Optimal Manganese Nutrition Increases Photosynthesis of Immature Pecan Trees

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Abstract. Southwestern U.S. pecan [*Carya illinoensis* (Wangenh.) K. Koch] orchard soils are typically alkaline and calcareous, making micronutrients such as manganese (Mn) poorly available for root uptake. Manganese is essential to the light reactions of photosynthesis (P_n), but the level of leaf Mn for optimum P_n in pecan is unknown. Our objective was to characterize the relationships of foliar Mn fertilizer applications and leaf Mn nutrition with P_n over a broad range of leaf Mn concentrations. Two experiments were conducted from 2011 to 2012 (Expt. 1) and in 2013 (Expt. 2) in immature, nonbearing ‘Pawnee’ and ‘Western’ pecan orchards near Las Cruces, NM. To create differential leaf tissue Mn concentrations, four Mn spray concentrations were applied foliarly: 0.00, 0.34, 0.68, and 1.3 g Mn/L (Control, Low, Medium, and High, respectively). In Expt. 2, we added a higher Mn concentration (2.7 g Mn/L). Repeated measurements of leaf P_n were made beginning 1 week following a Mn application using a portable P_n system. Across treatments in both studies, final leaf Mn concentrations ranged from 21 to 1488 µg·g⁻¹. Leaves treated with 0.68 g Mn/L had higher P_n than the other treatments in each experiment. In 2013, P_n rates of the leaves treated with 0.68 g Mn/L increased 7.1% and 10.4% over the Control for ‘Pawnee’ and ‘Western’, respectively. Our data confirm an association between leaf tissue Mn and P_n; the leaf tissue Mn concentration at which P_n rates are optimized in immature pecan trees was estimated to be 151.64 (±17.3 SE) µg·g⁻¹ Mn.

Pecan (*C. illinoensis*) is the only widely grown commercial nut tree species native to North America. The native distribution of

pecan is primarily along the Mississippi River Valley as far north as southeastern Iowa and south to Mississippi and Louisiana. Isolated populations of native pecans exist in Mexico, extending even as far south as Zaachila, Oaxaca, and Mexico (Janick and Paull, 2008; Thompson and Grauke, 1991). The native pecan region includes humid and semiarid areas with harsh to mild winters and annual rainfall between 660 and 1300 mm (Sparks, 2002, 2005).

In the southwestern United States, improved pecan cultivars have become a major driver for the agricultural economy with combined in-shell production value of \$182 million in 2014 for New Mexico and Arizona (USDA-NASS, 2015). One important way in which the growing environment in the southwestern pecan production region differs from that of the pecan native range and southeastern

U.S. growing areas is soil pH. Southwestern pecan orchard soils typically have alkaline pH and are calcareous, whereas southeastern U.S. soils tend to be neutral to acidic (Chang, 1953; Worley, 1994). Plant available Mn, more specifically Mn²⁺, and other micronutrients are less soluble and poorly available for root uptake by pecan trees in those alkaline soils (Chang, 1953; Lindsay, 1979; Sims, 1986; Sparks, 2000) and this results in micronutrient deficiency symptoms that are commonly visible in southwestern U.S. pecan orchards. The complexity of Mn availability in soil was suggested by Millaleo et al. (2010) and Rule and Graham (1976) who found soil Mn availability increased with high pH under white clover (*Trifolium repens* L.), but Mn availability decreased with higher pH under tall fescue (*Festuca elatior* L.), indicating that soil chemistry, plant species responses at the plant root to soil interface, and microorganism activity also may affect Mn availability (Barker and Pilbeam, 2015).

Zinc nutrition has been the subject of much intensive research in pecan since the early 1930s, when the “little leaf” and “rosette” symptoms were first identified as symptoms of zinc (Zn) deficiency in pecan (Alben et al., 1932a, 1932b). Commercial pecan producers in the southwest today typically make multiple Zn fertilizer applications each growing season as part of their regular mineral nutrition programs (Heerema, 2013; Walworth and Pond, 2006). Manganese, on the other hand, has received relatively little attention from pecan researchers or growers, despite the roles it plays in plant carbon fixation.

Manganese is best known for its essential role in P_n, specifically on the oxidation side of the PS II complex in the oxygen reaction center, and as a coenzyme for biosynthesis of chlorophyll (Epstein and Bloom, 2005; Marschner, 1995). According to Kriedemann et al. (1985), inhibition of P_n occurs in wheat even with moderate Mn deficiencies but chloroplast ultrastructure and chlorophyll breakdown (leading to visible symptoms of deficiency) occurs only with severe deficiencies. In the 1960s, Mn deficiency and its negative impact on P_n was measured in spinach and tomato where there was a 50% to 70% reduction in photophosphorylation in isolated chloroplasts and rapid declines in Hill reaction activity (Spencer and Possingham, 1960, 1961).

Sufficient Mn concentration ranges in pecan leaflet tissue have been reported to be between 200–500 µg·g⁻¹ (Jones et al., 1991) and 150–500 µg·g⁻¹ (Robinson et al., 1997). Smith et al. (2001) published the first report of Mn deficiency in pecan. In their study, they showed that trees growing in a Texas soil with pH 7.2 had suppressed Mn uptake such that leaf concentrations were only 1 to 18 µg·g⁻¹ and “shoot growth was short with pale green foliage but no discernible pattern of chlorosis.” Foliar-applied Mn sulfate fertilizer increased leaf Mn concentrations to

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138 $\mu\text{g}\cdot\text{g}^{-1}$ and eliminated the chlorotic symptoms (Smith et al., 2001). Henriques (2003) showed that with decreasing Mn availability in field grown ‘Kiowa’ pecan trees, total chlorophyll content and P_n rates decline dramatically, below 11 $\mu\text{g}\cdot\text{g}^{-1}$ Mn leaf tissue concentrations. In that study, without any Mn fertilizer applications, the “mildly deficient” plants (mean leaf Mn 11 $\mu\text{g}\cdot\text{g}^{-1}$) showed a 44.3% reduction of P_n and the “severely deficient” plants (mean leaf Mn 7 $\mu\text{g}\cdot\text{g}^{-1}$) showed a 62.9% reduction of P_n when compared with plants with mean leaf Mn concentration of 79 $\mu\text{g}\cdot\text{g}^{-1}$. In a second study, Henriques (2004) suggested that pecan adapt to Mn deficiency by reducing the chloroplast numbers while preserving PS II function in existing chloroplasts, which in turn reduces metabolic costs associated with low Mn concentrations. Other plant species form disorganized chloroplasts with low chlorophyll content, such as *Spinacia oleracea* and *Senna obtusifolia* (Homann, 1967).

Visible Mn toxicity is very rare in the southwestern United States, but Núñez-Moreno (2009) reported reduced shoot growth and fruiting of ‘Western’ pecan in Arizona because of increased leaf Mn levels. Severely affected trees in that study had leaf Mn concentration of 4034 $\mu\text{g}\cdot\text{g}^{-1}$, whereas unaffected trees had 1620 $\mu\text{g}\cdot\text{g}^{-1}$ leaf Mn. Similarly, in a study involving potted ‘Desirable’ pecan trees, the toxic Mn levels in leaf tissue were 4525 $\mu\text{g}\cdot\text{g}^{-1}$ (O’Barr et al., 1987). In Oklahoma, leaf Mn levels in ‘Western’ pecan trees have been reported as high as 2244 $\mu\text{g}\cdot\text{g}^{-1}$ with no visible phytotoxicity symptoms (Smith and Cotton, 1985). Among other plant species, maize has a much lower tolerance to Mn toxicity with a critical threshold of only 200 $\mu\text{g}\cdot\text{g}^{-1}$ resulting in leaf death (Marschner, 1995). Soybean has a threshold of up to 600 $\mu\text{g}\cdot\text{g}^{-1}$, but sunflower can tolerate as high as 5300 $\mu\text{g}\cdot\text{g}^{-1}$ Mn in plant tissue before leaf tissue becomes chlorotic and eventually dies (Edwards and Asher, 1982; Marschner, 1995).

The NMSU Cooperative Extension Service recommends 100–300 $\mu\text{g}\cdot\text{g}^{-1}$ Mn in July sampled leaflet tissue for New Mexico pecan trees and University of Arizona Cooperative Extension recommends 104–674 $\mu\text{g}\cdot\text{g}^{-1}$ (Heerema, 2013; Walworth et al., 2011). New Mexico pecan orchards showed, on average, only 85 $\mu\text{g}\cdot\text{g}^{-1}$ Mn in leaf tissue (McCaslin and Boyse, 1999; Pond et al., 2006), but the level of Mn at which P_n is optimum is not known. Symptoms of micronutrient deficient plants are not always visible even when the plant is deficient and this well-documented phenomenon is known as “hidden hunger” (Brady and Weil, 2007; Heerema, 2013). On a nutrient response curve, hidden hunger occurs when the concentration of the nutrient in the leaf is low enough to suppress plant performance (e.g., growth, yield) but not low enough for visible symptoms to be expressed.

The objective of our study was to investigate P_n rates in relation to increasing Mn concentrations. To accomplish this, we characterized the effect of foliar Mn fertilizer applications on leaf Mn and P_n and related

physiological parameters over a broad range of leaf Mn concentrations.

Materials and Methods

Study site, experimental design, and treatments. Two experiments were conducted at the NMSU-Linwood Nursery Research Orchards at the Leyendecker Plant Science Research Center in Las Cruces, NM (lat. 32°11’39” N, long. 106°44’18” W; elevation 1174 m). The soils in the areas of the orchards used in the experiments are primarily Armijo clay loam (Aw; fine, smectitic, thermic Typic Torrierts), Harkey clay loam (Hk; coarse-silty, mixed, calcareous, thermic Typic Torrifluvents), Anapra clay loam (Ao; fine-silty over sandy or sandy-skeletal, mixed, superactive, calcareous, thermic Typic Torrifluvents), and Agua silt loam (Ag; coarse-loamy over sandy or sandy-skeletal, mixed, superactive, calcareous, thermic Typic Torrifluvents). These soils are all characterized as having pH >7.5 and good drainage, with a restrictive layer depth >200 cm (NRCS, 2014). The soils in the orchard were naturally stratified with a $\approx$ 1 m layer of fine textured soils overlaying coarser textured soils, but, before planting, the soil layers were mechanically mixed in the tree rows to a depth of $\approx$ 2–2.5 m for a more homogenous texture. Expt. 1 was conducted during the 2011 and 2012 growing seasons and Expt. 2 was conducted in the 2013 growing season. Twenty-four ‘Pawnee’ (on seedling ‘VC1-68’ rootstock) pecan trees planted in 2010 were used for Expt. 1. Trees were irrigated, pruned, and supplied other macro- and micronutrients according to standard cultural practices for the New Mexico pecan industry (New Mexico State University, 2016).

In Expt. 1, four treatments were assigned to trees (six single-tree replications per treatment) according to a completely randomized design. Treatments consisted of foliar applications of varying concentrations of amino acid chelate Mn fertilizer (Manganese Metalosate®; Albion Plant Nutrition, Clearfield, UT) solution. Increasing applications per concentration rate of Mn was to create greater differences between Mn treatments. Expt. 1 treatments were as follows:

- 1) High: 1.3 g Mn/L; applied three times in 2011 (25 May, 8 June, and 22 June) and five times in 2012 (25 May, 8 June, 24 June, 14 July, and 27 July).
- 2) Medium: 0.68 g Mn/L; applied two times in 2011 (25 May and 8 June) and four times in 2012 (25 May, 8 June, 24 June, and 14 July).
- 3) Low: 0.34 g Mn/L; applied one time in 2011 (25 May) and three times in 2012 (25 May, 8 June, and 24 June).
- 4) Control: 0.00 g Mn/L (H_2O only); applied three times in 2011 (25 May, 8 June, and 22 June) and five times in 2012 (25 May, 8 June, 24 June, 14 July, and 27 July).

Expt. 2 was conducted in 2013 and consisted of 30 ‘Pawnee’ (planted in 2010) and 30 ‘Western’ cultivar (syn. ‘Western Schley’;

planted in 2011) pecan trees. Trees were irrigated, pruned, and supplied other macro- and micronutrients using standard cultural practices for the New Mexico pecan industry. Five treatments were assigned to trees (six single-tree replications per cultivar and treatment) according to a completely randomized design; however, blocks were formed based on measurement sequence. Thus, using blocks based on measurement time accounted for environmental factors which are variable throughout the morning. Five Mn treatments were applied to both cultivars on the same dates and the concentrations are as follows (the same Mn fertilizer was used as in Expt. 1):

- 1) Ultra High: 2.7 g Mn/L; applied six times (4 June, 19 June, 4 July, 20 July, 8 Aug., and 21 Aug.).
- 2) High: 1.3 g Mn/L; applied five times (4 June, 19 June, 4 July, 20 July, and 8 Aug.).
- 3) Medium: 0.68 g Mn/L; applied four times (4 June, 19 June, 4 July, and 20 July).
- 4) Low: 0.34 g Mn/L; applied three times (4 June, 19 June, and 4 July).
- 5) Control: 0.00 g Mn/L (H_2O only); applied six times (4 June, 19 June, 4 July, 20 July, 8 Aug., and 21 Aug.).

Leaf gas exchange. Leaf gas exchange [including P_n , stomatal conductance (g_s), intercellular CO_2 (c_i)] and photosynthetic active radiation (PAR) were measured using the LI-6400XT portable P_n system equipped with the 6400-02B Red/Blue Light Source (LI-COR Biosciences, Lincoln, NE) 1 week after every Mn application when possible for repeated measurements throughout the season. Except on the initial measurement date, gas exchange measurements were sequenced to ensure that each treatment’s measurements were spaced throughout the morning.

Fully sun-exposed lateral leaflets of leaves located midway between the shoot apex and the transition of current-season to previous-season shoot growth were used for gas exchange measurements. On the south side of each tree, gas exchange measurements were made between 0800 and 1300 hr with light intensity set to “track PAR ” so that the leaflet in the chamber was exposed to the same irradiance as the leaflet was receiving just before measurement. On days where scattered, thin clouds had potential to shade sunlight, PAR was set for constant 1700 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. In Expt. 2, time clusters were created as a blocking factor and PAR -in was adjusted between replications on each measurement date to account for the variability of ambient PAR measurement. Lombardini et al. (2009) and Anderson (1994) reported light saturation for pecan P_n at 1500–1700 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Average PAR in Expt. 1 was 1986 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and in Expt. 2 was 1821 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Carbon dioxide (CO_2) concentration (reference CO_2) was held constant in the chamber at 390 $\mu\text{L}\cdot\text{L}^{-1}$, near ambient atmospheric CO_2 levels.

In both experiments, midday stem water potential (Ψ_{swp}) was measured from one

completely shaded fully expanded leaf (sealed in a reflective plastic bag for ≈ 20 –40 min) from each experimental tree on the same dates as gas exchange was measured using the Scholander pressure chamber (PMS Instrument Company, Albany, OR) to determine plant moisture stress levels, since P_n is negatively impacted when tree water potential is less than -0.9 MPa (Othman et al., 2014). In Expt. 1, on every date Ψ_{swp} was -0.9 or higher with the exception for one tree on 18 June 2012, six trees on 29 June 2012, and four trees on 20 July 2012; however, on none of these did Ψ_{swp} measure lower than -1.0 MPa. In Expt. 2, there was one tree on 13 June 2013 that measured no lower than -1.02 MPa Ψ_{swp} and on 27 June 2012 two trees measured no lower than -1.0 MPa for Ψ_{swp} . For the cultivar Pawnee in Expt. 2, on no dates did any tree have Ψ_{swp} go lower than -0.9 MPa.

Chlorophyll. In both experiments, a portable chlorophyll meter [Soil Plant Analytical Development (SPAD)] (SPAD 502; Konica Minolta, Ramsey, NJ) was used to measure greenness of the interveinal regions of the same leaflets measured for P_n .

Leaf tissue chlorophyll content was measured in Expt. 2 at the end of the growing season and after all applications of Mn had been administered (20 Sept.). The leaflet was chosen using the same protocol for leaf sampling. On the same day, ≈ 50 mg of fresh weight (FW) of leaf from 28.27-mm^2 discs were taken from the interveinal regions of leaflets. Leaf tissue discs were stored at -80°C before grinding with a mortar and pestle in ≈ 2 mL liquid nitrogen. The 50 mg ground samples were each placed in 10 mL of 99.6% acetone and centrifuged at $2700 g_n$ for 10 min. Absorbance of the supernatant was made at 662, 645, and 470 nm using a SpectraMax M2 spectrophotometer running SoftMax Pro, version 5 software (Molecular Devices LLC, Sunnyvale, CA). Tissue chlorophyll a, chlorophyll b, chlorophyll a + b, and carotenoid contents were calculated using the equations of Lichtenthaler and Wellburn (1983) and Zhang et al. (2005) as follows:

$$\text{Chl a} = (11.24 \times \text{absorbance at } 662 \text{ nm}) - (2.04 \times \text{absorbance at } 645 \text{ nm})$$

$$\text{Chl b} = (20.13 \times \text{absorbance at } 645 \text{ nm}) - (4.19 \times \text{absorbance at } 662 \text{ nm})$$

$$\text{Chl a} + \text{b} = (7.05 \times \text{absorbance at } 662 \text{ nm}) + (18.09 \times \text{absorbance at } 645 \text{ nm})$$

$$\begin{aligned} \text{Carotenoids (xanthophylls and beta-carotene)} \\ = [(1000 \times \text{absorbance at } 470 \text{ nm}) - (1.90 \times \text{Chl a} - 63.14 \times \text{Chl b})] \div 214 \end{aligned}$$

Leaflet tissue nutrient analyses. Leaflet tissue samples were collected from 12 non-fruitlet shoots per tree on 25 July 2011, 25 Aug. 2012, and on 24 Aug. 2013 (after foliar Mn applications were completed), according to the method described by Heerema

(2013). Leaflet tissue samples were also collected on 24 May 2012 (before any 2012 foliar Mn applications) in Expt. 1 to determine if there were any carryover effects from 2011 Mn applications. Leaflet samples were washed briefly in a phosphorous-free detergent bath, rinsed once with deionized water, washed in a 0.1 M hydrochloric acid bath, and then rinsed twice with deionized water. Samples were dried at 60°C for 48 h in paper bags. In 2011 and 2013, nutrient analyses were performed using the methods developed by Gavlak et al. (1994) for determining macro- and micronutrients, Jones et al. (1991) for protocol on microwave digestion, and U.S. Environmental Protection Agency (1982) Staff for determination of trace elements using a Perkin Elmer Optima 4300 DV Inductively Coupled Plasma/Optical Emission Spectrometer (PerkinElmer, Waltham, MA). Total Kjeldahl nitrogen (TKN) was determined using a Technicon AutoAnalyzer (SEAL Analytical, Southampton, UK). In 2012, leaf analyses were performed using the methods developed by Gavlak et al. (1994) for determining macro- and micronutrients using a Perkin Elmer 5300 Inductively Coupled Plasma/Atomic Emissions Spectroscopy (PerkinElmer) and TKN using a LECO 528 (LECO, St. Joseph, MI).

Statistical analyses. For all statistical analyses, sensitivity of findings to extreme data points was examined using the outlier strategy with outliers identified as those observations with studentized residual magnitude >2.5 (Ramsey and Schafer, 2002).

Statistical significance was defined as $P \leq 0.05$ and Fisher's protected least squares difference was used to compare the magnitude of difference between treatments and protect the experiment-wise error. SAS software, version 9.3 (SAS Institute Inc., Cary, NC, 2010) was used for all analyses.

Leaf Mn concentrations were analyzed separately by year (Expt. 1) and cultivar (Expt. 2) with Mn treatment as the explanatory variable using a mixed model that fit separate treatment group variances to account for increasing variance.

Gas exchange data (P_n , g_s , and c_i) and SPAD for Expt. 1 were analyzed with years 2011 and 2012 combined as a completely randomized design with repeated measures. The mixed model included fixed effects for treatment, date, and their interaction. A heterogeneous compound symmetric variance structure was fitted to the repeated measures. For Expt. 2, the datasets for 'Pawnee' and 'Western' cultivars were analyzed separately as randomized complete block designs with repeated measures. Random effects for block and block by date were added to the model used for Expt. 1.

Chlorophyll content was analyzed as a two-way factorial with Mn treatment and cultivar as factors.

P_n data collected on the date closest to leaflet sampling in each season from Expts. 1 and 2 were combined to characterize the relationship between leaf Mn concentration and the P_n rate. Both mixed model regressions of P_n on polynomial functions of leaf Mn and

Fig. 1. Leaf manganese (Mn) concentrations per dry weight of tissue in 2011, 2012, and 2013 showing treatment application effect of the Control, Low, Medium, High, and Ultra-High treatments in 'Pawnee' and 'Western' pecan cultivars. Data are least squares means and bars correspond to the model-based standard error. New Mexico Cooperative Extension recommendation of leaf Mn concentration is shown in gray shaded region. Within year and cultivar, leaf Mn concentration means not accompanied by the same lower case letter are significantly different ($P \leq 0.05$).

design-based analyses using a four-level factor corresponding to combinations of year and cultivar, the treatment factor, and random blocks corresponding to measurement clusters. Because the Ultra-High treatment level was added in Expt. 2, a generalization of the methods of Piepho et al. (2006) was used to account for the missing cells and explicitly accommodate the $4 \times 4 + 2$ treatment structure by creating a three-level variable with one level corresponding to the 4×4 factorial portion of the design, another level for the Ultra-High-treated 'Western' cultivar and the third level for the Ultra-High-treated 'Pawnee' cultivar. For leaf Mn, separate variances were fit to each treatment group. For P_n , Mn treatment means ($\pm$ SE) were reported and formally compared. If the interaction test or the test comparing 'Western' and 'Pawnee' cultivars at the Ultra-High treatment level was significant then this was noted.

The mixed model polynomial regressions used only data corresponding to leaf Mn values below 500 units (Mn range 21.25–472.2 $\mu\text{g}\cdot\text{g}^{-1}$) (i.e., data from the Ultra-High treatment group were removed). Initial models included leaf Mn polynomial terms up to and including the cubic term, the year-cultivar factor, the year-cultivar by polynomial term interactions, and a random effect for measurement cluster block within year and cultivar. A reduced model was obtained using Type I (sequential) hypotheses to eliminate insignificant terms. The final model included separate cultivar intercepts and a cubic polynomial but no interactions. The leaf Mn value corresponding to optimal P_n was estimated by taking the derivative of the cubic polynomial, then applying the quadratic formula to find the root of the derivative corresponding to the maximum. The standard error of the estimated optimal leaf Mn was obtained using the delta method (Casella and Berger, 2002). Scale-related numeric problems were resolved by dividing the leaf Mn concentrations by 100.

Results and Discussion

Leaflet tissue Mn and chlorophyll concentrations. Average leaf Mn concentrations for the Medium Mn treatment in all years and both cultivars were consistently within the normal/recommended range of 100–300 $\mu\text{g}\cdot\text{g}^{-1}$ (mean range of 147–176 $\mu\text{g}\cdot\text{g}^{-1}$; Fig. 1). Leaf tissue Mn concentration in the Control trees in both experiments was well below the normal/recommended range. In Expt. 2, the Ultra-High Mn treatment had average leaf Mn concentrations between 1000 and 1300 $\mu\text{g}\cdot\text{g}^{-1}$ (Fig. 1) with no visible signs of toxicity. These Mn concentrations indicate that Mn treatments created a broad range of leaf Mn concentrations for observing differences in P_n and other physiological parameters. For all treatments, average leaf concentration of other macro- and micro-nutrients were within the recommended/normal ranges of Heerema (2013) in both experiments (data not presented).

Fig. 2. Mean (A) leaflet photosynthesis, (B) intercellular CO_2 , and (C) stomatal conductance, respectively, on cultivar Pawnee across the season in Expt. 1. A total of three measurement dates in 2011 and five dates in 2012. Foliar applied manganese (Mn) treatments ranged from 0.0 to 1.3 g Mn/L. Data are least squares means and bars correspond to the model-based standard error. Means not accompanied by the same lower case letter are significantly different ($P \leq 0.05$).

Leaf Mn concentrations were not significantly different across treatments on May 2012 (before 2012 Mn applications), suggesting little to no carryover in the trees of remobilized leaf Mn from 2011 (data not shown). If Mn carried over from the previous season comprised an appreciable part of leaf Mn in May 2012, we would expect to see some similar pattern of differential leaf Mn concentrations among the treatments early in

2012 before Mn applications had begun in that season.

Even at Mn concentrations as low as 24 $\mu\text{g}\cdot\text{g}^{-1}$ in the Control, which is well below the level considered deficient for Mn in pecan, no visible signs of Mn deficiency such as interveinal chlorosis were noted at any point during the experiments. There were no significant differences for SPAD ($P \leq 0.05$) among treatments in Expt. 1 or 2 (data not shown). The

SPAD (or greenness) means across Mn treatments in Expt. 1 ranged from 41.9 to 42.6 and in Expt. 2 ranged from 43.9 to 45.8 for 'Pawnee' and 40.1 to 41.9 for 'Western'.

There were no statistically significant differences ($P \leq 0.05$) for total chlorophyll content measured in Expt. 2 (data not shown). Total chlorophyll ($a + b$) means ranged from 8.3 to 10.2 $\mu\text{g}\cdot\text{g}^{-1}$ FW for the 'Pawnee' and 5.7–7.2 $\mu\text{g}\cdot\text{g}^{-1}$ FW for the 'Western'. Chlorophyll a/b ratio means ranged from 2.0 to 2.1 for both cultivars.

Gas exchange. The Mn treatment main effect on P_n was significant in both experiments. Specifically, across dates, the Medium Mn treatment had significantly higher P_n than the other treatments (Figs. 2A and 3A). In both experiments, the date effect was also significant for these gas exchange parameters, but the interaction between treatment and date was not significant. The percentage increase in P_n between the Medium Mn treatment and the Control was 7.1% in Expt. 1. In Expt. 2, the P_n rate increase between the Medium and Control treatments was 6.5% and 8.6% for the 'Pawnee' and 'Western' cultivars, respectively. In Expt. 1, P_n for the Low and High treatments were not significantly different from the Control. In Expt. 2, however, the High and Ultra-High treatments were significantly lower in P_n than the Control in both cultivars. Henriques (2003) research indicated significant decrease in P_n as Mn in leaf tissue became more deficient ($<11 \mu\text{g}\cdot\text{g}^{-1}$) and severe enough to produce visible symptoms. Our results indicate that even with slight deficiencies in Mn (not yet inducing visible symptoms), P_n is negatively impacted. We speculate that with optimum level of Mn in the medium treatment there is higher electron transport rates, light reactions, and NADPH turnover for the dark reactions, therefore, increased assimilation of CO_2 .

Intercellular CO_2 did not consistently show significant differences among the Mn treatments during the two experiments. In Expt. 1, the Medium Mn treatment had higher c_i than the other treatments (Fig. 2B). For Expt. 2, however, the c_i did not show any treatment effect differences for the 'Pawnee' and 'Western' (data not shown) despite increased g_s . Means of c_i in the 'Pawnee' and 'Western' cultivar in Expt. 2 ranged from 243 to 250 $\mu\text{mol mol}^{-1}$.

g_s followed a similar pattern to that of P_n , with the Medium Mn treatment having significantly higher g_s (250 $\text{mol H}_2\text{O m}^{-2}\cdot\text{s}^{-1}$) than all other treatments in Expt. 1 (Fig. 2C). In Expt. 2, g_s for the Medium Mn treatment was higher than all other treatments in the 'Western' but not different from the low Mn treatment in the 'Pawnee' (Fig. 3B). The percent increase of g_s between the Medium Mn treatment and Control was 12% in Expt. 1. In Expt. 2, percentage increase in g_s between the Medium and Control treatments was 13.8% for the 'Western' and 8.6% for the 'Pawnee'. In Expt. 2, the mean g_s for the Medium Mn treatment in 'Pawnee' and 'Western' was 350 and 360 $\text{mol H}_2\text{O m}^{-2}\cdot\text{s}^{-1}$, respectively (Fig. 3B). In Expt. 1, g_s for the Low

Fig. 3. Mean (A) leaflet photosynthesis and (B) stomatal conductance, respectively, on 'Pawnee' and 'Western' pecans across the season in Expt. 2. Six measurement dates total of each cultivar. Foliar applied manganese (Mn) treatments ranged from 0.0 to 2.7 g Mn/L. Data are least square means and bars correspond to the model-based standard error. Within cultivar, means not accompanied by the same lower case letter are significantly different ($P \leq 0.05$).

and High treatments were not significantly different from the Control; however, in Expt. 2, the High and Ultra-High treatments showed significantly lower g_s than the Control.

Similarly, Henriques (2003) showed a pattern of increasing g_s with increasing leaf Mn concentration when leaf Mn was below 100 ppm. Furthermore, in our study, g_s was lower for the High and Ultra-High Mn treatments, where mean leaf Mn exceeded 300 $\mu\text{g}\cdot\text{g}^{-1}$, than in the Medium Mn treatment where leaf Mn values were between 149–176 $\mu\text{g}\cdot\text{g}^{-1}$.

This suggests that stomata of pecan respond to increased demand for CO_2 in the leaves of trees with optimal Mn levels. However, in agreement with Henriques (2003), interpretation of the relationship between CO_2 uptake, g_s , and c_i is difficult since they vary with internal and environmental conditions. In addition to stomatal responses to light intensity and quality, leaf water status, mesophyll metabolites (i.e., ABA), root metabolites (i.e., cytokinins), salinity, and humidity, g_s can be affected by intercellular and boundary

Fig. 4. Relationship of photosynthesis rate of leaflets to leaf tissue manganese (Mn) concentration by dry weight. Leaf tissue sampled 1 week following photosynthesis measurements. The data from both experiments were fitted to the cubic polynomial equation $y = 4.6546 (Mn/100) - 2.1219 (Mn/100)^2 + 0.2581 (Mn/100)^3$ with y intercepts 13.66 for 'Pawnee' and 10.94 for 'Western'. Significance level set at $P \leq 0.05$.

layer CO_2 concentrations (Farquhar and Sharkey, 1982). It is not conclusive, however, that Mn deficiency affects stomatal limitation to net P_n as confirmed in previous studies on other plant species (Henriques, 2003; Ohki, 1985; Terry and Ulrich, 1974; Weiland et al., 1975). For example, Terry and Ulrich (1974) indicated findings in beet where even though P_n declined by one-third in Mn-deficient leaves compared with control the stomatal openings remained unchanged. Weiland et al. (1975) further observed in soybean a 50% reduction in P_n of Mn-deficient third and fourth leaf nodes and 70% reduction in P_n in the fifth node but no difference in stomatal aperture or size compared with controls under microscopic examination. It may be that the leaf stomatal response in trees with optimal Mn (and therefore greater leaf photosynthetic capacity) would allow increased transfer of CO_2 from the atmosphere into the leaf intercellular spaces to better supply the additional demands for CO_2 by the Calvin cycle and, furthermore, mitigate photorespiration.

Leaflet Mn to P_n relationship. The design-based analysis using the P_n sampling closest to the leaflet sampling date did not detect a Mn treatment by cultivar-year combination interaction ($P = 0.5706$) but did detect both Mn treatment and cultivar-year main effects ($P = 0.0066$ and <0.0001 , respectively). P_n was higher for the Medium Mn treatment [$16.17 (\pm 0.41) \mu mol CO_2 m^{-2} s^{-1}$] than for the Control, Low, High, or Ultra-High Mn treatments [$14.84 (\pm 0.41)$, $14.42 (\pm 0.41)$, $14.28 (\pm 0.41)$, and $13.51 (\pm 0.58) \mu mol CO_2 m^{-2} s^{-1}$, respectively].

Although reanalysis with two outliers removed detected a significant interaction, overall means were changed little with estimates for the Low and Medium treatments increasing by 0.5 and $0.45 \mu mol CO_2 m^{-2} s^{-1}$, respectively. The reanalysis reduced variance estimates, however, and as a result the Ultra-High P_n estimate was found to be significantly lower than the Control, Low, and Medium Mn treatment P_n estimates. Consistent with analyses using the data from all sampling dates, within each cultivar-year combination, the Medium Mn treatment had the numerically highest estimated P_n .

Leaf tissue Mn concentration was different between the treatments and findings from the design-based analysis combining data from all cultivars and years were similar to findings from the by cultivar and year analyses (Fig. 1). Although the Mn treatment by cultivar-year combination interaction P value was 0.0158, within cultivar and year, the concentration of Mn in leaf tissue climbed steadily with increasing Mn applications, even though the P_n rates did not climb correspondingly. The design-based analyses indicate optimum P_n rates in the Medium Mn treatment, which had estimated leaf Mn concentrations ranging from 147.4 (in 2012) to 176.9 (for 'Western' in 2013) $\mu g \cdot g^{-1}$; averaging across cultivars and years produced an estimated leaf Mn concentration for the Medium Mn treatment of $158.5 (\pm 4.4 \text{ SE}) \mu g \cdot g^{-1}$ (Fig. 1).

A common cubic polynomial function of leaf Mn over the range 21.25–472.2 $\mu g \cdot g^{-1}$ with separate cultivar intercepts was fitted to P_n and produced the cubic polynomial

equation $y = 4.655 (Mn/100) - 2.122 (Mn/100)^2 + 0.2581 (Mn/100)^3$ with y intercepts 13.66 for 'Pawnee' and 10.94 for 'Western' (Fig. 4). The estimated Mn leaf concentration where P_n was highest was at $151.64 (\pm 17.3 \text{ SE}) \mu g \cdot g^{-1}$ Mn; using the outlier strategy did not change this estimate appreciably ($154.59 \pm 10.99 \text{ SE } \mu g \cdot g^{-1}$).

In summary, these data provide strong evidence of improved tree photosynthetic performance in immature nonbearing pecan trees and we predict a similar response in mature, fruit-bearing trees and the possibility of improvement on flowering, fruit set, nut yield, and nut quality with foliar applied Mn resulting in leaflet Mn tissue concentrations around $150 \mu g \cdot g^{-1}$. Although the Control treatment in our experiment was within deficient levels of leaf tissue Mn concentrations (Heerema, 2013), there were no visible symptoms of Mn deficiency. That pecan is a hyperaccumulator of Mn (Núñez-Moreno, 2009; O'Barr et al., 1987) is evidenced by the fact that visible Mn toxicity symptoms were not apparent in the Ultra-High treatment even though concentrations were above $1000 \mu g \cdot g^{-1}$. In addition, the high foliar application rates of Mn do not confer any additional gas exchange benefit. Nevertheless, P_n was significantly lower for the Control compared with the Medium treatment and this indicates that pecan trees in the study may have "hidden hunger" for Mn and could benefit from foliar application of Mn. Our data, therefore, confirm a relationship in pecan between P_n and Mn nutrition and, furthermore, indicate that low Mn availability may be one factor that limits P_n in pecan orchards.

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