Shade and Fertilizer Affects Yield and Quality in a Clonal Plantation of Yaupon Holly

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ABSTRACT

Yaupon holly (*Ilex vomitoria* Ait.) is the only native American source of caffeinated tea and the small amounts of tea product that is available is currently wild-collected from diverse populations. A clonal field plantation of yaupon was grown under shading and fertilizer treatments and harvested three times in one season to observe changes in yield and phytochemistry. The June and September harvest produced more mass than the July harvest for all treatments. Shading and fertility had interactive effects on increasing fresh mass of the pooled annual harvest, whereas the providing 30% shade and increased fertilizer application (from 567 to 1163 mg/N plant) raised yield 58%. Fertility of 1163 mg/N per plant with 60% shade increased yield another 13% to approximately 1070 kg/ha. This experimental plantation contained 467 plants per ha and was at about half the density of commercial fields (882 plants per ha). Leaves were smaller in July and larger in June and September. Shade greatly increased the leaf size and water content. Caffeine content increased with leaf size over the duration of the experimental treatments and 60% shade treatments in September produced the highest caffeine content (1.21 ± 0.17% of dry mass). In general alkaloids were promoted by shading, and phenylpropanoids were promoted by bright light. This report from one season of observation showed that genetically uniform yaupon holly plantations were manipulated for yield and quality using shade and fertilizer.

INTRODUCTION

Ilex vomitoria Ait. (yaupon holly) is the only North American native plant that contains caffeine. Yaupon is native to the southeastern coast of the US and local consumption of yaupon tea for stimulant properties goes back to native people circa 750-1400 (Crown et al., 2015). Although yaupon tea was used in rituals that included vomiting, there are no known emetic compounds in yaupon tea and one is as likely to vomit from the over-consumption of coffee (Wainwright and Putz, 2014). Spanish settlers in Florida learned of yaupon tea in the 1500's from the Timucauan Indians (Hudson, 1979). In the 1700's, English settlers came to know yaupon tea as cassina, or the black drink in South Carolina (Hudson, 1995) and yaupon in North Carolina (Hale, 1891). In the 1800's it also gained acceptance in Europe as "Carolina tea" or Appalachina (Hudson, 1979). During the Civil War, harbor blockades of the Confederate states inhibited the import of coffee and tea, raising yaupon consumption. However, by the late 1800's consumption in the US and abroad ceased without any apparent reason (Hudson, 1979; 1995). During the early 20th century the USDA encouraged yaupon cultivation as a replacement for imported coffee and tea (Wainwright and Putz, 2014).

Ilex (holly) is a large genus with 600 species, most being natives of the tropical Americas and East Asia, but only three contain caffeine. The other two caffeine-containing *Ilex* species are also new world plants. South America's *I. paraguariensis* (yerba mate) and *I. guayusa* have been in cultivation as teas for hundreds of years. Along with caffeine, both contain theobromine, the "calming effect" in green

tea. Yerba mate and guayusa tea hold a reasonable share of their respective local markets (Argentina and Ecuador) for caffeinated beverages, alongside coffee and common tea (*Camellia sinensis*).

Currently in the US, yaupon holly is grown exclusively as an ornamental shrub and small tree. The species is dioecious, with extreme variations in plant size, branching pattern, leaf size, berry color with upright and weeping forms (Dirr, 2009). There has not been commercial selection for plantation culture and tea production. The small amount of yaupon tea available in the artisanal food trade comes from wild collection of heterogenous native populations.

A field of clonal plants from cuttings was assembled in Mettler GA to study the effects of shade and fertigation on yield and quality of yaupon tea. This paper reports on this 3-year-old clonal planting, in its first year under treatment conditions.

MATERIALS AND METHODS

A native selection of *I. vomitoria* was propagated by cuttings, and nursery stock in 18.9-liter containers was planted in 48.8 m rows, 1.52 m apart with 6.5 m between rows, in a sandy loam soil without fertilization in 2015. The field was mulched and mechanically cultivated under organically certified methods (USDA, 2021). A small portion of the field was covered with shade cloth creating three shade treatments under 36.6 m x 30.5 m sections (30%, 0% and 60% shade density provided by spectral-neutral black polyethylene netting; SunBlocker, Growers Supply, Windsor CT). Fertigation by injection into a trickle irrigation system was established in three zones of (four rows each) under each of the three shade treatments (Figure 1). Every two weeks, April 29 – Sept 15, Neptune's Harvest Hydrolyzed Fish Emulsion (2-4-1; Neptune's Harvest, Gloucester MA) was delivered at rates of 567, 1134 and 1701 mg N per plant (1x, 2x and 3x, respectively).

Three successive harvests were conducted June 13, July 25 and September 21, 2018. The nine combinations of shade (3) x fertilizer rates (3) were sampled with five pre-selected subplots (1-5), each containing four shrubs, and 6.1 meters or more from any field space in a different shade-fertilizer treatment. The southeastern plant in each subplot was marked for morphometric data collection and hand-harvested to determine the length of new growth, number of leaves, length of longest leaf on branch, and leaf area (using Image J, https://imagej.nih.gov/ij/index.html). The remainder of the subplot was harvested by shearing the new growth, stems were separated from leaves, and fresh mass recorded. Dry mass was determined for each subplot using an approximately 20g sample of leaves in paper envelopes, dried for 48 hours at 40 °C and stored in desiccator prior to extraction.

From each even numbered sub-plot, a small sample of leaves were dried to absolutely remove all moisture, in steel cans in convection (100 °C) until stable weight was attained. 1.6 g of normally dried leaves was powdered and steeped in 80 ml of boiled water for six minutes. The supernatant was filtered through Whatman 4 filter paper and stored at -20 °C awaiting biochemical analysis. Analytical data was adjusted to absolute dry mass, although normally dried tea leaves were extracted and analyzed.

Using the aqueous extract, caffeine and theobromine concentration in the hot water extract was quantified using Ultra High Pressure Liquid Chromatography (UHPLC) coupled to a Diode Array Detector. The compounds were separated on a reverse phase column (Kinetic C18 XB, 3.0 x 150 mm, 2.6-µm particle size) using acetonitrile and 0.1% formic acid as the solvents. The compounds were detected based on the absorbance at 274 nm, and the quantification was done using the external standard curve prepared from commercial standards. Tentative identification of the separated components was made by matching UV-Vis spectra and retention time match with commercial standards. Each sample was analyzed in duplicate. The identity of the compounds was further confirmed using an ultra-high resolution mass spectrometer (Orbitrap Fusion; Thermo Scientific) with an electrospray ionization interface following the above UHPLC condition. The identification was based on accurate mass and fragmentation pattern of the compound/peak of interest.

A hydrophilic-$ORAC_{FL}$ assay was performed on the samples according to Robbins, et al. (2015). A

phosphate buffer (0.075 M, pH 7.4) used as the blank and diluent. Fluorescein (0.1 μM) was the reaction probe and 2,2′-azobis(2-amidino-propane) dihydrochloride, (AAPH, 80 μM in phosphate buffer), was used as the radical initiator. Both working solutions were held at 37 °C for the duration of the experiment. The phenolic extract was diluted to 0.5 mg/mL with 95% (v/v) ethanol. The ethanolic solution was further diluted with the phosphate buffer to a final concentration of 0.025 mg/mL. A standard curve based on five different Trolox concentrations (12.5, 25, 50, 80, and 100 μM in the phosphate buffer) was constructed. The area under the kinetic curve (AUC) was determined and following blank correction, samples and standards (*i.e.,* 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid {Trolox}) was compared. Final values were reported as mmol Trolox eq./100-g sample from triplicate measurements.

A metabolomics approach putatively identified the variation in small molecules that were readily extractable from the plant tissues to provide an unbiased, comprehensive data of cellular metabolism (Patti et al., 2012). The analysis procedure was similar to that of de Vos et al. (2007). The diversity of compounds in the plant extract used multiple liquid chromatography workflows for compound separation, and the use of multiple mass analyzers for the identification and precise quantification of metabolites. Compounds were separated on a C18-reverse phase chromatography. Following separation, the identification of compounds was performed using a quadrupole-iontrap-orbitrap platform (Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer) coupled to an electrospray ionization interface (ESI). Data was collected in both positive and negative ESI ionization modes. The iontrap-orbitrap analyzer combination was utilized for identification of the unknown compounds through accurate mass measurements (resolution of 450,000 (FWHM) at m/z 200) with sequential fragmentation (MS^n) experiments. The LC-MS data was processed using MetAlign, MZmine and XCMS, and the unknown compounds were annotated by comparing the accurate mass and MS/MS spectra with online mass spectral databases including MassBank, METLIN and Glom, and compound databases including KEGG, ChEBI, and PubChem (Lommen et al., 2009). Molecular ion networks using GNPS were employed. Statistical analysis was conducted using JMP 14.1 (SAS Inst., Cary NC).

RESULTS AND DISCUSSION

The June and September harvest dates had two to three times the mass of the July harvest for fresh and dry mass, across all treatment conditions. Total yield of the three harvests, by fresh and dry mass, were expressed as a pooled summation of harvest dates. The amount of harvested fresh mass was affected by both the level of shading and the amount of fertilizer applied. Shading increased the fresh harvest mass at all fertility rates and the increase was related to the rate of fertilizer applied (Fig 2A). Plants grown in full sun had the lowest yield (approximately 700 kg/ha). However, 30 and 60% shade both increased yield to about 950 kg/ha with 1x fertilizer concentration. In 2x fertilizer application, 30% shading was ineffective but 60% shading increased yield to about 1070 kg/ha. The regressed curves indicate with a 1x fertilizer rate (567 mg N per plant), shading at about 40% would be near optimal, but with 2x fertility yield is likely to increase with shading even greater than 60%. Treble fertilizer rate yielded less than 2x and would have a greater cost.

Yaupon teas are usually sold on a dry mass basis, and the yield in dry mass is an important measure of productivity. Harvested dry mass followed similar trends as fresh, and shade increased the amount of harvested dry mass. At the 1x fertilizer application, dry mass increased from 250 to 350 kg/ha, when full sun was compared to 30% shade (Fig. 2B), but shade greater than 30% likely decreased the yield of dry mass. With 2x fertilizer, 60% shade was required to produce 350 kg/ha, and greater shading is likely to increase dry mass further. Treble fertilizer yielded no better than 2x, and the greater cost, had no measured advantage. This plantation contained 467 plants per ha, whereas a more standard density of 882 plants per ha was not used (and a direct per plant adjustment of yield would not be accurate).

The percentage of dry matter in harvested tissue

was greatly affected by shading (Fig. 3A) and tissues with more shade had lower % dry mass (or greater % of water). That effect was not fully expressed during the first harvest when the plants had been exposed to the shade treatment for a short period of time. It is likely that shade levels greater than 60% would have less dry mass, or greater water mass. Fertilizer had some effect but was less important than shading and date.

Leaf size on new growth increased with greater shading. The 30% shade may have had a slight effect, but leaves under 60% shading were much larger, and shading greater than 60% should produce still larger leaves (Fig. 3B). It should be noted that the mid-season harvest date had the smallest leaves. This is logical, since shade was applied as a fraction of solar radiation, and during the mid-season growth period, June 14- July 24, sunlight was most intense, so greater shade factor would be needed for the plants to receive a comparable solar intensity to earlier or later growing seasons. Fertilizer had some effect on leaf size but was less important than shading and date. The largest leaves were measured on pre-determined branches where new growth had occurred during that growth period. Other anticipated measurements on new growth (the length of new stem and the number of branch points) were disregarded after the first harvest because rank growth on young shrubs being forced into a hedging system created erratic branching patterns. However, on the new growth, leaf size increased with increasing shade up to 60% during all the harvest dates. Large leaves are desirable because they aid in harvest efficency. The current practice is to select clones for large leaves during wild crafting. During this experiment, opinions were rendered by field personnel that the shade grown plants, especially in the 60% group, appeared to be “better”.

A quantitative assessment of responses showed large leaves were generally related (47% correlation) to more mass at harvest (Table 1). Larger leaves were also related to greater dry mass (41% correlation), but to a lesser extent. This was because larger leaves had a lower % dry mass (-38% correlation). Larger leaves had more caffeine (38% correlation), and less of the alkaloid theobromine (-32% correlation). Leaves with lower % dry (more water) had more caffeine (-69% correlation). This level of correlation is moderately predictive, and it is possible to get improved leaf qualities in one treatment considering specific treatment factor combinations for several responses. Antioxidant capacity did not have any correlation with other responses.

Caffeine content (expressed on a dry leaf mass basis as % dry mass) greatly increased with more shading (Fig. 4A). At the first harvest, shade had the least effect because the short duration of the treatment was not adequate for full effect. During the second harvest, shade increased the caffeine content of the tea from about 0.31±0.17 to about 1.14±0.17 % dry mass. The caffeine content in the third harvest increased with the shade from 0.11±0.17 to 1.21±0.17 % dry mass. The higher caffeine levels in the experiment were much higher than that reported by Palumbo et al. (2009; 0.22% dry mass) but fertilizer had no effect. Caffeine concentration in the dry mass was increased with increased shade when applying the least fertilizer. In Palumbo et al. (2009) caffeine increased 2.65x when nitrogen fertilizer was added to non-fertilized plants, but the lowest rate of fertilizer applied in our experiment (567 mg N per plant, every two weeks) was much greater than Palumbo’s application of 88 mg per week.

Theobromine content (expressed on a dry leaf mass basis as % dry mass) was reduced with greater shading at the two first harvests (Fig. 4B). At the first harvest, 60% shade reduced theobromine from 0.02±0.01% to 0.004±0.01% dry mass and during the second harvest, 60% shade reduced theobromine to about 0.04±0.01% to 0.02±0.01% dry mass. At the third harvest, shade had no effect on theobromine where full sun or more shading had 0.02±0.01% dry mass. Low fertilizer reduced theobromine in the first and second harvests but only the highest rate fertilizer rate in the third harvest reduced theobromine. Increasing theobromine past a certain point is not desirable because of its bitter taste and growing plants under 40-60% shade reduced theobromine to less than 0.02% of the dry mass.

The antioxidant capacity per dry leaf mass basis

(ORAC value) was not affected by the treatments (data not shown). The total ORAC value per g of dry mass was 3678.14 µM TE/g and that was higher than ORAC value of fertilized yaupon (Nana) cultivar and wild type reported by Palumbo et al. (2009; 670 and 1034 µM TE/g respectively). It was also higher than green tea_*Camellia sinensis* (1346 µM TE/g) and *Ilex paraguariensis* (1239 µM TE/g) reported by Chandra and de Mejia-Gonzalez (2004). ORAC is no longer recommended by USDA as a measure of potency for bioactive phytochemicals.

A metabolomics' profile was run on a small set of plants to determine if the light response had other effects on tea quality (Fig. 5). There was an obvious dichotomy in the alkaloids vs phenylpropanoids when it comes to the light treatments; and they were inversely related. This may cause an inverse relation of antioxidant activity and caffeine content. Unlike *Camellia sinensis* (common tea), the flavan-3-ols (eg. catechins) and their glycosides were very low in yaupon, and yaupon teas are rich in flavonols and their glycosides (eg. quercetin). Another aspect was the differential regulation of the isomeric form within the same compound class (eg. on chlorogenic and dicaffoelquinics) by the treatments, where one of the isomers showed a differential response to the light treatments.

Yaupon leaves had been documented to contain various isomers of chlorogenic acid, coumaric acid, and an array of flavonoids. Yaupon holly can offer anywhere from fifty to one hundred percent of the antioxidant benefits of green tea (Palumbo, 2009). In *I. paraguarensis*- mate tea, shifting the metabolic profile toward phenylpropanoid compounds to include chlororgenic acid, dicaffoelquinones quercitin and rutin enhanced biological activities to include analgesic, antiatheroscloretic, antibacterial, antidiabetic, antitumor, antiulcer, choleretic, and vasiodiolator properties (Heck and deMeija 2007).

Within a clone, light treatments give the tea master choices to blend for flavor and health promoting compounds. Theobromine imparts a bitter flavor but has many purported health benefits. The blends of phenolic compounds in yaupon are complex. The preliminary metabolomic profile suggested our treatments alter many compounds that may alter the perceived qualities of the tea. Sophisticated flavor appreciated by tea aficionados can be better served when shading is understood and used a tool in culture to bring out the desired array of compounds. Health claims are harder to make but may come with successful practice of plantation culture. It will be re-iterated that this was single season observation of flowering stage shrubs actively growing to fill the rows in a plantation. It will take several years to know how a fully grown hedgerow plantation system would respond. The mature plantation would be required for an accurate economic analysis.

Table 1. Correlation among measured responses in the field and laboratory were shown by Pearson's correlation. Blue values were positively related, red values were negatively related and greyed values show no significant relationship.

	Largest leaf	Harvested fresh mass	Harvested dry mass	% dry mass	Caffeine	Theobromine	Antioxidant capacity
Largest leaf	1.0000	0.4739	0.4052	-0.3768	0.3821	-0.3150	-0.1406
Harvested fresh mass	0.4739	1.0000	0.9810	-0.1407	-0.0062	-0.3510	-0.2032
Harvested dry mass	0.4052	0.9810	1.0000	0.0301	-0.1155	-0.3817	-0.1995
% Dry mass	-0.3768	-0.1407	0.0301	1.0000	-0.6918	-0.0736	0.0919
Caffeine	0.3821	-0.0062	-0.1155	-0.0736	1.0000	0.2547	0.3234
Theobromine	-0.3150	-0.3510	-0.3817	-0.6918	0.2547	1.0000	-0.1422
Antioxidant capacity	-0.1406	-0.2032	-0.1995	0.0919	-0.1422	0.3234	1.0000

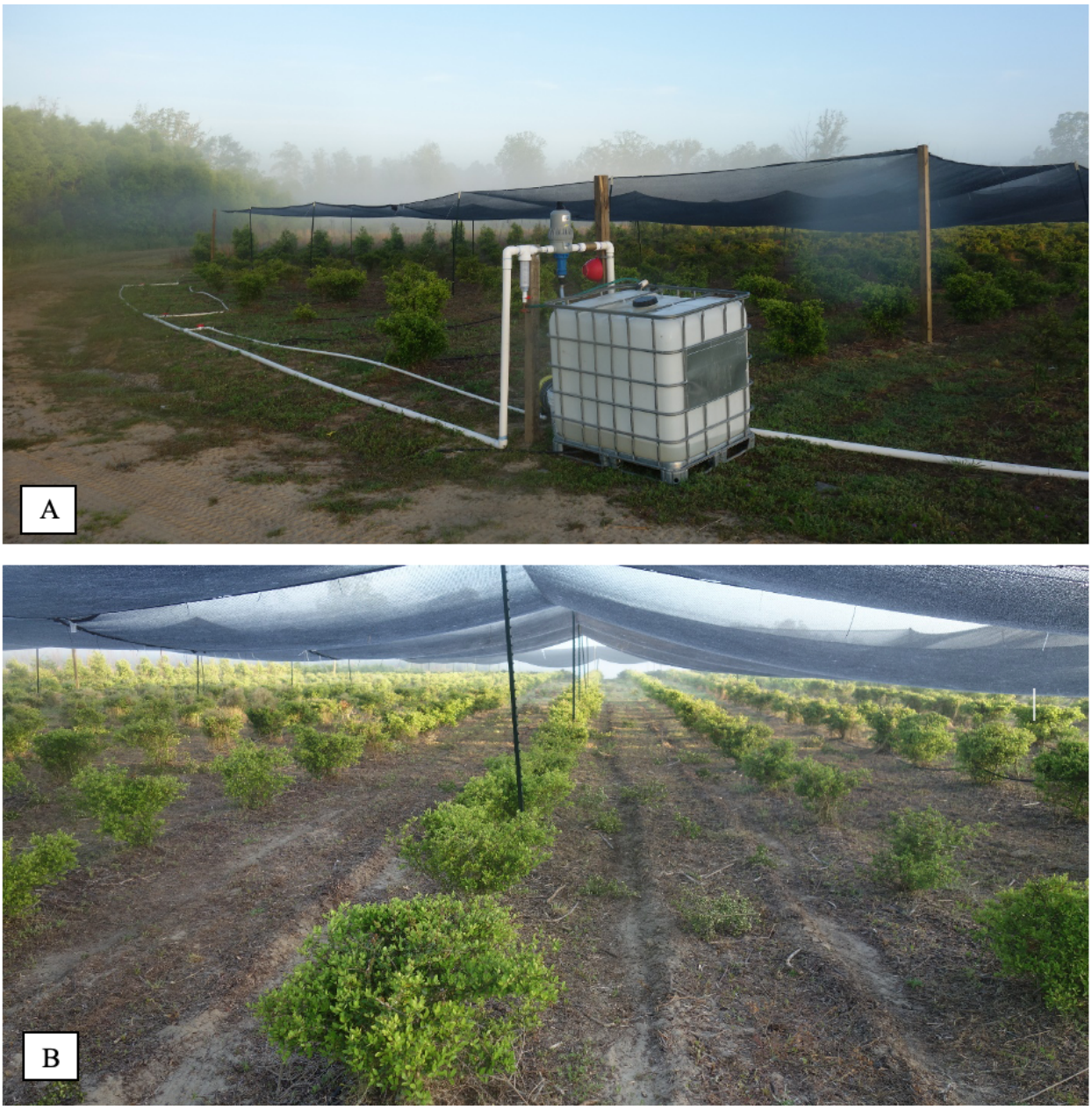

Figure 1. Three fertigation headers created rows with different fertilizer rates running the entire length of the field and spanning the three shade treatments (A). The 30% shading treatment shown in the foreground (B), is followed by the brightly lit full sun treatment, with the 60% shade treatment in the distant background.

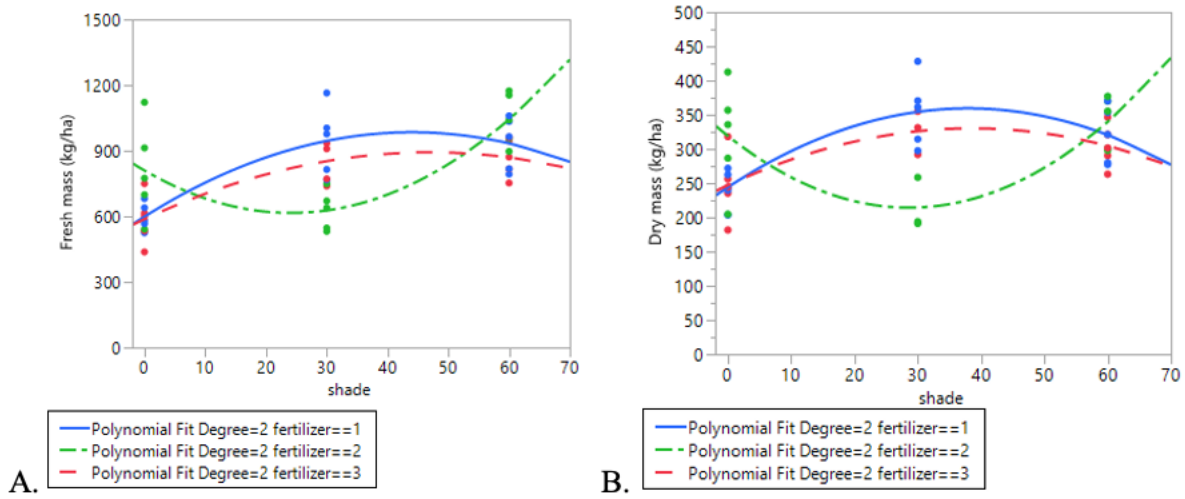

Figure 2. Fresh and dry masses (kg/ha) of yaupon harvests in the 2018 season are shown affected by shade and fertilizer treatments.

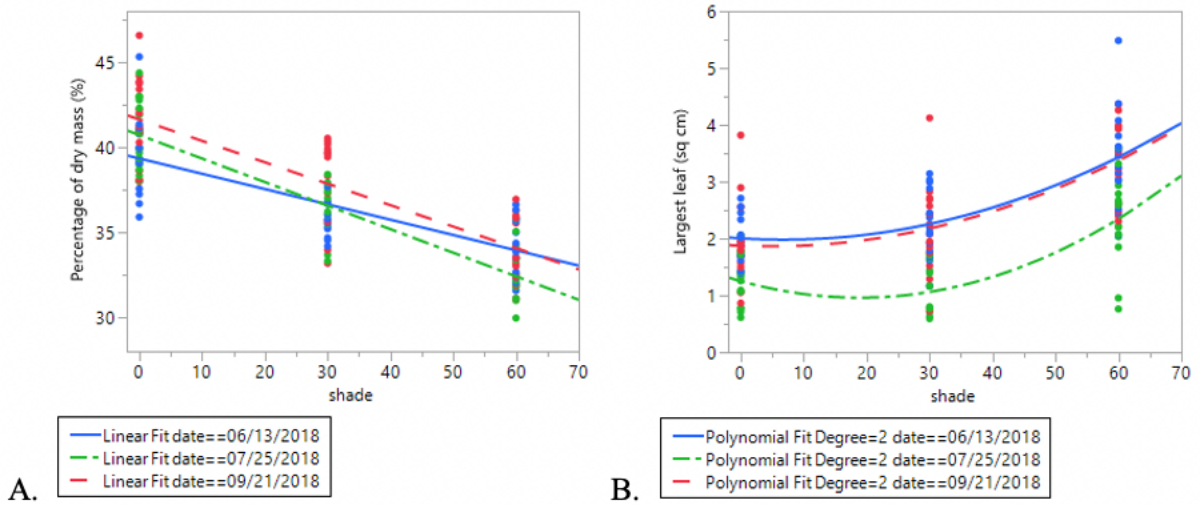

Figure 3. The percentage of dry/fresh mass (A) and the size of the largest leaf (B) on the new growth are presented for the 2018 growing season. Linear and polynomial fits show the effects of shading at the three harvest dates, with data pooled for the 3 fertilizer treatments.

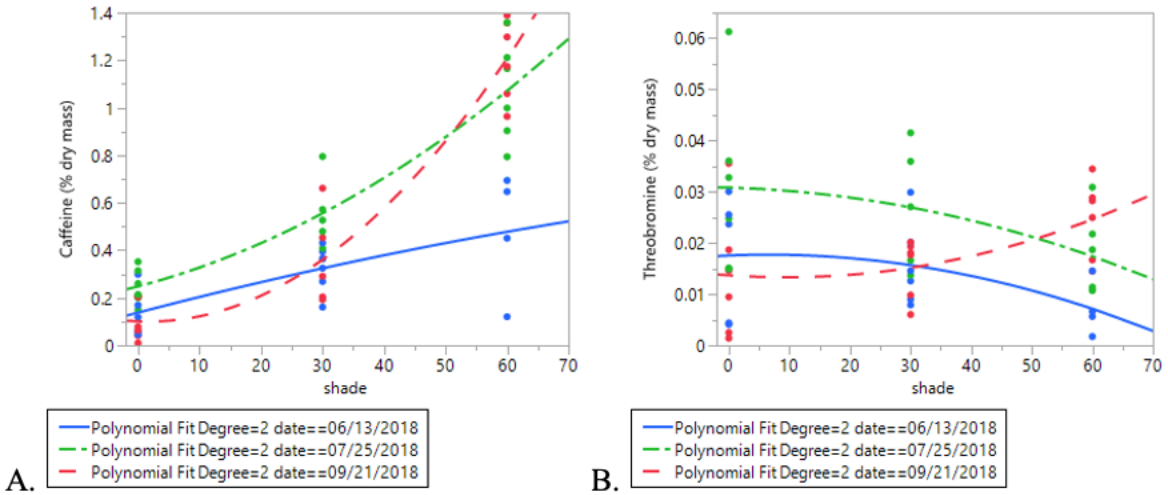

Figure 4. The concentration of caffeine (A) and theobromine (B) are presented from aqueous extracts of treatments from the 2018 growing season. Linear and polynomial fits show the effects of shading at the three harvest dates, with data pooled for the 3 fertilizer treatments.

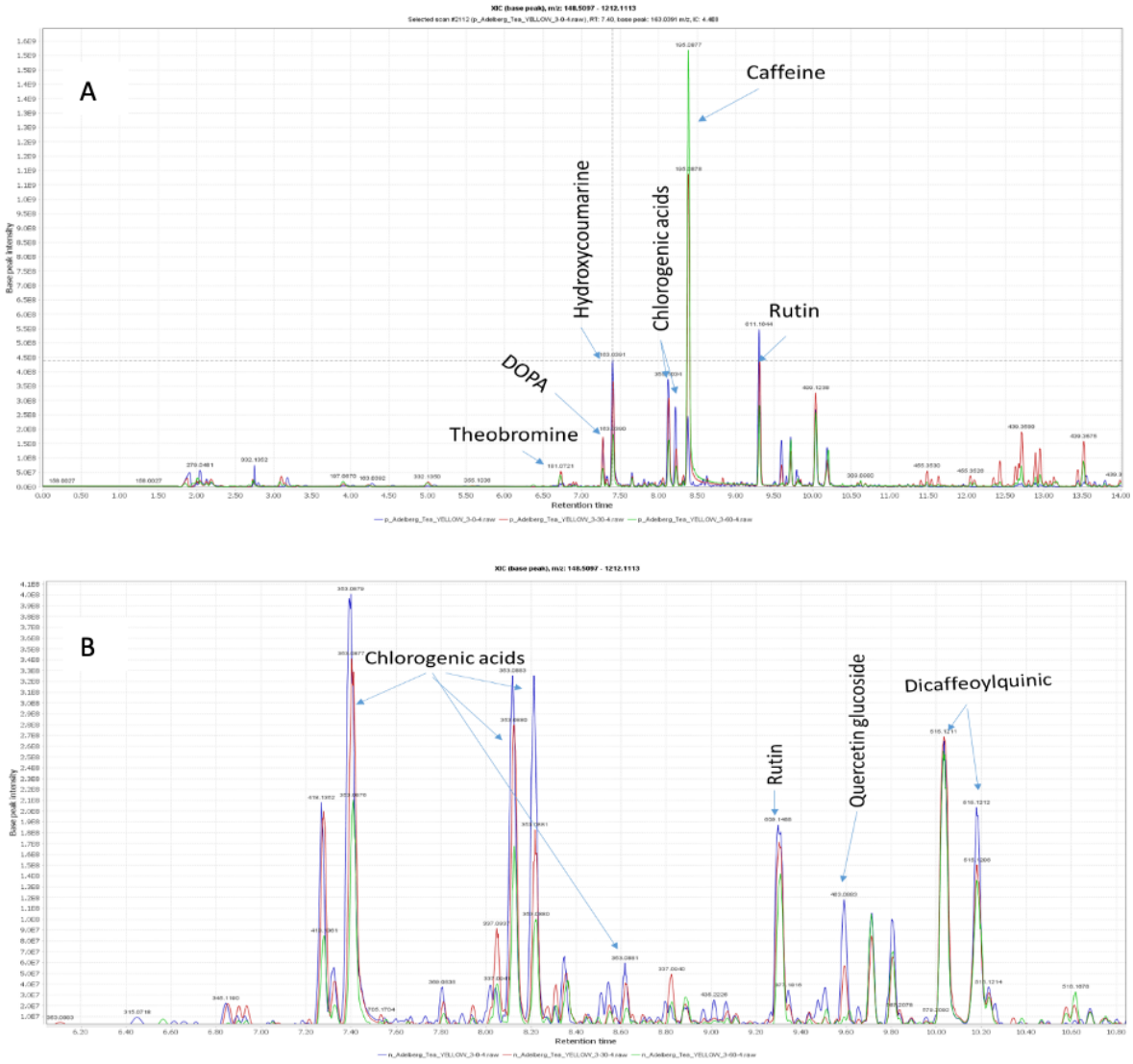

Figure 5. Yaupon tea metabolomic profiles are shown for treatments on the last harvest date, at treble fertilizer rate. The blue lines are without shade, the red lines 30% shade, and the green lines 60% shade. Only a fraction of the putatively identified compounds are labeled. Identification was based on accurate mass (< 2ppm error) and fragmentation match >80% similarity. Alkaloids are shown from positive ionization overlay (A.) and phenylpropanoids are shown with negative ionization overlay (B.)

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