 2.3, 1.4 and 1.7-fold in the activities of the leaf extract enzymes CAT, SOD and GSH-Px, respectively (Fig. 3).

Similar increases under Zn deficiency have previously been found in mulberry tree leaves (Tewari *et al.*, 2008). However, Zn deficiency has been reported to cause significant decreases in the activities of SOD and GSH-Px in maize (*Zea mays* L.), though CAT activity was not significantly affected (Wang and Ji-Yun, 2007). Increases in the activities of enzymes involved in ROS detoxification,

such as of SOD, CAT and POD, have been reported in assessments of antioxidant responses to Zn deficiency in pea genotypes (*Pisum sativum* L.) (Pandey *et al.*, 2012). Also, in *Pistacia vera* (Tvallali *et al.*, 2010) when under oxidative stress. The evidence indicates that oxidative damage to critical cell components resulting from reactive oxygen underlies various disturbances in plant growth caused by Zn deficiency (Cakmak, 2000). In the present study, H_2O_2 concentration increased in parallel with the level of Zn deficiency. This indicates the synthesis of H_2O_2 due to SOD activity exceeds the capacity of the CAT and GSH-Px to degrade it (Fig. 3).

Interestingly, compared to the control, Zn deficiency also decreased total N and NO_3^- by 39% and 36%, respectively (Fig. 4), with samples suffering severe Zn deficiency showing the lowest NO_3^- concentrations. It has been reported that the net acquisition of NO_3^- is suppressed by Zn deficiency (Ojeda-Barrios *et al.*, 2012). A similar correlation of total N with Zn deficiency has been observed in rice (Chen *et al.*, 2007).
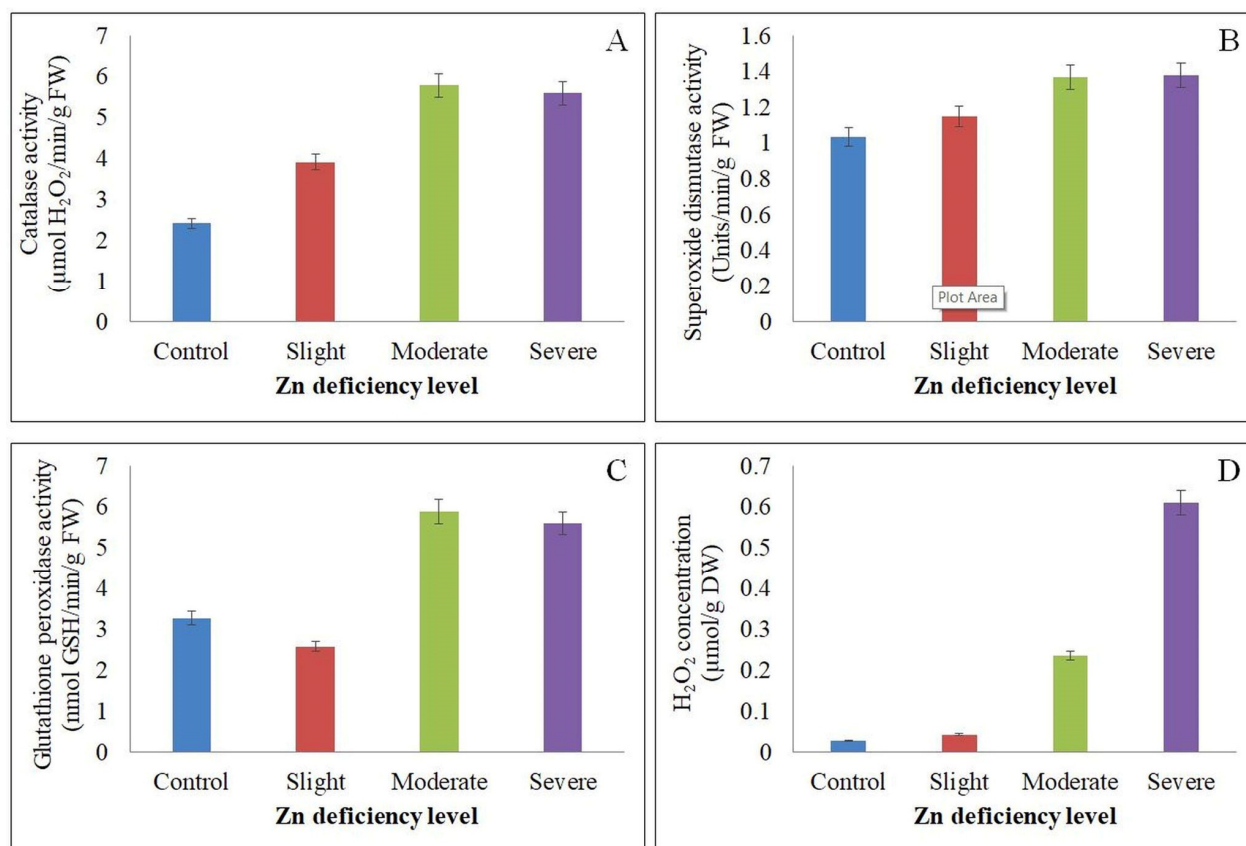

Fig. 3. Effects of slight, moderate and severe Zn deficiency on the activity of Zn-dependent enzymes in pecan tree leaf extracts. (A) CAT, (B) SOD, (C) GSH-Px activities and (D) H_2O_2 concentration. Data are means ± SE (n = 6). Means were compared using the Tukey's test (p ≤ 0.05)
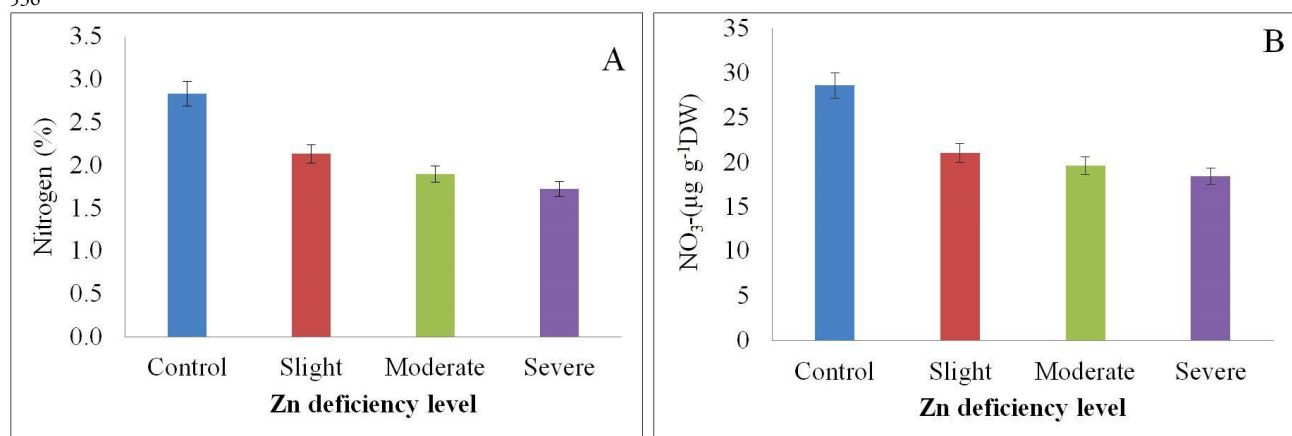
Fig. 4. Effects of slight, moderate and severe Zn deficiency on the concentrations of (A) N and (B) NO_3^- in leaves of pecan tree. Data are means ± SE (n = 6). Means were compared using the Tukey's test (p ≤ 0.05)

Conclusions

Our results showed a significant increase in the enzymatic activity of SOD, CAT and GSH-Px in the moderate level of Zn deficiency. On the other hand, it is observed that under conditions of severe Zn deficiency, there is a decrease in the production and quality of nuts that coincides with the increase in the activity of the enzymes SOD, CAT and POD, involved in ROS detoxification. As a result of this study, Zn deficiency in pecan trees regulates the enzymatic activities involved in the detoxification of reactive oxygen species in response to the high accumulation of H_2O_2 in the leaves. These results show that SPAD values, total N and NO_3^- content, metabolism of oxidative stress, production and nut quality could be bioindicators of Zn deficiency in pecan tree.

Acknowledgements

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