

Sodium sulfate exposure slows growth of native pecan seedlings

El sulfato de sodio reduce el crecimiento en plántulas nativas de nogal pecanero

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Abstract. Pecan [*Carya illinoensis* (Wanngenh) K. Koch] is one of the most important nut crops in arid and semiarid regions of Mexico. Here, most pecans are grown in saline soils having poor permeability which are further degraded by the use of low-quality irrigation water. Salinity adversely affects both pecan nut quality and yield. Little work has been done to explore the physiological effects of salinity on native pecan trees. Here we examine physiological changes determined by exposure of pecan seedlings to sodium sulfate (Na_2SO_4) at four concentrations: 1000, 2000, 3000 and 4000 mg/L applied twice weekly over a 70 d period. Control plants were similarly irrigated but with water free of Na_2SO_4 . The aim was to identify and quantify the putative salinity damage to native pecan seedlings growing in Chihuahua, Mexico. Seedlings exposed to Na_2SO_4 were of reduced height and stem diameter. At the highest exposure level (4000 mg/L), proline concentration in the leaflets was 820% higher (2.63 mg/g) than in the controls (0.32 mg/g), and chlorophyll was 35% lower (23.4 mg/L) than in the controls (36 mg/L). Meanwhile, sulfate ion concentration was increased by 104% from 84.47 to 172.5 mg/g. Root biomass decreased by 310% (from 30.5 to 9.5 g) and foliar biomass decreased by 260% (from 26.7 to 10 g). No disease symptoms were apparent in any seedlings suggesting that these changes were induced by Na_2SO_4 stress alone. Of the physiological parameters measured, proline, chlorophyll and sulfate ion concentration, as well as root and shoot biomasses were strongly affected by irrigation with Na_2SO_4 at concentrations of 2000 mg/L and above.

Keywords: Biomass; *Carya illinoensis*; Chlorophyll; Proline; Salinity.

Resumen. El nogal pecanero [*Carya illinoensis* (Wanngenh) K. Koch] es uno de los cultivos de nuez más importantes de las regiones áridas y semiáridas en México. La mayoría de las áreas donde se cultiva el nogal están establecidas en suelos salinos. Estos suelos tienen pobre permeabilidad y baja calidad en el agua de riego afectando la calidad y producción de nuez. Los cambios fisiológicos debido al estrés por salinidad han sido poco estudiados en plántulas de nogal pecanero nativo. En este trabajo de investigación se estudiaron los cambios fisiológicos inducidos por sulfato de sodio en plántulas de nogal, las cuales se expusieron a cuatro concentraciones de Na_2SO_4 : 1000, 2000, 3000 y 4000 mg/L y el control sin Na_2SO_4 . Las dosis se aplicaron dos veces por semana durante 70 días con el objetivo de evaluar el posible daño en plántulas de nogal nativo de Chihuahua, México. Las plántulas que se expusieron a Na_2SO_4 presentaron menor altura y desarrollo del diámetro del tallo. La concentración de prolina en los folíolos fue 820% más alta en la dosis de 4000 mg/L (2,63 mg/g) que en el control (0,32 mg/g). En esta dosis, la clorofila se redujo en un 36% con respecto al control, de 36 a 23,4 mg/L. La concentración de sulfato aumentó en 104%, de 84,47 a 172,5 mg/g. La biomasa de las raíces disminuyó en 310%, de 30,5 a 9,5 g. La biomasa foliar también disminuyó de 26,7 a 10 g. No se presentaron síntomas visibles de enfermedad. El estrés salino indujo estos cambios metabólicos en las plántulas. A partir de 2000 mg/L de Na_2SO_4 los parámetros fisiológicos concentración de prolina, clorofila y sulfatos, así como la biomasa de raíz y foliar fueron fuertemente afectados en plántulas nativas de nogal pecanero.

Palabras clave: Biomasa; *Carya illinoensis*; Clorofila; Prolina; Salinidad.

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INTRODUCTION

Soil salinity is a common problem throughout the world. It is often exacerbated by irrigation with water containing high levels of dissolved mineral salts. It has been estimated that more than 20% of irrigated areas are affected by salinization (Yamaguchi & Blumwald, 2005). Many pecan orchards have been established in saline soil regions in northern Mexico. Here, drought conditions are common due to low and highly-seasonal precipitation patterns and high evapotranspiration rates. Irrigation water is drawn from underground sources at depths of 200-500 m. Use of deep, low quality, irrigation water commonly results in salt accumulation in the topsoil with the situation especially exacerbated in areas in which soil permeability is limited (Thompson & Walworth, 2006). In northern Mexico, water quality is decreased by high levels of sulfate, which cause reductions in nut production and also in nut quality (Miyamoto & Nesbit, 2011).

Pecan trees [*Carya illinoensis* (Wanngenh.) K. Koch] are a profitable crop in Mexico. The cultivated area in the Chihuahua State has increased from 30000 to 60000 ha in the last five years (SIAP, 2012). This crop is highly sensitive to salt (Miyamoto & Nesbit, 2011). Sodium salts, such as NaCl, Na₂SO₄ and Na₂CO₃, are the most common salts in the soils of arid and semiarid regions (Peleg et al., 2011). These salts tend to accumulate in top soils having poor drainage properties, and this further contributes to soil salinity (Miyamoto & Nesbitt, 2011). Plants exposed to salt suffer decreases in chlorophyll synthesis and growth due to a reduction in the supply of water to the tissues. They also suffer long-term effects caused by the continual uptake of mineral ions and the gradual accumulation of these materials in the plant (Munns et al., 2006).

It is well known that proline concentration exhibits an interesting behavior under salinity stress with plants increasing their proline concentrations as a protective mechanism (Avendaño et al., 2005). Proline is an amino acid which is present at low concentration in plants growing under optimal conditions, but, under stressed conditions, its concentration increases sufficiently to serve as an osmotic agent, helping to protect the plant from dehydration (Avendaño et al., 2005).

Biomass accumulation rate is an indicator of plant vigor (Smith & Wood, 2006) and this can be affected by excess sulfate. Limited studies of the effects of salinity have been conducted on a number of commercial pecan cultivars, and little attention has been paid to its effects on native pecan seedlings.

In this work, we examine key physiological parameters in native pecan seedlings grown outdoors in containers under a range of levels of salinity stress induced by irrigation with Na₂SO₄. The aim was to determine their responses to Na₂SO₄ salinity.

MATERIALS AND METHODS

Native Pecan seeds were obtained from Jimenez, Chihuahua, Mexico, and these were sown in February 2012. In March

2013, 50 one-year-old seedlings were transplanted into 30 x 20 cm polyethylene containers each containing 4 kg of sandy soil. One seedling per container constituted a single experimental unit. The fifty containers were placed outdoors in Chihuahua (28° 39' 26" N, 106° 05' 15" W, 1446 m.a.s.l.), Mexico.

Solutions of Na₂SO₄ were prepared as follows: solution T1 (control): 0 mg/L; T2: 1000 mg/L; T3: 2000 mg/L; T4: 3000 mg/L, and T5: 4000 mg/L Na₂SO₄. The range was determined based on previous studies by Miyamoto & Nesbit (2011) and Thompson & Walworth (2006). The solutions were incorporated in the irrigation tap water, from April to June 2013. A standard volume (0.85 L) of each treatment solution (ten replicate plants) was added twice a week. The frequent applications were required because of the coarse (1.5 - 2 mm), sandy soil has large pores through which salts are rapidly leached. Natural rainfall to which the plants were exposed was 20 mm during the 70 day experimental period and potential evapotranspiration was 3 mm.

Seedling height and stem diameter were measured twice, once at the beginning and once at the end of the experiment. Biomass was measured once at the end of the experiment. Proline concentration was determined in the middle leaflets of the leaves as described by Bates (1973). It consisted of a spectrophotometer determination in the solution after reaction with ninhydrin acid, measured at 520 nm absorbance of the product dissolved in toluene in a UV/VIS (Lambda 25, Perkin Elmer). The calibration curve was made using L-proline (Sigma). A total of 20 leaflets were selected per replication (x10) per treatment (x5). Chlorophyll levels were measured *in situ* with a chlorophyll meter (Minolta-SPAD-502). Quantities of total chlorophyll were obtained by extraction with 100% methanol, and thereafter using a spectrophotometer measuring at 652 nm absorbance in a UV/VIS (Lambda 25, Perkin Elmer) as described by Ojeda-Barrios et al. (2012). Sulfates were determined from the same leaflet sample using the technique proposed by Rennenberg (1984), which measured absorbance at 420 nm with a UV/VIS spectrophotometer (Lambda 25, Perkin Elmer). The whole seedlings were harvested to determine the dry biomasses of the above-ground (shoots) and below-ground (roots) components. Roots were washed thoroughly with water. For biomass determination, all roots and foliage were rinsed three times with distilled water and non-ionic detergent (1% aqueous 'Mistol' dishwashing liquid, Henkel, Spain) and then carefully blotted dried with paper tissue and weighed. They were then dried to constant weight over 72 h in a circulating air furnace as described by De la O-Quezada et al. (2011).

The seedlings were frequently inspected throughout the study to record the possible appearance of root diseases and pests. Previous studies have reported that salinity stress can increase the incidence and/or severity of root rot by pathogens such as *Phytophthora* spp. (DiLeo et al., 2010). Statistical analyses were carried out (IBM SPSS Statistics) to describe the

relationships between Na_2SO_4 stress and the measured variables (plant height, stem diameter, proline concentration, total chlorophyll, chlorophyll SPAD units, sulfate ion concentration, root dry weight, shoot dry weight). Experimental design was completely randomized and included four treatments and a control with ten replications per treatment. For laboratory analysis, ten plants per treatment were used. Each seedling was used as an experimental unit.

A regression analysis was carried out between sulfate dose and each of the variables evaluated.

RESULTS

Regression analyses for most of the variables evaluated exhibited high values for R^2 - the exception was for seedling height.

Control seedling height increased from a mean of 16.8 to 24.5 cm - an average increase of 7.6 cm. Seedlings watered with the 2000 mg/L Na_2SO_4 solution grew by only 5.5 cm in this period (from 18.7 to 24 cm) and those watered with

the highest concentration (4000 mg/L) grew by only 3.37 cm. These reductions showed a quadratic relationship ($R^2 = 0.95$) (Fig. 1A). The 4000-mg/L Na_2SO_4 treatment diminished growth by more than 50% (from 7.6 cm to 3.37 cm).

Stem diameter growth was affected by all Na_2SO_4 treatments with diameter growth decreasing as Na_2SO_4 concentration increased. These reductions showed a lineal relationship ($R^2 = 0.71$) (Fig. 1B). While the controls increased by 1.5 mm over the 70 d period, the growth increment was reduced to 1.0 mm (66%) in the 1000 mg/L treatment and to 0.75 mm (50%) in the 2000 mg/L treatment. The 3000 and 4000 mg/L treatments did not seem to be much more severe than the 2000 mg/L.

The accumulation of proline in the seedlings increased strongly with rising Na_2SO_4 concentration (Fig. 1C). This variable had a quadratic regression with an increase of 0.42 mg/g of proline per 1000 mg of Na_2SO_4 ($R^2 = 0.98$). Compared to the controls (0.42 mg/g) (100%), at 1000 mg/L the proline level increased to 0.68 mg/g (213%), at 2000 mg/L to 1.4 mg/g (438%), and at 4000 mg/L to 2.63 mg/g (822%).

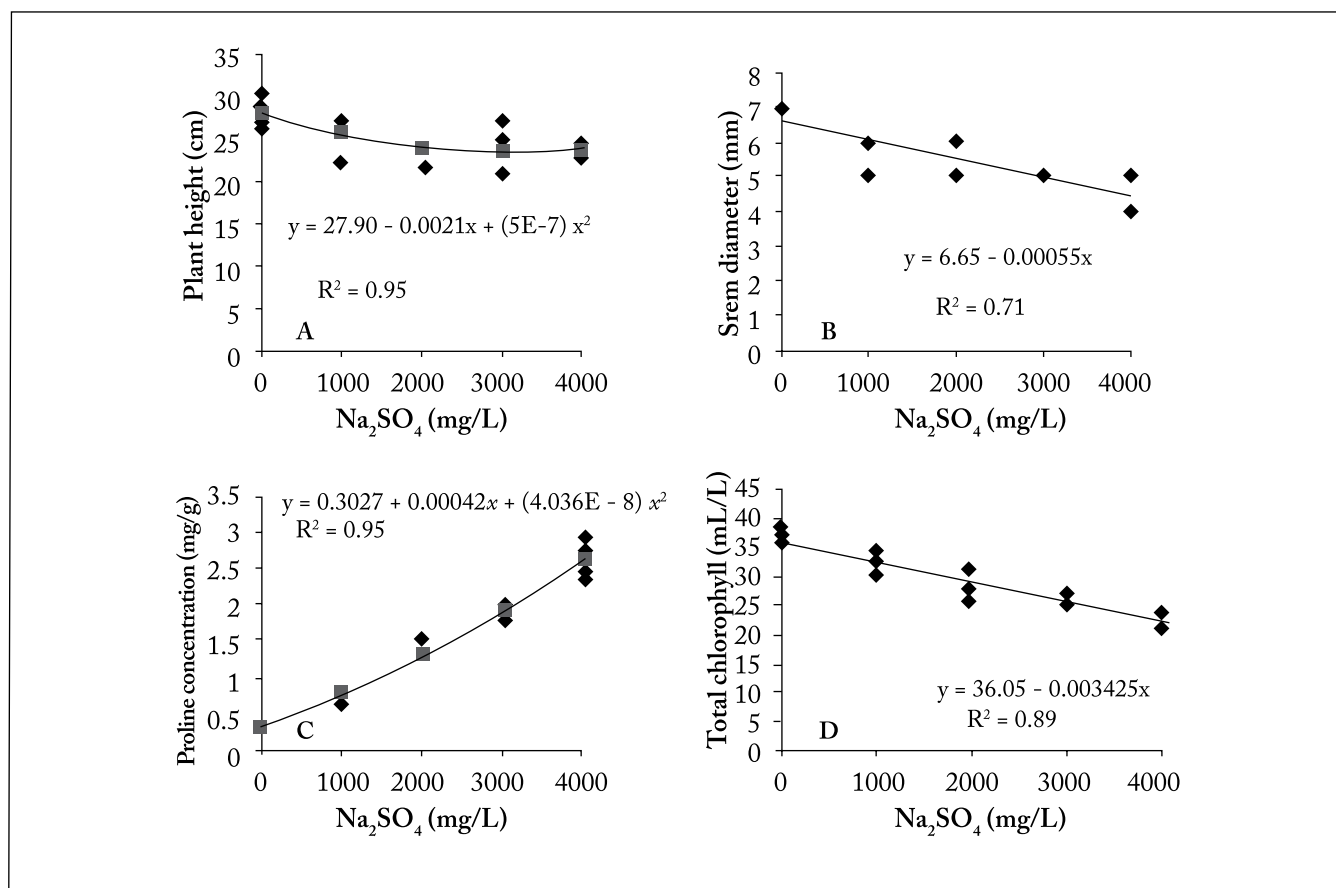


Fig. 1. Changes in physiological variables of container-grown, native pecan seedlings following additions of Na_2SO_4 . A. Plant height (cm), B. Stem diameter (mm), C. Leaflet proline concentration (mg/g), D. Total chlorophyll (mg/L).

Fig. 1. Cambios en las variables fisiológicas de plántulas de *Carya illinoensis* nativas que crecieron en macetas luego del agregado de Na_2SO_4 . A. Altura de planta (cm), B. Diámetro del tallo (mm), C. Concentración de prolina del foliolo (mg/g), D. Clorofila total (mg/L).

Total chlorophyll content (Chl. a + Chl. b) decreased as Na_2SO_4 level increased. There was a decrease of 3.4 ml/L chlorophyll per 1000 mg of Na_2SO_4 , the relationship followed a negative linear regression ($R^2 = 0.89$). The control seedlings synthesized an average of 36 mg/L of chlorophyll; whereas plants exposed to 2000 mg/L Na_2SO_4 synthesized an average of 28.8 mg/L. Treatment with Na_2SO_4 reduced the ability of the seedlings to synthesize chlorophyll to as low as 23.4 mg/L (4000 mg/L) (Fig. 1D). Total chlorophyll decreased in seedlings by 64.6% with respect to the controls (36.19 mg/L) with 4000 mg/L Na_2SO_4 (23.4 mg/L).

Chlorophyll (SPAD units) decreased by 7.25 units, for 1000 mg/L Na_2SO_4 compared to the controls. These reductions showed a lineal relationship ($R^2 = 0.72$) (Fig. 2A). The controls increased by 0.25 SPAD units, from 45.5 to 45.75 units, while the seedlings treated with 4000 mg/L Na_2SO_4 decreased by 7.75 SPAD units, from 39 to 31.25 units.

Sulfate ion content in the leaflets and Na_2SO_4 concentration had a positive linear regression where there was an increase of 21.4 mg/g of sulfate per 1000 mg/L of Na_2SO_4 (R^2

$= 0.95$) (Fig. 2B). Sulfate ions increased from 84.5 to 172.5 mg/g, an increase of 104% with 4000 mg/L Na_2SO_4 , and from 84.5 to 131.5 mg/g (an increase of 55.6%) with 2000 mg/L Na_2SO_4 . Symptoms of chlorosis and necrosis were observed in leaves for all Na_2SO_4 treatments; symptoms were most severe in leaflets with 3000 and 4000 mg/L Na_2SO_4 .

Foliar dry biomass decreased 4.0 g per each 1000 mg of Na_2SO_4 . This relationship followed a negative quadratic regression ($R^2 = 0.97$) (Fig. 2C). The relationship between root dry weight and Na_2SO_4 concentration showed a negative quadratic regression ($R^2 = 0.98$) with a root dry weight decrease of 5 g per 1000 mg/L of Na_2SO_4 (Fig. 2D).

In this experiment, the seedlings exhibited no disease symptoms or signs of pathogens or pests.

DISCUSSION

Studies have been conducted to measure the physiological responses of plants to soil salinity, including the degree of growth damage, dry biomass variations and chlorophyll

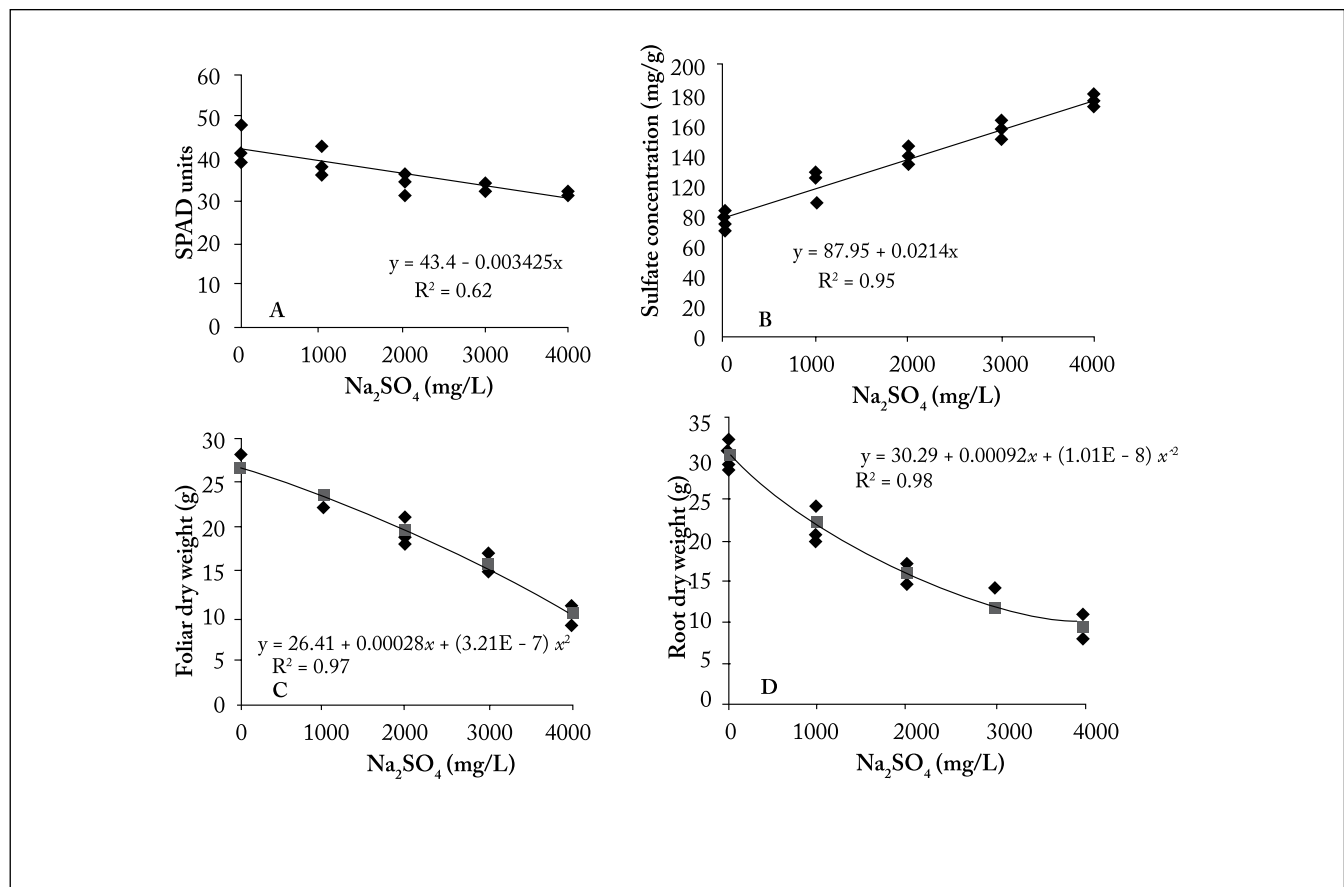


Fig. 2. Changes in physiological variables of container-grown, native pecan seedlings following additions of Na_2SO_4 . A. SPAD units, B. Sulfate concentration (mg/g), C. Foliar dry weight (g), D. Root dry weight (g).

Fig. 2. Cambios en las variables fisiológicas de plántulas de *Carya illinoensis* nativas que crecieron en macetas luego del agregado de Na_2SO_4 . A. Unidades SPAD, B. Concentración de sulfato (mg/g), C. Peso seco foliar (g), D. Peso seco radical (g).

reduction, as well as proline accumulation (Avendaño et al., 2005; Karimi et al., 2009; Hatami et al., 2012).

We found that the addition of 4000 mg/L of Na_2SO_4 caused severe damage to the seedlings, resulting in growth suppression, proline and sulfate ion accumulation, and chlorophyll and biomass loss. In addition, interveinal chlorosis of young leaves was evident, which is usually an indication of soil mineral toxicity. Previous research confirms that salinity stress build up of specific anions such as Cl , SO_4 and cations (usually Na) directly inhibits plant growth and reduces photosynthesis (Pal et al., 2004). In pecan, which is susceptible to salinity, it has been reported that a dose of 2332 mg/L reduces growth in 25% of adult pecan plants (Miyamoto & Nesbit, 2011). Other studies show that a salinity level of 3.5 dS/m in the soil (measured in a saturated paste equivalent to 2200 mg/L) represses growth in 25% of pecan trees (Thompson & Walworth, 2006). In this study, a similar Na_2SO_4 treatment exceeded this percentage (25%) of growth reduction, most likely because seedlings are more susceptible to toxic salts than adult trees. It has been reported that for crops such as grapes (*Vitis vinifera* L.), salt tolerance varies with the vegetative stage (Hatami et al., 2012) with plants becoming more tolerant as they develop (Yamaguchi & Blumwald, 2005). It has been reported that plants are particularly sensitive to salinity during the seedling and early vegetative growth stages. In this experiment, the height and stem diameter of one-year-old native pecan seedlings were severely affected by Na_2SO_4 . Research reports indicate that cells dehydrate and immediately shrink after salt absorption, but recover their original volume a few hours later. Nevertheless, rates of cell elongation and cell division both decrease, resulting in reduced growth rate of roots and leaves (Läuchli & Grattan, 2007). As a response to concentration increments in Na_2SO_4 treatment, seedlings modify their cell elongation and division, causing slowed growth, chlorosis and necrosis in the leaves (Thompson & Walworth, 2006). This response is similar to that observed in this study for all Na_2SO_4 treatments.

Proline concentration in our native pecan seedlings increased strongly in response to increases in Na_2SO_4 concentration. This behaviour reflects the operation of a defense mechanism in these seedlings. High concentrations of proline (>2 mg/g) have also been reported for pecan seedlings when exposed to water stress (De la O-Quezada et al., 2011).

Some reports indicate that proline accumulation is a plant response after exposure to a large number of stresses including salinity stress (Arshi et al., 2005). Increases in proline protect the plant from dehydration by acting as an osmotic agent (Avendaño et al., 2005; Karimi et al., 2009). An observed increase from 0.16 to 0.18 mg/g of proline in apple trees under water stress (Parra-Quezada et al., 2002) may be due to the contribution of this amino acid to cell osmoregulation for the maintenance of tissue turgor. Alternatively, proline could also be a source of nitrogen.

The reduction in total chlorophyll observed in salt-treated plants may be attributed to an increase in chlorophyllase activity which degrades chlorophyll (Karimi et al., 2009). The results of this study are consistent with those reported in mulberry *Morus alba*, pistachio *Pistacia vera* and citrus *Citrus* spp. (Das et al., 2002; Ranjbar et al., 2002; Bajwa & Anjum, 2007; Tavallali et al., 2008), where chlorophyll concentration decreased under saline conditions. Based on our chlorophyll content results and on previous studies (Bajwa & Anjum, 2007), it can be assumed that salinity stress affects the biochemistry of photosynthesis by disrupting the chloroplasts which lose their integrity, thereby reducing photosystem activities (Läuchli & Grattan, 2007).

Compared to the control, the 104% increase in sulfates observed in the leaf in the 4000 mg/L Na_2SO_4 treatment is evidence of the typical response of a salt-sensitive plant, which does not have the ability to prevent or mitigate the accumulation of leaf salts to toxic levels. One-year-old native pecan seedlings accumulate high levels of sulfates in their leaflets, and are therefore very sensitive to soil salinity.

Salinity involving anions such as Cl , SO_4 and cations (usually Na) and its effect on biomass production have been studied by several researchers (Mehari et al., 2005; Hatami et al., 2012). According to Smith & Wood (2006), biomass is an important indicator related to a plant's nutritional physiology, its absorption efficiency for nitrogenous reserves and its partitioning of carbon.

Plants subjected to salinity stress are predisposed to infections from root pathogens. Studies show that salinity increases the incidence of root infections from *Phytophthora* in plants, such as chrysanthemum (DiLeo et al., 2010) and tomato (Triky-Dotan et al., 2005). No symptoms of disease or signs of pests were observed in this study. This was likely because the soil where seedlings were planted was relatively free of pathogen spores. Our findings show that increases of soil Na_2SO_4 levels determine physiological alterations in pecan seedlings, which disrupt growth and development.

In particular, our results provide compelling evidence for Na_2SO_4 involvement in determining native pecan disorders. Future work should include the study of plant variables such as carotene content, fresh weight, total nitrogen, nitrates, and relative water content. Addition to the soil of *Phytophthora* zoospores could be used to challenge the saline stressed seedlings of native pecan with a common root disease.

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