Pecan Kernel Phenolics Content and Antioxidant Capacity Are Enhanced by Mechanical Pruning and Higher Fruit Position in the Tree Canopy

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ADDITIONAL INDEX WORDS. free radicals, human health-promoting components, sunlight management

ABSTRACT. Pecan (*Carya illinoensis*) is a tree nut native to North America. Although inhibited light exposure (most specifically as a result of overlapping tree canopies) has been shown to impair yield, the effect of this factor on nut antioxidant properties remains unknown. This study investigated effects of mechanical pruning and canopy height position of fruit on pecan kernel antioxidant contents and capacity. Beginning in 2006, trees in a ‘Western’ pecan orchard in New Mexico were subjected to three mechanical pruning frequency treatments (annual, biennial, and triennial) paralleling conventional practices, while other trees were maintained as unpruned controls. During the 2012 to 2014 seasons, pecans were sampled at fruit maturity from three canopy height zones (“low,” “middle,” and “high,” corresponding to 1.5 to 3.0 m, 3.0 to 4.5 m, and 4.5 to 6.0 m above the orchard floor). In vitro phenolics contents and antioxidant capacities of the nutmeats were evaluated by total phenolics content (TPC) and oxygen radical absorbance capacity (H-ORAC_{FL}), respectively. Soluble ester- and glycoside-bound phenolics were quantified by reversed-phase high-performance liquid chromatography (HPLC). For both TPC and H-ORAC_{FL}, results determined pruned samples had significantly higher values than unpruned samples ($P < 0.001$ for both comparisons), and that samples of “high” canopy height were significantly greater than those of “middle” height, which were in turn greater than those of “low” height ($P < 0.001$ for all comparisons). HPLC findings showed that in all three phenolic fractions (free, esterified, and glycoside-bound phenolics), nuts acquired from pruned trees had substantially greater concentrations of ellagic acid and its derivatives. Our findings indicate mechanical pruning of pecan trees and higher tree canopy position of fruit increase nut antioxidant properties.

Pecan (*Carya illinoensis*) is a heterodichogamous, monoecious, and deciduous nut-bearing tree species in the Juglandaceae family indigenous to North America (Sparks, 2005). Among the 18 species in the genus *Carya*, only the pecan is now a widely planted and economically important horticultural crop. The native pecan growing range extends from the alluvial basins in the south-central United States northward to southern Illinois and Indiana and southward to southern Texas. Smaller native populations of pecan trees are also found scattered

throughout many parts of Mexico (Hall, 2000; Reid and Hunt, 2000; Sparks, 2005). Pecans were highly valued as a staple food and important article of trade for centuries within its area of origin.

Today, the vast majority of pecan nuts are produced in improved cultivar orchards rather than native groves, but the pecan industry is still largely centered in North America. The United States and Mexico are the largest pecan producing nations, each with total in-shell production averaging about 120,000 t per year during the period 2011–16 [Servicio de Información Agroalimentaria y Pesquera, 2018; U.S. Department of Agriculture (USDA), 2018a]. Smaller, but expanding, pecan industries are found in Australia, South Africa, Argentina, and China.

Pecan kernels are an excellent source of energy and dietary plant protein with a wide array of known human health-promoting components, such as soluble dietary fiber, indispensable amino acids, vitamins, minerals, tocopherols, phytosterols, and phytochemicals (Eitenmiller and Pegg, 2009; Gong et al.,

Received for publication 14 Aug. 2019. Accepted for publication 31 Jan. 2020. Published online 13 March 2020.

We thank Joshua Sherman, Marisa Thompson, and Sara Moran for assistance with fieldwork; the Salopek family for use of orchards for this study; and the U.S. Department of Agriculture, National Institute of Food and Agriculture, Specialty Crop Research Initiative (Award No. 2011-51181-30674) for funding this research.

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2017; Jia et al., 2018; Robbins et al., 2011, 2015). Compositional analysis has revealed that pecans contain a sizable quantity of lipids, which are predominantly triacylglycerols, as well as relatively small amounts of diacylglycerols and monoacylglycerols. Pecan oil is low in saturated fatty acids and rich in monounsaturated fatty acids, particularly oleic acid. The fatty acid profile of pecan oil is similar to that of olive (*Olea europaea*) oil, which is recognized for human health-promoting properties (Alasalvar and Shahidi, 2009; Kornsteiner et al., 2006).

Over the past 2 decades, a number of epidemiological studies and clinical trials have revealed an inverse relationship between nut consumption and status of chronic diseases. These studies confirmed the expected favorable impacts of pecan consumption on major risk factors for cardiovascular disease, namely blood low-density and high-density lipoprotein cholesterol levels, triacylglycerol levels, and other lipoprotein profiles (Bao et al., 2013; Domínguez-Avila et al., 2015; Kris-Etherton, 2014; Morgan and Clayshulte, 2000; Rajaram et al., 2001; Ros, 2010). These proposed cardiovascular health benefits are likely attributed to the unique package of nutrient-dense healthful lipids and phytochemicals, which have proven bioavailability in humans (Amarowicz et al., 2017; Eitenmiller and Pegg, 2009; Hudthagosol et al., 2011; Pegg and Wells, 2012; Wu et al., 2004).

While relatively limited information is available regarding the variation in health-promoting components among pecan cultivars (Robbins et al., 2015; Villarreal-Lozoya et al., 2006; Wood, 2009), it has been shown in other crops that health-promoting lipid and phytochemical components in plants can be influenced by sunlight intensity and other environmental factors (Atkinson et al., 2005; Procházková and Wilhelmová, 2011; Re et al., 2019). Pecan is well adapted to high sunlight conditions (Andersen, 1994). Even where trees are planted at relatively low density per unit area, inadequate sunlight distribution in pecan orchard canopies eventually appears due to cross-shading among overcrowded trees and self-shading among excessively tall trees. In turn, when sunlight distribution is poor, pecan orchards can exhibit declining average annual yields and nut quality (Lombardini, 2009; Stein and McEachern, 2007). Also, poor canopy sunlight distribution in pecan is associated with more severe alternate bearing intensity; i.e., greater magnitude of season-to-season fluctuations in nut production measured at the orchard or tree level (Wood, 1991). Effective canopy sunlight management is, therefore, of utmost importance to pecan growers striving to sustain high levels of orchard performance over the long term.

Orchard thinning (i.e., tree removal) has long been a common orchard light management practice. Although orchard thinning is effective in opening up crowded orchard canopies and increasing sunlight penetration, it also may temporarily decrease total nut yield on a land area basis (Wells and Harrison, 2017). In the western U.S. pecan production region where sunlight intensity is high, mechanical pruning has become the most common orchard management practice to improve sunlight penetration into tree canopies. Specifically, mechanical pruning has been demonstrated in some studies to effectively increase light penetration and distribution in pecan tree canopies under varying growing conditions (Lombardini, 2009; Malstrom et al., 1982). Furthermore, a number of studies have shown that mechanical pruning can at least partially alleviate unfavorable horticultural impacts of canopy crowding

and self-shading in production of pecans (Heerema et al., 2012; Lombardini, 2009; Walworth, 2012; Wood, 2009; Wood and Stahmann, 2004) and other tree nut species (Ferguson et al., 1995; Ramos et al., 1992). In a long-term study, Wood and Stahmann (2004) showed that ‘Wichita’ and ‘Western’ (synonym ‘Western Schley’) pecan orchards gave 44% and 13% higher in-shell nut yields, respectively, when mechanically pruned on a 2-year cycle, than if subjected to tree thinning (i.e., removal of 50% of the trees in 1 year, followed by removal of 50% of the remaining trees in the subsequent year for a total of 75% of the original trees removed in two phases). Compared with orchard thinning, mechanical pruning also significantly reduced alternate bearing intensity for ‘Wichita’.

As antioxidant biosynthesis in plants is generally considered as a response to physiological stresses (Sharma et al., 2012), it is possible that greater exposure to solar radiation could result in higher quantities and potentials of antioxidants in crops. To this point, solar radiation, specifically ultraviolet B (i.e., $\lambda = 280$ to 315 nm), has been shown to upregulate phenylalanine ammonia lyase and chalcone synthase, which are key factors in the initiation of phenolics synthesis in plants and the bioaccumulation of phenolics to boost oxidative stress tolerance in pecan (Heck et al., 2003; Sharma et al., 2012). The authors are unaware of specific investigations on relationships of crop antioxidant properties with sunlight exposure in crop canopies during crop growth or horticultural practices (e.g., pruning) used to manage sunlight distribution in crop canopies.

Phenolic compounds can be segregated into free or insoluble-bound forms (with esterified and glycoside-bound types as a subset), depending on whether they exist free or are covalently bound to other plant constituents. Most insoluble-bound phenolics bind to cell wall materials including pectin, cellulose, arabinoxylan (i.e., a hemicellulose), and structural proteins (Shahidi and Yeo, 2016). These phenolics are “unextractable” or “insoluble” without a pretreatment (either alkaline, acid, or enzymatic), because of the covalent and hydrogen bonds associated with the structural polysaccharides. If not liberated, then the phenolics content from any analytical determination is an underestimation of the true value, on account of the bound phenolic cell wall material that is not released by standard extraction protocols (Ma et al., 2014). In an effort to liberate these phenolic derivatives, the plant tissue (i.e., defatted pecan kernels, in this study) is subjected to base followed by acid hydrolysis before conventional extraction.

The objective of the present study was to evaluate the impact of mechanical pruning and canopy height position of fruit on antioxidant components and potential in pecan kernels. Pecan samples collected during the course of three consecutive harvest years were analyzed for phenolic concentrations (free, esterified, and glycoside-bound forms), TPCs, and antioxidant capacities. It is anticipated that these data will provide insight and practical information as to whether pecan growers can adapt such horticultural practices to better meet consumer demand for foods with superior antioxidant qualities.

Materials and Methods

STUDY SITE, PRUNING TREATMENTS, AND NUT SAMPLING. Pecan nuts in this study were hand-harvested over 3 consecutive years from 2012 to 2014 from a commercial orchard located in the Mesilla Valley, NM (lat. 32°14′00.5″N, long. 106°47′56.9″W; elevation 1182 m). The trees in the study were mature (>30

years old) ‘Western’ pecan trees. The seed source for the tree rootstocks was unknown. ‘Wichita’ trees were present in the orchard as pollinizers, but only ‘Western’ trees were used in this study. Trees were arranged in a square planting pattern, spaced at 9.1 × 9.1 m. Tree rows were all at least 250 m in length and oriented in the northwest-southeast direction, which determined the normal direction of all orchard operations, including mechanical pruning.

Soils in the orchard were mostly Anapra clay loam [mixed, superactive, calcareous, thermic Typic Torrifluvents (USDA, 2018b)]. As needed, the orchard was basin flood irrigated with water from the Elephant Butte Irrigation District or irrigation wells. Pest and mineral nutrient management practices were implemented according to the common commercial standards for the southwestern United States. A clear biennial bearing cycle was evident in the orchard with “off” (i.e., relatively low nut production) seasons in 2012 and 2014 (average in-shell nut yields of 1015 and 1216 kg·ha⁻¹ across all treatments, respectively) and an “on” (i.e., relatively high nut production) in 2013 (average in-shell nut yields of 2594 kg·ha⁻¹ across all treatments).

All trees in the study were mechanically pruned in Winter 2005–06 and then for the following 9 years were subjected to one of four different mechanical pruning frequency treatments: annual pruning (i.e., every year), biennial pruning (i.e., every other year), triennial pruning (i.e., once every 3 years), and unpruned control (no pruning since the 2005–06 dormant season). During the 3 years of data collection (2012–14), the biennially pruned treatment was pruned in the dormant seasons before the 2012 and 2014 growing seasons and the triennially pruned treatment was pruned only in the dormant season before the 2012 growing season. All pruning treatments and the unpruned control were replicated four times within the commercial orchard in a randomized complete block experimental design (i.e., the orchard was divided spatially into four blocks, and each block was further divided into four plots (each ≈16 trees × 16 rows), among which each of the four treatments were assigned randomly.

Each time the trees were pruned, the tree rows were mechanically topped with large circular saws on two sides at a 45° angle and to a maximum height of ≈9.1 m. As needed, the tree rows were also similarly side-hedged at a 5° angle from vertical at a distance of ≈2.7 m from each side of the tree row centers. During the study, the tree rows were maintained as continuous hedgerows (i.e., they were never cross-pruned in the northeast-southwest direction). During the 3 years of data collection, canopy coverage in the unpruned control was complete, that is, the canopies of adjacent tree rows were touching each other and, besides scattered sunpatches, the orchard floor was virtually entirely shaded at midday. For each of the three pruning treatments in the seasons immediately following mechanical pruning, canopy coverage was visually estimated based on orchard floor shade at midday to be ≈50%.

Roughly 0.5 kg (in-shell weight) of pecan nut samples were hand-picked each year of the study from selected trees away from the edges of each plot, so as to avoid influence of adjacent plots or the outside of the orchard. Nuts were collected at the beginning of the commercial pecan harvest season (29 Nov. 2012, 19 Nov. 2013, and 1 Dec. 2014) at three height positions in the canopies: “low” (1.5 to 3 m above the orchard floor), “middle” (3 to 4.5 m), and “high” (4.5 to 6 m). In-shell nut

samples were air-dried at room temperature, shipped to the University of Georgia’s Department of Food Science and Technology (Athens), vacuum packed, and stored at –80 °C until analyzed.

EXTRACTION OF PHENOLIC COMPOUNDS (AS LYOPHILIZED HYDROPHILIC EXTRACTS). Before all analyses [TPC, hydrophilic-oxygen radical absorbing capacity (H-ORAC_{FL}), and HPLC], a lyophilized hydrophilic extract powder was obtained from the pecan nuts for use in the analyses. This extraction procedure was replicated for each sample analysis (i.e., the replicate assessments described in this paper refer to replicates that began at this extraction level). Pecan nuts were shelled by hand, refrozen at –80 °C, and then ground in a coffee grinder to a very fine powder. Ground nutmeat (≈20 g) was defatted using a Soxhlet apparatus with ≈350 mL of hexanes for ≈18 h. The defatted pecan meal was removed from the cellulose thimbles, air-dried for 6 h in a fume hood, weighed, and transferred into 500-mL erlenmeyer flasks. As described by Wu et al. (2004), a 100-mL portion of extraction solution [(CH₃)₂CO/H₂O/CH₃COOH, 70:29.5:0.5, v/v/v] at a material-to-solvent ratio of ≈1:6 (w/v) was used to extract the phenolic compounds. Extraction procedures were performed according to Craft et al. (2010). Briefly, the slurry in each flask was placed in an orbital-shaking water bath (OLS Aqua Pro; Grant Instrument, Royston, UK) and heated at 60 °C for 30 min. The contents were then filtered through a Büchner funnel using Whatman No. 1 filter paper (Whatman, Maidstone, UK) and the extract was collected in a round-bottom flask. Phenolics were reextracted from the solids residue with fresh solvent two more times and the extracts were pooled. Acetone was then removed (and sent to waste) from the pooled extract using a rotary evaporator (Rotavapor R-210; Büchi Corp., New Castle, DE) connected to a vacuum pump (V-700; Büchi Corp.) with a vacuum controller (V-850, Büchi Corp.) at 45 °C. The remaining aqueous residue was transferred to a crystallization dish (100 mm diameter × 50 mm height), covered with filter paper, and placed in a –80 °C freezer until completely frozen. The frozen aqueous residue samples were then lyophilized using a freeze dryer (Freezone 2.5 L; Labconco Corp., Kansas City, MO) and the mass of each lyophilized extract was weighed, transferred into an amber vial, capped, and stored at –20 °C until analyzed. These lyophilized extract powders were used for all subsequently described analytical methods.

TPC ASSAY. TPC was determined with 12 repetitions per treatment (triplicate assessment within each of the four geographic blocks) using a method employing Folin and Ciocalteu’s phenol reagent. Lyophilized extract powders from each sample were dissolved and diluted to 0.20 mg·mL⁻¹ with CH₃OH. The assay was performed in borosilicate glass test tubes containing 1 mL of each methanolic extract, 7.5 mL of deionized water, 0.5 mL of Folin and Ciocalteu’s phenol reagent, and 1 mL of saturated sodium carbonate. The contents in each test tube were vortexed for 30 s. A quiescent period of 60 min was used to allow for optimal color development. Absorbance of the resulting chromophore was measured at λ = 750 nm using a ultraviolet-Vis diode array detection (DAD) spectrophotometer (8453; Agilent Technologies, Santa Clara, CA). A standard curve was prepared from working solutions of ellagic acid (1.6 to 8.0 µg·mL⁻¹), and TPC values were reported as milligrams ellagic acid equivalents (EAE) per 100 g nutmeat.

HYDROPHILIC-ORAC_{FL} ASSAY. An H-ORAC_{FL} assay was performed on the lyophilized extract with 12 repetitions per

treatment (triplicate assessment within each of the four geographic blocks). The methodology was as described by Prior et al. (2003) with slight modifications. Phosphate buffer (0.075 M, pH 7.4) was used as the blank and diluent. Fluorescein (0.1 μ M) was used as the probe and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; 80 mM in 0.075M phosphate buffer) was used as the radical initiator. Both fluorescein and AAPH working solutions were prepared on the day of use and maintained at 37 °C before and during the experiment. The phenolic extract was diluted to 0.5 mg·mL⁻¹ with ethanol (95%). The ethanolic solution was further diluted with the phosphate buffer to a final concentration of 0.025 mg·mL⁻¹.

A microplate reader (FLUOstar Omega; BMG Labtech, Cary, NC) equipped with two internal 500- μ L reagent pumps was used for the analysis. Fluorescent detection was set with excitation/emission wavelengths of 485/520 nm, the temperature in the plate reader compartment was maintained at 37 °C, and the run time was set for 60 cycles (i.e., 3 h). A 96-well clear, nonsterile, nontreated microtiter plate (CoStar; Washington, DC) was used for each analysis. An aliquot of 20 μ L was pipetted into each corresponding well for sample, blank, or standard. Before initiating each analysis, 200 μ L of fluorescein working solution was pipetted manually into well A1 for gain adjustment. During the second cycle of the analysis, 200 μ L of fluorescein and 20 μ L of AAPH were automatically added into each well via the internal dual pump, separated by one cycle between each reagent. At the end of the cycle, a standard curve was constructed based on five different Trolox concentrations (12.5, 25, 50, 80, and 100 μ M in the phosphate buffer). The area under the kinetic curve was determined following blank correction. Final values were reported as mean mmol Trolox equivalents per 100 g nutmeat.

REVERSED-PHASE HPLC CHARACTERIZATION. For HPLC analysis, samples of all three canopy tree heights (low, middle, high), all 3 sample years, and all four blocks were combined by equal masses before extraction. This was done separately for unpruned samples and for pruned samples. In the case of pruned samples, there was an additional pooling of all three pruning frequencies. Reported values were determined in triplicate. To facilitate phenolic compounds identification by HPLC-quadrupole time-of-flight-electrospray ionization-mass spectrometry (HPLC-QToF-ESI-MS) and subsequent quantitation, crude phenolic extractions were fractionated into three fractions, or classes (i.e., free, soluble ester, and glycoside-bound) from the lyophilized extract powders by the method described by Weidner et al. (1999) with slight modifications. Briefly, 0.8 g of each extract was dissolved in 20 mL of acidified water (pH 2.0, using 6 M HCl). With a separatory funnel, the free phenolic compounds were extracted with diethyl ether (5 $\times$ 20-mL portions). The extractant was composed of acetone, water, and acetic acid. The organic phase (acetone) was removed using the rotary evaporator at 45 °C and then redissolved in CH₃OH and injected into the HPLC. To the aqueous-phase residue 2 M NaOH (20 mL) was added, the vials were flushed with N₂, and the mixture was hydrolyzed for 4 h at room temperature. After hydrolysis, the pH was adjusted to 2.0 using 6 M HCl and then the liberated phenolic compounds from soluble esters were recovered with diethyl ether (5 $\times$ 30-mL portions). The organic phase was once again evaporated and the solids redissolved in CH₃OH, before injection. The aqueous-phase residue was combined with 15 mL of 6 M HCl, flushed

with N₂, and placed in a 100 °C forced-air convection oven for 60 min. The liberated phenolic compounds from the glycosides were then extracted from the cooled sample using 5 $\times$ 45-mL portions of diethyl ether. The organic phase was once again evaporated and the solids redissolved in CH₃OH before HPLC injection.

The reversed-phase (RP)-HPLC method reported by Gong and Pegg (2017) was modified to characterize the crude extracts of the pecan cultivars. An HPLC system (1200 series, Agilent Technologies) equipped with a Kinetex pentafluorophenyl fused-core column (4.6 $\times$ 150 mm, 2.6 μ m particle size, 100 Å; Phenomenex, Torrance, CA) was used to analyze the pecan crude extracts. The system consisted of a quaternary pump with degasser, autosampler, thermostated column compartment, ultraviolet-Vis DAD with standard flow cell, and 3D ChemStation software (Agilent Technologies). A volume of 20 μ L was injected for each pecan extract (10.0 mg·mL⁻¹ in mobile phase A and then a 1:1 dilution) after being filtered through a 0.45- μ m polytetrafluoroethylene membrane filter (Phenomenex). Detection wavelengths were λ = 255 nm (ellagic acid, and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids, notably of the *trans*-cinnamic acid family), and 360 nm (flavonols). Mobile phases A and B were prepared with H₂O/CH₃CN/CH₃COOH (94:5:1, v/v/v) and H₂O/CH₃CN/CH₃COOH (59:40:1, v/v/v), respectively. A linear gradient elution at a flow rate of 0.8 mL·min⁻¹ was used as follows: 0 to 30 min, 0% to 60% B; held for 2 min; 32 to 33 min, 60% to 100% B; 33 to 35 min, 100% to 0% B. The thermostat was set at 25 °C during the entire period of analysis. Tentative identification of separated components was made by matching ultraviolet-Vis spectra and retention time (*t*_R) mapping with commercial standards.

Identifications of the separated phenolic compounds were confirmed using HPLC-QToF-ESI-MS. Briefly, an HPLC system (1100 series; Agilent Technologies) coupled to a mass spectrometer (MS) with an ESI interface (QToF micro; Waters Corp., Milford, MA) was used for the identifications. The MS was operated in both positive- and negative-ion modes using capillary voltages of +3.5 kV and -2.5 kV, respectively. The HPLC conditions of separation were the same as previously described. The microchannel plate detector voltage was set at +2.35 kV. Nitrogen was used as the desolvation gas at a temperature of 100 °C and a flow rate of 150 L·h⁻¹. Argon was used as the collision gas. For normal MS, the collision voltage was set at 5 V, but for MS/MS, the collision voltage was increased to 30 V. Detection was carried out within a mass range of 50 to 1100 *m/z* for low-molecular-weight compounds and 300 to 3000 *m/z* for high-molecular-weight compounds. The MS/MS analyses were performed by automatic fragmentation, in which the three most intense mass peaks were fragmented. The MS was calibrated using a Glu-fibrinogen peptide (Waters Corp.) in the MS/MS mode and MassLynx 4.1 software (Waters Corp.) was used for control and analysis. Comparison of parent molecular ions [M-H]⁻ with known commercial standards was used to assist with elucidation of the identities of the phenolic compounds. When necessary, comparisons of *t*_{RS}, [M-H]⁻ *m/z* values and fragmentation patterns of phenolic compounds to those reported in the literature were used.

DATA ANALYSIS. Significant differences were assessed by techniques of analysis of variance (ANOVA) combined with Tukey's multiple comparisons test (α = 0.05 for all assessments),

with use of IBM SPSS Statistics for Windows (version 24.0; IBM Corp., Armonk, NY).

For evaluation of each of the treatments, the data from the randomized geographical blocks were combined and treated simply as additional replicate trials. This was done after determining the blocks were not a significant factor for any output data (one-way ANOVA; $\alpha = 0.05$). Furthermore, data from the three individual pruning treatment types are also presented in this paper as a pooled category of “pruned” (as opposed to “unpruned”). The pooling of pruning treatments was done partly in accordance with study intentions (to specifically examine the binary effect of presence/absence of pruning), but also due to examination of the acquired data, which showed minimal and nonsignificant variation between the pruning treatments for the assessed factors (one-way ANOVA; $\alpha = 0.05$; discussed in the Results and Discussion section). As a consequence, mean values of pruned data represent three times greater sample sizes than those of unpruned data.

For direct comparison of the samples of different treatments, ANOVA (with Tukey’s test) was implemented for TPC and H-ORAC_{FL} with consideration of a composite factor of canopy position and pruning treatment (“low” and unpruned, “middle” and unpruned, “high” and unpruned, “low” and pruned, “middle” and pruned, “high” and pruned), controlling for the factor of harvest year (2012, 2013, 2014).

For examination of significant differences in TPC and H-ORAC_{FL} values according to sample treatment factors (i.e., without treating canopy position and pruning treatment as a composite factor, as described previously), ANOVA (with Tukey’s test) was implemented. This test used consideration of the factors of pruning treatment type (pruned, unpruned) and canopy positions (“low,” “middle,” “high”), and again controlled for harvest year (2012, 2013, 2014). For RP-HPLC data, statistically significant differences between pruned and

unpruned samples were determined by the unpaired *t* test ($\alpha = 0.05$).

Results and Discussion

TOTAL PHENOLICS CONTENT. The kernel TPCs, according to presence of mechanical pruning treatments and canopy height position of nuts, are depicted in Table 1 and Fig. 1. The comparison of individual pruning treatments (annual, biennial, triennial) was not a statistically significant factor for TPC values ($P > 0.05$), nor did it exhibit significant interaction effects with the other assessed factors ($P > 0.05$). Therefore, data of the pruning treatments were pooled (as “pruned”) for use in further analysis and depiction. Table 1 shows the comparison of TPC values for the samples according to combined consideration of pruning treatment and canopy position. The results reveal a clear pattern of higher kernel TPCs associated with both pruning and higher canopy height position. For all three canopy positions (“low,” “middle,” and “high” heights), the kernel samples from pruned trees had significantly higher TPC values than their unpruned counterparts [e.g., nuts from the “low” height position on pruned trees had significantly higher TPCs than those from “low” positions on unpruned trees ($P < 0.001$ for all comparisons)]. Also, within samples from both unpruned and pruned trees, there was a consistent pattern of the “high” canopy position yielding nuts with significantly greater kernel TPCs than the “middle” canopy position, which in turn had significantly greater TPCs than those from the “low” position ($P < 0.001$ for all comparisons).

Figure 1 shows the results for the individual harvest years of 2012, 2013, and 2014. Note that the purpose of showing each of these years’ results graphically is not due to interest in a comparison of the years, but rather to demonstrate the clear and consistent pattern in which pruning and higher canopy position

both are positively associated with increases in kernel TPCs. Three-way ANOVA analysis affirmed the effect of the pruning and canopy factors, showing that pruned samples (collectively across canopy height positions) had significantly greater values (1740 EAE per 100 g nutmeat) than unpruned samples [1516 EAE per 100 g nutmeat ($P < 0.001$)]. Three-way ANOVA also disclosed that samples of “high” canopy height (collectively across pruning treatments) were significantly greater (1855 EAE per 100 g nutmeat) than those of “middle” height (1682 EAE per 100 g nutmeat), which were in turn greater than those of “low” height (1514 EAE per 100 g nutmeat) ($P < 0.001$ for both comparisons). The data show $\approx 14\%$ greater TPC values for samples from pruned trees rather than unpruned trees, and $\approx 23\%$ greater TPC values for pecans of the “high” canopy position rather than the “low” position.

Table 1. Total phenolics contents (TPC) and hydrophilic-oxygen radical absorbance (H-ORAC_{FL}) values of ‘Western’ pecan kernels according to tree pruning treatment and canopy position.

Pruning treatment ^z	Canopy position ^y	TPC [EAE (mg/100 g)] ^x	H-ORAC _{FL} [TE (mmol/100 g)] ^x
		(mean $\pm$ SD)	
Unpruned	Low	1,392 $\pm$ 22.39 e ^w	10.38 $\pm$ 0.808 d
	Middle	1,537 $\pm$ 23.73 d	11.45 $\pm$ 0.563 d
	High	1,619 $\pm$ 56.11 c	13.02 $\pm$ 1.097 c
	Avg	1,516	11.62
Pruned	Low	1,555 $\pm$ 40.94 d	15.71 $\pm$ 0.873 b
	Middle	1,730 $\pm$ 33.52 b	17.89 $\pm$ 0.962 a
	High	1,934 $\pm$ 59.14 a	19.00 $\pm$ 1.180 a
	Avg	1,740	17.53

^zUnpruned mean values represent 36 data points each; 12 replications for each sample determined following three growing seasons ($n = 12 \times 3 = 36$). Pruned mean values represent 108 data points each; 12 replications for each sample for each of three different pruning frequencies (annually, biennially, and triennially) following three growing seasons ($n = 12 \times 3 \times 3 = 108$).

^yLow refers to pecan kernels harvested from canopy positions 1.5 to 3 m above the orchard floor; middle refers to kernels harvested 3 to 4.5 m above the orchard floor; high refers to kernels harvested 4.5 to 6 m above the orchard floor.

^xData are combined means from the 2012–14 seasons. All TPC values are given in milligrams ellagic acid equivalents (EAE) per 100 g nutmeat and all H-ORAC_{FL} values are given in millimoles Trolox equivalents (TE) per 100 g nutmeat.

^wFor the six sample means for each assessment, those without the same letter in a column are significantly different ($\alpha = 0.05$) as determined with analysis of variance combined with Tukey’s multiple comparisons test for factor of pruning treatment type $\times$ canopy position ($n = 6$), controlled for year of harvest ($n = 3$).

Fig. 1. Total phenolics contents (TPC) of kernels harvested from three canopy positions of pruned and unpruned ‘Western’ pecan trees in (A) 2012, (B) 2013, and (C) 2014. For pruned trees, data from three different pruning frequency treatments (annually, biennially, and triennially) were pooled. “Low” refers to pecan kernels harvested from canopy positions 1.5 to 3 m above the orchard floor; “Middle” refers to pecan kernels harvested 3 to 4.5 m above the orchard floor; “High” refers to pecan kernels harvested 4.5 to 6 m above the orchard floor. All TPC values are given in milligrams ellagic acid equivalents (EAE) per 100 g nutmeat. Data are means and error bars indicate 95% confidence intervals.

Fig. 2. Hydrophilic-oxygen radical absorbance capacities (H-ORAC_{FL}) of kernels harvested from three canopy positions of pruned and unpruned ‘Western’ pecan trees in (A) 2012, (B) 2013, and (C) 2014. For pruned trees, data from three different pruning frequency treatments (annually, biennially, and triennially) were pooled. “Low” refers to pecan kernels harvested from canopy positions 1.5 to 3 m above the orchard floor; “Middle” refers to pecan kernels harvested 3 to 4.5 m above the orchard floor; “High” refers to pecan kernels harvested 4.5 to 6 m above the orchard floor. All H-ORAC_{FL} values are given in millimoles Trolox equivalents (TE) per 100 g nutmeat. Data are means and error bars indicate 95% confidence intervals.

Fig. 3. Reversed-phase high-performance liquid chromatography at $\lambda = 255$ nm of the fractionated free phenolics, soluble esters liberated by alkaline treatment and glycoside-bound phenolics liberated by acid hydrolysis from a defatted acetonetic crude extract of pecan kernel samples from (A) unpruned and (B) pruned trees. For pruned trees, data from three different pruning frequency treatments (annually, biennially, and triennially) were pooled.

The positive effects of pruning and higher canopy position are consistent with our hypothesized expectations. The consistency and magnitude of these observed effects are worth exploring further. To our knowledge, the effects found in this study have not been previously reported for tree nuts or any similar crop. Also notable is the persistence of significant pruning effects on kernel TPC for at least 3 years. These findings suggest that pruning is a significant factor when only considering TPCs. These findings also suggest that to prune more frequently than every 3 years may not have benefits with regard to kernel TPC. The TPC findings of this study also suggest that the local light environment in the canopy may be an important factor for phenolic production in developing pecan kernels. Further study of these effects is merited.

HYDROPHILIC-ORAC_{FL} ASSAY. The kernel H-ORAC_{FL} values (measuring antioxidant capacity) based on mechanical pruning treatments and position of the nuts in the tree canopies are depicted in Table 1 and Fig. 2. As with TPCs, the comparison of individual pruning treatments (annual, biennial, triennial) was not a significant factor for H-ORAC_{FL} values ($P > 0.05$), and therefore pooled data of those treatments (as “pruned”) were selected for further analysis and depiction. Also, as with TPCs, the H-ORAC_{FL} results establish a very

clear pattern of the positive effect of pruning and higher canopy position on antioxidant potential.

Table 1 shows the comparison of H-ORAC_{FL} values for the samples by combined consideration of pruning treatment and canopy position. For the effect of pruning, we see a clear divide in which all pruned samples (harvested from “low,” “middle,” and “high” canopy locations) had significantly higher H-ORAC_{FL} values than all unpruned samples ($P < 0.001$ for all comparisons). For the effect of canopy position, we see a consistent pattern that samples of higher canopy positions had higher H-ORAC_{FL} values, although the difference between samples was not always statistically significant. Specifically, for unpruned trees, H-ORAC_{FL} values of kernels sampled from the “high” canopy position (average of 13.02 mmol Trolox equivalents per 100 g nutmeat) were significantly higher than those of samples taken from “middle” [11.45 mmol Trolox equivalents per 100 g nutmeat ($P = 0.007$)] and “low” [10.38 mmol Trolox equivalents per 100 g nutmeat ($P < 0.001$)] canopy positions, but the low and middle unpruned samples were not significantly different from each other ($P = 0.139$). For pruned trees, the average kernel H-ORAC_{FL} value from “high” (19.00 mmol Trolox equivalents per 100 g nutmeat) and “middle” (17.89 mmol Trolox equivalents per 100 g nutmeat) positions were not statistically significantly different from one another ($P = 0.112$), but were both significantly higher than the “low” canopy position sample (15.75 mmol Trolox equivalents per 100 g nutmeat) ($P < 0.001$ for both comparisons).

Figure 2 depicts the results for the individual harvest years of 2012, 2013, and 2014. As with TPCs, the purpose of showing each of these years’ results graphically is to demonstrate the clear and consistent pattern with which pruning and higher canopy location both are positively associated with increases in kernel H-ORAC_{FL}. Three-way ANOVA affirmed these effects, showing kernel samples from pruned trees (collectively across canopy heights) had significantly higher values (17.53 mmol Trolox equivalents per 100 g nutmeat) than samples from unpruned trees [11.62 mmol Trolox equivalents per 100 g nutmeat ($P < 0.001$)]. Three-way ANOVA also demonstrated that samples of “high” canopy position (collectively across pruning treatments; 17.51 mmol Trolox equivalents per 100 g nutmeat) were significantly greater than those of “middle” height position (16.28 mmol Trolox equivalents per 100 g nutmeat), which were in turn greater than those of “low” height position (14.38 mmol Trolox equivalents per 100 g nutmeat) ($P < 0.001$ for both comparisons). The data indicate $\approx 51\%$ greater H-ORAC_{FL} values for samples from pruned trees rather than unpruned trees, and $\approx 22\%$ greater H-ORAC_{FL} values for pecans of high canopy position rather than low position.

As with TPCs, noteworthy in these findings is the consistency and magnitude of the effects of these factors. Also as with TPCs, it is notable that any benefit associated with pruning more frequently than triennially was not observed in our results. Particular to the H-ORAC_{FL} assessments is the substantial magnitude of the observed effect of pruning (51% higher values than unpruned), which suggests pruning had a greater effect on antioxidant potential than it did on phenolics contents. These results have useful implications for orchard light management and for understanding the development of antioxidant potential within crops in response to sunlight. Furthermore, investigations into the mechanisms contributing to the observed differences in effect between TPC and H-ORAC_{FL} may well be warranted as a topic for future research.

Table 2. Tentative identification and quantitation of phenolic compounds isolated from extracts of pecan kernels harvested from unpruned and pruned trees.

Peak	Retention time (min)	[M-H] [−] (<i>m/z</i>) ^y	MS ² (<i>m/z</i>) ^x	Tentative identification	Concn of identified compounds (μg·g ^{−1} acetonetic crude extract) ^z	
					Unpruned	Pruned
					(mean ± sd)	
Free phenolics						
1	4.45	303	169-125	Gallic acid derivative	6.31 ± 0.25	5.02 ± 0.45*
5	7.37	577	425-289-245	Proanthocyanidin B-type dimer	3.67 ± 0.12	3.10 ± 0.20*
9	12.10	333	247-217	No identification	4.90 ± 0.33	3.10 ± 0.27*
11	13.96	577	425-289-245	Proanthocyanidin B-type dimer	3.10 ± 0.71	3.91 ± 0.41
13	16.93	469	425-301-217	Valoneic acid dilactone	4.55 ± 0.89	4.83 ± 0.91
15	20.96	433	301-217	Ellagic acid pentose	13.61 ± 2.10	15.07 ± 2.19
16	22.60	301	217	Ellagic acid	35.98 ± 4.03	56.41 ± 5.31*
19	25.40	315	301	Methylellagic acid	21.07 ± 4.91	16.11 ± 3.02
20	26.21	585	301	Ellagic acid galloyl pentose	5.91 ± 0.23	3.09 ± 0.70*
21	27.43	447	315-301	Methyl ellagic acid galloyl	13.09 ± 3.20	7.96 ± 1.53
23	28.90	585	301-217	Ellagic acid galloyl pentose	4.01 ± 0.83	2.30 ± 0.91
24	29.56	329	314-301	Dimethylellagic acid	12.91 ± 2.43	11.81 ± 2.03
Soluble esters						
1	4.45	303	169-125	Gallic acid derivative	48.76 ± 4.09	30.49 ± 2.16*
8	10.52	289	245-205-179	(+)-Catechin	3.35 ± 0.89	2.97 ± 0.99
11	13.96	577	425-289-245	Proanthocyanidin B-type dimer	3.10 ± 0.71	3.91 ± 0.41
13	16.93	469	425-301-217	Valoneic acid dilactone	120.4 ± 7.27	107.98 ± 9.03
15	20.96	433	301-217	Ellagic acid pentose	5.11 ± 1.04	6.79 ± 0.97
16	22.60	301	217	Ellagic acid	42.61 ± 3.12	67.22 ± 5.21*
17	23.12	397	223	Sinapoylquinic acid	6.12 ± 1.70	2.91 ± 0.37*
Glycoside-bound						
1	4.45	303	169-125	Gallic acid derivative	31.21 ± 1.87	22.45 ± 1.59*
2	5.10	169	125	Gallic acid	48.08 ± 2.92	101.2 ± 8.50*
3	5.62	577	425-289-245	Proanthocyanidin B-type dimer	2.10 ± 0.71	1.77 ± 0.31
4	6.38	153	153	Protocatechuic acid	5.91 ± 1.10	3.88 ± 0.76
6	8.30	577	425-289-245	Proanthocyanidin B-type dimer	4.91 ± 0.82	3.65 ± 0.67
7	9.47	137	125	<i>p</i> -Hydroxybenzoic acid	6.71 ± 0.20	3.06 ± 0.24*
8	10.52	289	245-205-179	(+)-Catechin	13.45 ± 2.33	14.12 ± 2.09
10	12.91	577	425	Proanthocyanidin B-type dimer	9.07 ± 2.11	8.69 ± 1.79
12	16.30	291	247-203	Brevifolin carboxylic acid	6.91 ± 1.02	2.30 ± 0.09*
13	16.93	469	425-301-217	Valoneic acid dilactone	189.60 ± 7.62	251.30 ± 9.79*
15	20.96	433	301-217	Ellagic acid pentose	12.10 ± 1.04	9.08 ± 2.11
16	22.60	301	217	Ellagic acid	31.60 ± 3.72	58.09 ± 5.81*
18	23.18	263	241-197	Syringic acid derivative	2.12 ± 0.08	1.90 ± 0.24
22	27.90	737	425-301-217	Ellagitannin derivative	8.16 ± 1.54	7.68 ± 1.01
23	28.90	585	301-217	Ellagic acid galloyl pentose	10.03 ± 3.01	14.59 ± 4.20
	SUM				731	859

^zAll quantitation is from reversed-phase high-performance liquid chromatography using a pentafluorophenyl fused-core column [4.6 × 150 mm, 2.6 µm particle size (Kinetex; Phenomenex, Torrance, CA)]. Commercial standards were used for caffeic acid, gallic acid, (+)-catechin, ellagic acid, protocatechuic acid, and *p*-hydroxybenzoic acid. All other compounds were quantified using the standard that is most similar. Kernel samples were analyzed in triplicate. For pruned trees, data from three different pruning frequency treatments (annually, biennially, and triennially) were pooled. Asterisks indicate a statistically significant difference ($\alpha = 0.05$) between the two values (unpruned and pruned) in the row as determined by unpaired *t* test.

^yMass to charge ratios (*m/z*) of molecular ions detected.

^xMass to charge ratios (*m/z*) of fragment ions detected.

RP-HPLC ANALYSIS OF FRACTIONATED PECAN PHENOLICS. Represented in Fig. 3 are overlaid RP-HPLC chromatographs of the three fractions of phenolics (i.e., free, soluble ester, and glycoside-bound) at $\lambda = 255$ nm; this wavelength is a characteristic maximum belonging to ellagic acid and its derivatives. It was apparent that the additional two hydrolyzes (i.e., the first

alkaline followed by an acid hydrolysis) successfully liberated phenolic compounds from their bound forms; however, one cannot be 100% certain that all bound forms of phenolics were extracted from the samples even though the best approach was used. A total of 24 phenolic compounds were separated and tentatively identified in the three fractions (i.e., free, soluble

esters, and glycoside-bound; Table 2). As aforementioned, identification of these compounds was based on t_R matching, the molecular ion $[M-H]^-$, and characteristic MS^2 fragment ions with available standards or information found in the literature. Protocatechuic acid (compound 4, $[M-H]^-$, m/z 153) and *p*-hydroxybenzoic acid (compound 7, $[M-H]^-$, m/z 137), which are not present in the free phenolic fraction, were identified in the soluble ester and glycoside-bound (i.e., hydrolysis) fractions by t_R and fragmentation pattern matching of commercial standards. The compound that eluted at $t_R = 23.12$ min (i.e., compound 17) was assigned as sinapylquinic acid based on the $[M-H]^-$ peak at m/z 397 and the product ion at m/z 223 (Clifford et al., 2010).

As expected, ellagic acid was confirmed in all three fractions at $t_R = 22.60$ min, showing a molecular ion at m/z 301 with intense MS^2 peaks at m/z 217. Several ellagic acid derivatives were also observed across the three fractions. Specifically, an ellagic acid pentose at $t_R = 19.98$ min was identified with a molecular ion of m/z 433 and fragment ion of m/z 301 $[M-H-pentose]^-$; methylellagic acid at $t_R = 25.40$ min and dimethyl ellagic acid at $t_R = 29.56$ min with parent ions at m/z 315 and 329, respectively. Peaks 1 and 13 of Fig. 3 noticeably increased in both the soluble ester and glycoside-bound fractions. Compound 2, detected at $\approx t_R = 3.84$ min showing an m/z of 303 with fragment ions at m/z 169 and 125, indicated that the compound might possibly be a derivative of gallic acid (i.e., whose molecular ion is at m/z 169 and fragment ion at m/z 125). It is speculated that the compound could be a gallic acid dimer with a C-C bridge and a free carbonyl group. Compound 13 at $t_R = 13.95$ min yielded an $[M-H]^-$ ion at m/z 469, two fragment ions at m/z 425 $[M-H-CO_2]^-$ and an ellagic acid fragment at m/z 301. This fragmentation pattern matched closely with that of valoneic acid dilactone, a hydrolyzable tannin that has been previously reported in english walnut (*Juglans regia*) phenolics (Regueiro et al., 2014). Peaks giving $[M-H]^-$ ions at m/z 577 ($t_R = 7.37$ and 13.96 min) with fragment ions at m/z 425 and 289 corresponded to the fragmentation patterns of a proanthocyanidin B-type dimer (Robbins et al., 2014). Similarly, another ellagic tannin-related compound identified in the present study was peak 22 at $t_R = 27.90$ min.

The quantitation of phenolics isolated in pruned and unpruned pecan kernels is listed in Table 2. Notable variations were evident in concentrations of a number of major phenolic constituents. For instance, gallic acid, which is found in the glycoside-bound fraction, increased from 48.08 $\mu\text{g}\cdot\text{g}^{-1}$ acetonetic crude extract for kernels sampled from unpruned to 101.20 $\mu\text{g}\cdot\text{g}^{-1}$ acetonetic crude extract for mechanically pruned pecan trees. One of the predominant phenolic compounds in pecan kernels, ellagic acid increased by 56%, 57%, and 83% in the free, soluble ester, and glycoside-bound fractions, respectively, as a result of the mechanical pruning treatments. Interestingly, a significant elevation of valoneic acid dilactone from 189.6 to 251.3 $\mu\text{g}\cdot\text{g}^{-1}$ acetonetic crude extract due to pruning treatment was only noted in the glycoside-bound fraction.

A comparison of the sums of observed phenolics (731 in unpruned and 859 in pruned) presents a 17.5% increase associated with pruning. This corresponds very well with the 14% increase in TPC observed with pruning (discussed previously). It is highly feasible that these quantitative differences are contributing substantively to the observed differences in antioxidant capacities of pecan kernels yielded from pruned vs. unpruned trees.

Conclusion

It was found that TPCs and antioxidant potentials within pecan kernels were significantly increased both by mechanical pruning and by higher nut position in the tree canopy. The largest magnitude of the observed effect was a 51% increase in antioxidant potential associated with mechanical pruning treatment. These data suggest that the level of sunlight exposure within the canopy zones surrounding developing nut clusters is an important consideration in the biosynthesis of antioxidant compounds within pecan kernels. Our findings support the practice of sunlight management by mechanical pruning as a viable tool for the production of pecans with enhanced nutrient and bioactive levels.

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