Identifying Chemical and Genetic Factors Influencing Walnut Oxidative Stability

By

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Abstract

Walnut kernels are rich in polyunsaturated fatty acids. These confer numerous health benefits, but are readily oxidized, leading to the development of off-flavors. Current commercial methods of reducing lipid oxidation in walnut add complication and expense to storage and distribution practices. To develop shelf-stable walnut cultivars, a better understanding of the influence of fatty acid and antioxidant content on lipid oxidation is necessary. In this study, kernels from a diverse population were measured for lipid content and oxidative stability using TD-NMR and Rancimat instruments, respectively. Genome-wide association studies (GWAS) were performed on oxidative stability and oil content data. Thirty-four trees were screened for lipid, tocopherol, and phenol profiles to determine which chemicals are associated with oxidative stability. More phenotypic and genetic variation was observed for oxidative stability than for oil content, and the two traits had a moderate negative correlation (r = -0.36). GWAS identified significant associations for lipid content and oxidative stability on chromosome 10. A supplemental file contains raw phenotypic data and best linear unbiased predictions for the two traits, parental information for the phenotyped taxa, and temperature data for the 2019-2021 growing seasons. Fatty acid, tocopherol, and phenol profiles in walnut also varied significantly. However, significant correlations with oxidative stability were found only for oleic acid, linolenic acid, moisture, and total phenols as measured by the Colin-Ciocalteu method. The associations suggest that

breeding for high-oleic, low-linolenic cultivars would be the best method of developing a cultivar with a long shelf life. Unfortunately, this may sacrifice some of the health-promoting benefits of walnut consumption. Further study is needed to resolve whether increasing tocopherol and phenol content in walnut kernels can improve stability.

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Chapter 1 - Inheritance of oxidative stability and oil content in a large walnut breeding population

Abstract

Walnuts contain high amounts of polyunsaturated fatty acids (PUFAs), which have numerous health benefits but are susceptible to oxidation and the development of rancidity. As a first step towards improving shelf life in walnut, we measured oxidative stability and oil content in a large, genotyped breeding population. The two traits show modest negative phenotypic and genetic correlations (r_P = -0.36; r_G= -0.28, $p < 0.00001$). However, there is considerably more phenotypic and genetic variation for oxidative stability than for oil content. Genome-wide association (GWAS) identified significant SNPs for both traits in the same region of chromosome 10, close to a steroleosin gene highly expressed during kernel development. GWAS for oxidative stability using oil content as a covariate did not result in novel significant associations. These results suggest that variation in oxidative stability in our walnut population is driven primarily by factors other than oil content, such as oil profile or antioxidant capacity of the kernel.

Introduction

Persian walnut (*Juglans regia* L.) contains high amounts of polyunsaturated fatty acids (PUFAs), predominantly linoleic acid and α-linolenic acid, which improve gut health, reduce cardiovascular disease risk, and enhance cognitive function (Hayes et al.,

2016; Kris-Etherton, 2014; O'Brien et al., 2014; Arab and Ang, 2015; Bamberger et al., 2018). PUFAs are readily oxidized, however, which both reduces their nutritional value and leads to the development of rancid flavors and odors. Limited shelf life negatively impacts the walnut industry by reducing consumer demand and by limiting the incorporation of walnuts into processed food products. Numerous packaging technologies have been developed to increase the shelf life of walnuts, ranging from nitrogen flushing of walnut packages to applying edible protein-based coatings on walnut kernels. However, these methods add additional costs to walnut processing and distribution (Maté and Krochta, 1997; Jensen et al., 2003), and they may not be applied in time to prevent rancidity. The development of walnut cultivars with longer shelf life would benefit the supply chain by reducing potential losses and by improving product quality without additional packaging costs. Walnut shelf life is potentially affected by total oil content, oil composition, and overall antioxidant activity of walnut kernels. Walnut oil is composed primarily of linoleic acid (polyunsaturated; >60%), with oleic (monounsaturated) and linolenic (polyunsaturated) acid each accounting for 10-20% of total oil, and minor amounts of saturated fatty acids (Kakfas et al., 2020). Walnut kernels consist of an oily inner embryo and a papery outer pellicle of maternal tissue; the primary antioxidants in walnut kernels are tocopherols in the embryo and phenols in the pellicle (Arcan and Yemenicioğlu, 2009; Wang et al., 2015; Savage et al., 1999; Ortiz et al., 2019). Because rancidity is slow to develop under typical storage conditions,

experimental methods to speed up the rate of lipid oxidation are typically used in studies of oxidative stability, including the active oxygen method, the Schaal oven method, and the Rancimat method (Liang and Schwarzer, 1998). The Rancimat apparatus heats and passes air through a sample to induce rapid lipid oxidation and measures the formation of rancid secondary oxidation products as a conductivity change in a volume of pure water above the headspace of each sample. Induction time, a measure of oxidative stability, is defined as the breakpoint of the conductivity curve. Grilo et al. (2020) demonstrated the feasibility of using ground walnut kernels instead of walnut oil for measuring induction time, allowing higher throughput. The primary objectives of this study were to study the genetic variation in oxidative stability and oil content of walnut varieties in the UC Davis walnut breeding program, to determine the influence of oil content on induction time, and to identify useful markers for breeding walnuts with a longer shelf life.

Materials and Methods

Walnut harvesting and processing

Walnuts were harvested by hand from trees in the UC Davis Walnut Improvement Program (WIP) in Davis, CA over three growing seasons from 2019 to 2021. A total of 875 taxa were phenotyped (Table S1), of which 515 were derived from three different crosses with 06-005-31, a line that yields kernels with high oxidative

stability. Phenotypic data are summarized by family in Table S1. Maximum, minimum, and mean daily temperatures during the 2019-2021 growing seasons were obtained from the nearest CIMIS station and are summarized by month in Table S2 (CIMIS, n.d.). Nuts were hulled, placed in mesh produce bags, oven-dried for 16 hours at 38 ± 3°C, air-dried for two weeks at 20-25°C, and finally stored at 0°C until shelling. Walnuts were shelled by hand using hammers, and kernels were stored in plastic bags at -20°C until analysis.

Oxidative stability analysis using the Rancimat

For each Rancimat sample, 6 grams of kernels were ground in a KitchenAid Blade Coffee Grinder (model BCG111, Benton Harbor, MI, USA) for 15 seconds. Larger unground fragments were removed by hand, the remaining ground material was thoroughly mixed, and one gram was placed into a reaction vessel. The reaction vessel was then inserted into an 892 Rancimat Apparatus (Metrohm AG, Herisau, CHE) set to heat samples at 120°C and to feed air to the samples at 20 L h^{-1}. Each measuring vessel was filled with 50 mL of ultrapure water. Raw data for oxidative stability are shown in Table S3.

Oil analysis using TD-NMR

Oil content measurements were performed using the Minispec mq10 TD-NMR Analyzer (Bruker Corp., Billerica, MA, USA). Calibration standards were made by

pipetting oil from *J. regia* cv 'Howard' walnuts onto Kimwipes packed at the bottom of NMR tubes. Tubes were left to warm for 20 minutes on a block heater set to 40°C before NMR measurements were made. The receiver gain was adjusted using the highest calibration standard before making measurements to maximize the signal to noise ratio. Whenever possible, kernel halves used for TD-NMR were from the same individual kernels used for Rancimat analysis. Raw data for oil content are shown in Table S4.

Genotyping and imputation

Leaf tissue was collected in spring from new walnut leaves into 1.1 mL strip tubes in 96-well plates, freeze-dried, and used for DNA extraction and GBS library preparation as previously described (Poland et al., 2012) using simultaneous restriction-ligation with HindIII-HF, MseI, and T4 DNA Ligase (NEB). Following the TASSEL GBS pipelineV2 (Glaubitz et al., 2014), BWA (Li and Durbin, 2009) was used to align 64 bp tags to the reference assembly for Juglans regia cv 'Chandler' (Marrano et al., 2020). Only tags that aligned uniquely (MAPQ>=20) were retained. The SNPQualityProfilerPlugin in TASSEL was used to remove candidate SNPs with low depth (log(depth)< -1) and low inbreeding coefficient (F < -0.05) before variant calling with the ProductionSNPCallerPluginV2 in TASSEL. An inbreeding coefficient (F) of less than -0.05 represents excess heterozygosity relative to Hardy-Weinberg equilibrium; this filter was used to remove putative homeo-SNPs from heterozygous data. Vcftools

(Danecek et al., 2011) was used to remove indels and taxa with >90% missing data, and for depth thresholding using a minimum depth of 4 (--minDP 4). Imputation with Beagle 5.4 (Browning et al., 2018) was performed on unphased genotypes with no reference panel and a window size and walk speed of 12 and 4 Mb respectively.

Phenotypic data analysis and genome-wide association

Best linear unbiased predictors (BLUPs) for both traits were calculated using the 'mmer' function in the R package 'sommer' (Covarrubias-Pazaran, 2023). An additive relationship matrix generated using 39,408 SNPs with minor allele frequencies > 0.05 was used as a random effect. Several models with various combinations of covariates were tested for each trait. For oil content, the year harvested (Year) and the planting area (Block) were tested as random effects. For induction time, Year, Block, and the date analyzed (Date) were tested as random effects. Date was tested as a covariate for induction time but not oil content following our observations of control samples run repeatedly on both the Rancimat and TD-NMR instruments. The Akaike Information Criterion (AIC) was used to identify the best model for each trait, leading to the inclusion of Year, Block, and Date as covariates for induction time and Year and Block for oil content. The 'GWAS' function in the R package 'sommer' was then used to perform one-step genome-wide association on the raw data using the lowest-AIC model for each trait. Manhattan plots were created using 'sommer', and quantile-quantile (QQ)

plots were created using the 'GWASTools' package (Gogarten et al., 2012). Significance of SNPs was defined using the false discovery rate with alpha = 0.05 (Benjamini and Hochberg, 1995). Marker-based narrow-sense heritability estimates were calculated using the 'mmer' and 'vpredict' functions in the R package 'sommer'. All data were analyzed using R (https://cran.r-project.org/) and RStudio (https://posit.co/products/open-source/rstudio/). Student's t-tests were used to determine the significance of the difference between different years for induction time and oil content after only considering taxa shared by the two years (Figure 1.4).

Analysis of gene expression in walnut kernels

Walnut kernel RNAseq data from a previous study (Huang *et al.,* 2021), consisting of 24 paired-end Illumina libraries representing 8 time points with 3 biological replicates each, were downloaded as gzipped FASTQ files from the European Bioinformatics Institute website. This study used a single Chinese walnut cultivar ("Linzaoxiang"), and time points at 49, 63, 77, 91, 105, 119, 133, and 147 days after pollination. Data were re-mapped to the ChandlerV2 genome using BBMAP, featureCounts was used to generate a counts table, and R was used to calculate TPM (transcripts per kilobase per million reads) values from the counts table.

Results

Phenotypic and genetic variation in walnut oxidative stability and oil content

Oxidative stability (induction time) measurements ranged from 3.30-8.36 hours with a mean of 5.86 hours across 1,487 Rancimat measurements representing 873 walnut accessions (Figure 1.1B and Table 1.1). Because our data are highly unbalanced across years, we confirmed our results using balanced subsets of data. Among taxa with induction time data for both 2019 and 2020, mean induction times were 6.26 and 5.31 hours, respectively, and the year effect was highly significant ($p < 0.0001$; Figure 1.4). Similarly, among taxa with induction time data for both 2020 and 2021, mean induction times were 6.13 and 5.96 hours, respectively, and the year effect was still significant ($p < 0.05$). No taxa have induction time data for both 2019 and 2021. Lower induction times in 2020 and 2021 compared to 2019 might result from higher daily maximum temperatures in these years, particularly in May and September (Table S2), and from differences in irrigation management.

Oil content measurements ranged from 54.92-74.33% with a mean of 67.06% across 877 TD-NMR measurements representing 796 walnut taxa (Figure 1.1A and Table 1.1). Oil content was negatively correlated with induction time (r = -0.36; Figure 1.1C). Among taxa with oil content data for both 2019 and 2020, mean values were 66.57% and 67.69%, respectively, and the year effect was significant ($p < 0.0001$; Figure 1.4). Among taxa with oil content data for both 2020 and 2021, mean values were 66.85%

and 66.86%, respectively, and the year effect was not significant. No taxa have oil content data for both 2019 and 2021. The coefficient of variation (CV) for oxidative stability phenotypes was 0.14, ~3.5-fold greater than the CV for oil content phenotypes. When comparing BLUPs for the two traits, the CV for oxidative stability was still ~2-fold greater than the CV for oil content, and the genetic correlation between the two traits was -0.28 (Table S5). In other words, genetic variation in oil content explains only 7.8% of the genetic variation in oxidative stability.

GWAS for oxidative stability and oil content

The best model for oxidative stability included Year, Block, and Date as random effects in addition to the additive relationship matrix. Five significant SNPs in high linkage disequilibrium were found for oxidative stability on chromosome 10 (Figure 1.2A), and the marker-based narrow-sense heritability estimate for this trait is 0.13. The best model for oil content included Year and Block as random effects in addition to the additive relationship matrix. One significant SNP was identified on chromosome 10; this SNP is in the same region as the SNPs for oxidative stability but is not in linkage disequilibrium with them (Figure 1.3A). The marker-based narrow-sense heritability estimate of this trait is 0.21. No significant SNPs were identified for oxidative stability using oil content as a covariate, or in single-year analyses for either trait (Figure 1.5). The common allele at each significant SNP is inferred to be the ancestral allele based on genotype data from a black walnut outgroup (Figure 1.3B).

Kernel-expressed genes near GWAS hits on chromosome 10

The region from 0-1.5 Mb on chromosome 10, which encompasses all significant GWAS hits for induction time and oil content in this study, contains 183 annotated genes in the ChandlerV2 genome. We took advantage of a large public RNA-Seq dataset, composed of 24 libraries from 8 time points during walnut kernel development, to identify genes in this interval that are expressed in walnut kernels (Huang et al., 2021). Of the 183 genes in this interval, 49 are not expressed at all in walnut kernels, 55 more have very low expression (TPM<10), and eight are highly expressed (TPM>100). Expression patterns and annotations of the eight highly expressed genes are shown in Figure 1.6. The three most highly expressed genes in this interval are annotated as a 60S ribosomal gene (mean TPM = 1385), a steroleosin (mean TPM = 581), and a seed maturation protein (mean TPM = 380). Whereas the ribosomal gene shows high expression across all 8 time points, the steroleosin and the seed maturation protein show much higher expression from 63-147 days after pollination when most kernel oil accumulation occurs.

Discussion

High levels of polyunsaturated fatty acids in walnut kernels confer health benefits but accelerate the development of rancidity. Determining the inheritance of walnut oil content and oxidative stability and how they relate to each other are

important steps towards the development of walnut cultivars with improved shelf life. Mean values for oil content in this study are somewhat higher than previously reported for California walnuts, though our range of oil content values is in general agreement with existing data (Kafkas et al., 2020; Prasad, 2003).

The first year of this study (2019) yielded kernels with significantly higher oxidative stability and significantly lower oil content than subsequent years (2020 and 2021). The impact that year had on both oxidative stability and oil content suggests that variation in management and weather between years influenced nut quality in this study. Deficit watering (33% of ET) was previously found to significantly reduce the proportion of PUFAs to total fatty acids in "Chico" walnuts (Greve et al., 1992). Similarly, higher minimum daily temperatures during nut development were previously found to reduce the proportion of PUFAs, increasing oleic acid at the expense of linolenic acid (Poggetti et al., 2018). Some genotypes in that study appeared more susceptible to compositional differences, with year-to-year differences in oleic acid being negligible in some cultivars and as high as 5% in others (Poggetti et al. 2018). Finally, minimum daily relative humidity was previously found to be negatively associated with oleic acid and positively associated with linoleic acid content (Yang et al., 2022). The strength of environmental and GxE influences on induction time and oil content suggest that management techniques may be used on specific genotypes to prolong the shelf life of walnuts.

Walnut induction time and oil content show modest negative phenotypic (r_P = -0.36) and genetic (r_G = -0.28) correlations, emphasizing the fact that oil content alone has a limited influence on oxidative stability. Further research is needed to determine the influence of other factors on oxidative stability, including oil composition (fatty acid profile) and antioxidant capacity of the kernel and pellicle. The relative proportions of the major fatty acids are of particular interest since walnut cultivars with high oleic acid would be expected to have longer shelf-life, making them better suited for oil extraction and incorporation into processed food products (Warner et al., 2001; Liu et al., 2002).

Induction time GWAS identified five significant SNPs from roughly 0.3-1.4 Mb on chromosome 10 (Table 2). These SNPs have minor allele frequencies close to 0.2 and are in high linkage disequilibrium with each other (Figure 1.3A). Oil content GWAS identified one significant SNP at ~1 Mb on chromosome 10. This SNP has a minor allele frequency of 0.05 and is not in linkage disequilibrium with any of the induction time SNPs (r^2 ~0; Figure 1.3A), although D' is high, suggesting limited evidence for recombination. Therefore, independent derived mutations in the same region of chromosome 10 reduce oil content and oxidative stability in walnut. The reduction in oxidative stability associated with induction time SNPs on chromosome 10 appears recessive, manifesting primarily in homozygotes for the minor allele, and many popular walnut cultivars are heterozygous at this locus with little apparent effect on induction time (Figure 1.3B). The reduction in oil content associated with the SNP at 1,052,502 bp

on chromosome 10 appears to be additive in effect. Interestingly, this SNP is heterozygous in walnut cv 'Franquette', which was previously reported to have lower PUFA concentration and longer shelf life than other common walnut cultivars (Nogales-Bueno et al., 2021).

Using a large public RNA-Seq dataset, we identified eight genes that were both highly expressed during walnut kernel development and in close proximity to our significant GWAS SNPs. One of these genes is annotated as a steroleosin, a membrane-anchored hydroxysteroid dehydrogenase (HSD) protein found in oil bodies (Lin *et al.*, 2002). Oleosin, caleosin, and steroleosin are structural proteins embedded in the phospholipid monolayer of oil bodies; whereas oleosin and caleosin are highly abundant and required for maintaining oil body integrity, steroleosin is much less abundant and is thought to play signaling role (Shao *et al.*, 2019), and has not previously been implicated in quantitative variation in yield and quality of oilseed crops.

This study was likely limited by the low heritability of induction time and oil content, and by our relatively low marker density. Larger phenotypic and genotypic datasets and high-throughput screening of oil profile and total antioxidant capacity of walnut kernels in this breeding population will be critical next steps towards both characterization of the chromosome 10 locus, and towards development of new walnut cultivars with improved shelf life.

Figures

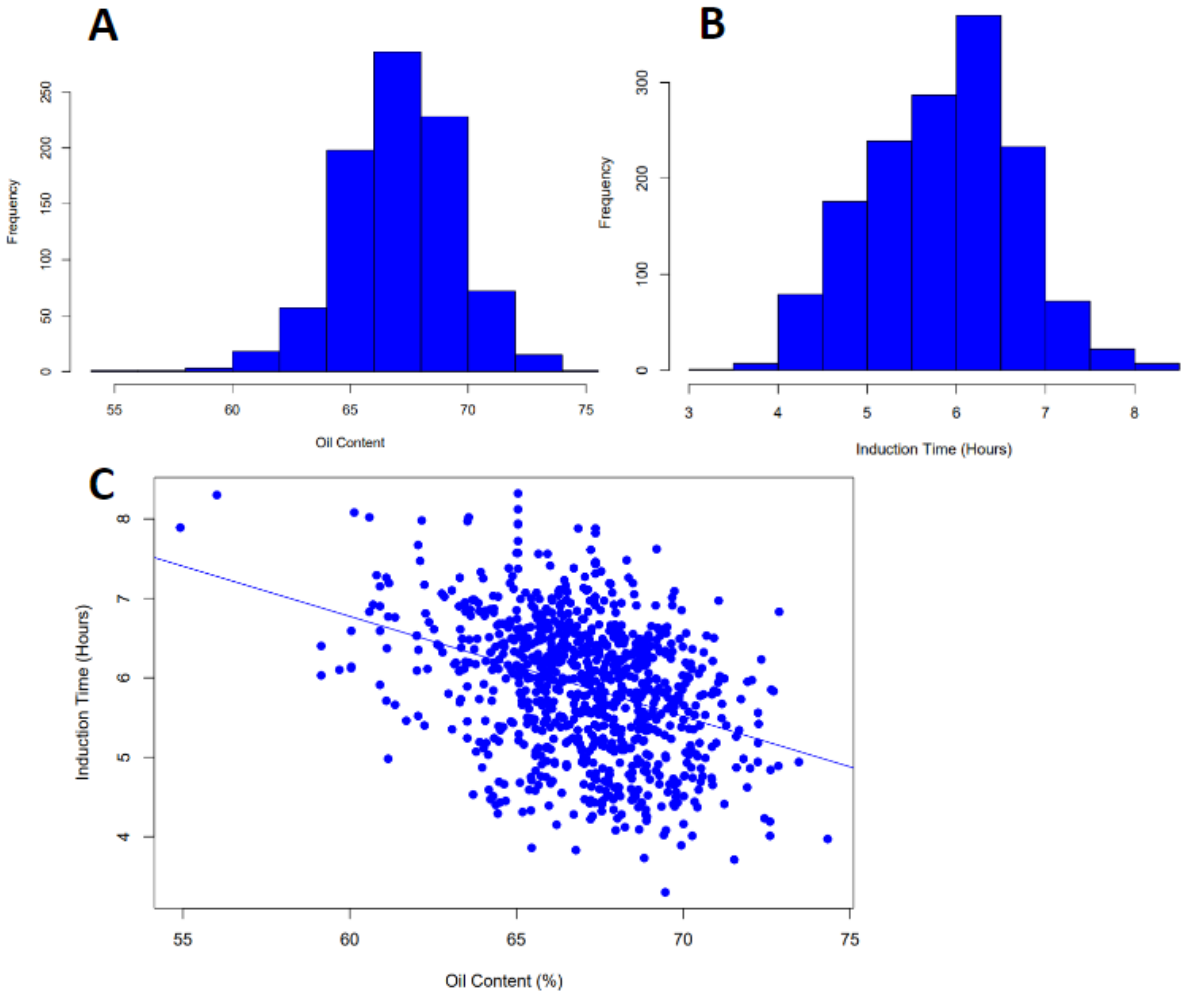

Figure 1.1: Oil content (A) and oxidative stability (induction time; B) variation of walnuts in the UC Davis breeding program. C) Oil content is negatively correlated with oxidative stability (r=-0.36, n=898, *P*<0.0001).

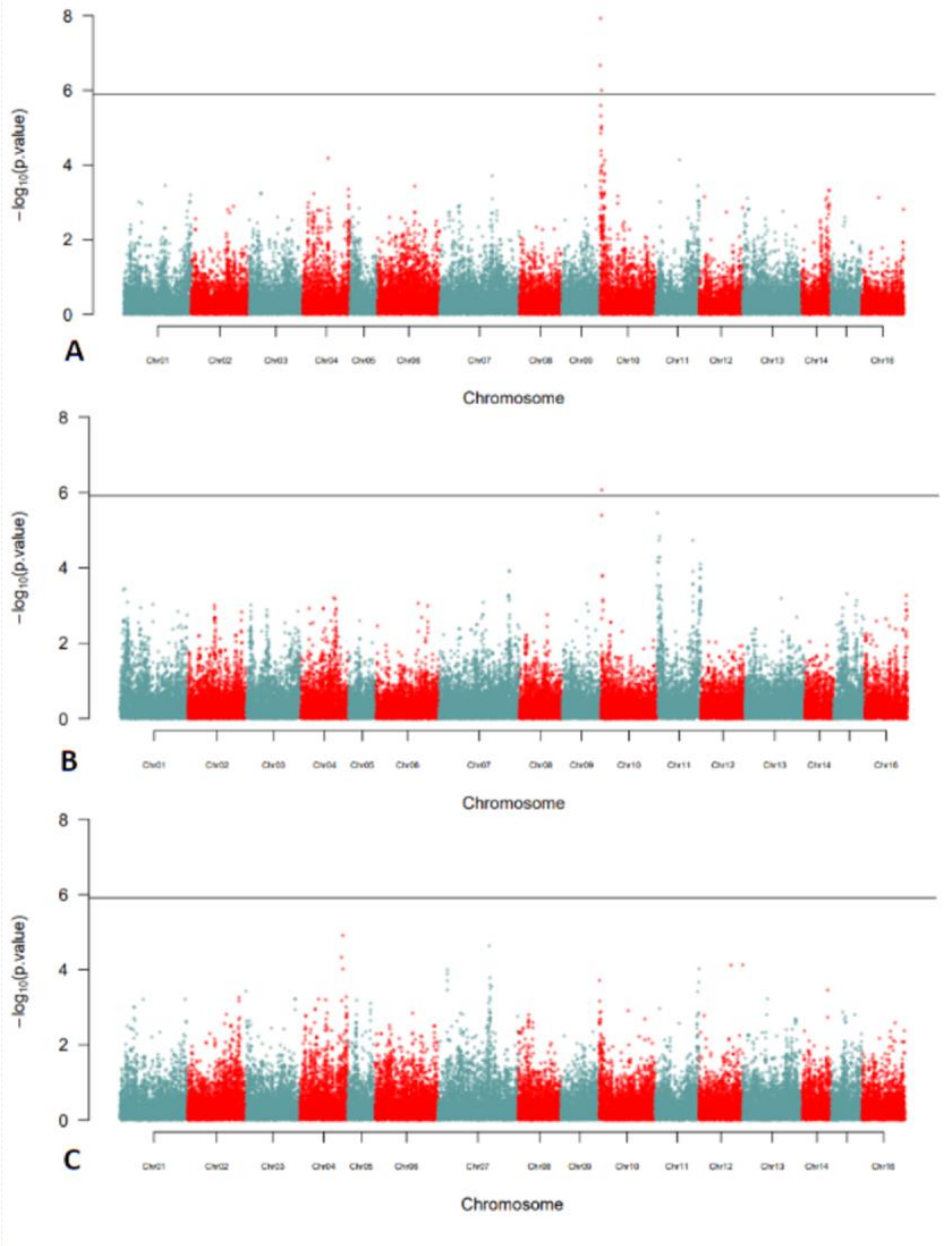

Figure 1.2: Genome-wide association for oxidative stability (A), oil content (B) and oxidative stability using oil content as a covariate (C). Horizontal lines represent Bonferroni significance thresholds at alpha=0.05. Use of the FDR threshold results in the discovery of two additional SNPs for oxidative stability.

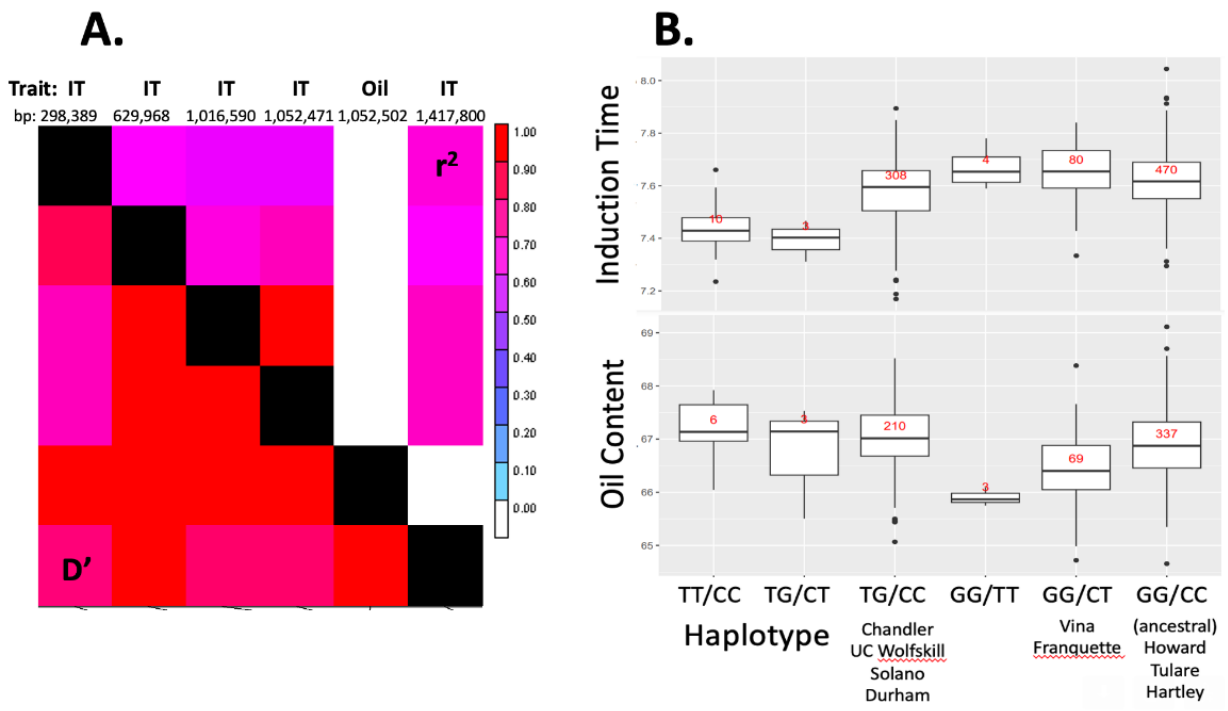

Figure 1.3: Linkage disequilibrium between significant SNPs on chromosome 10 (A) and phenotypic effects of the 1417800/1052502 SNP haplotype (B). Boxplots in (B) are constructed using BLUPs for each trait, with numbers representing the number of walnut taxa that carry a given haplotype. Haplotypes carried by several popular walnut cultivars are shown along the x-axis. The GG/CC haplotype is inferred to be ancestral based on genotyping of black walnut (Juglans section Rhysocaryon) outgroups.

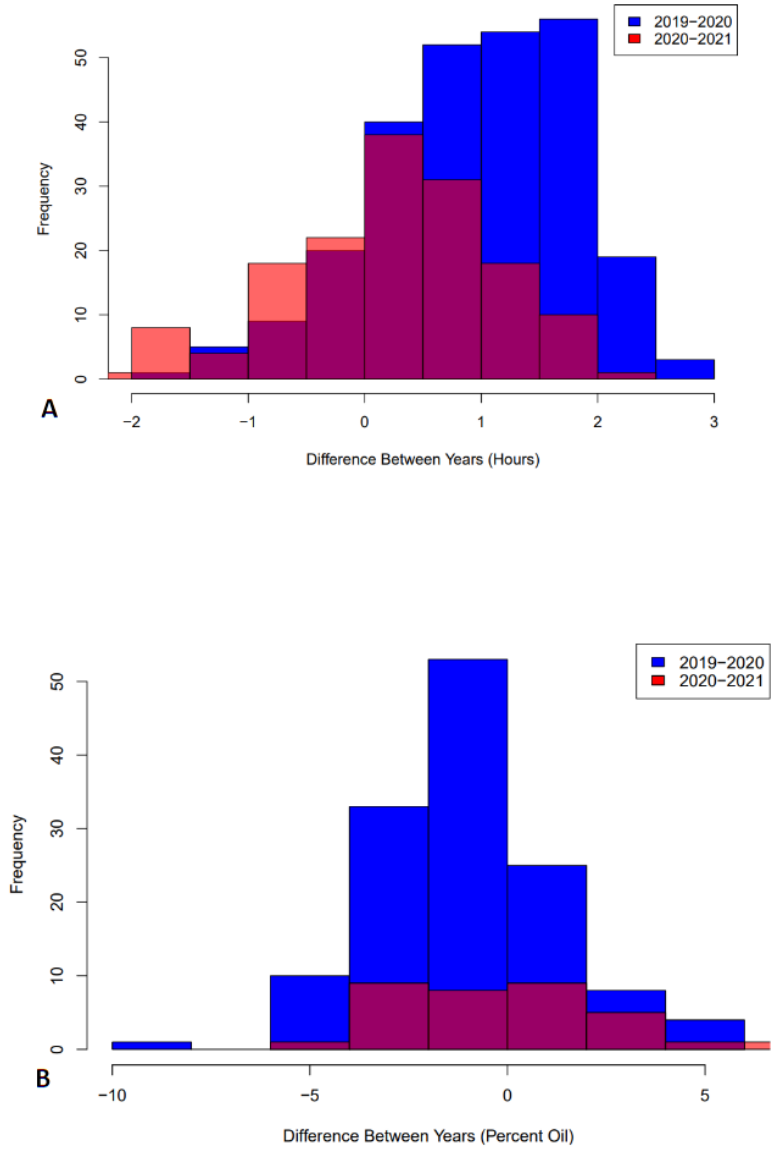

Figure 1.4: Distribution of phenotypic differences between years. A. Induction time differences of individual taxa between 2019 and 2020 (blue) and between 2020 and 2021 (burgundy) are mostly positive. B. Oil content differences of individual taxa between 2019 and 2020 (blue) and between 2020 and 2021 (burgundy) are mostly negative.

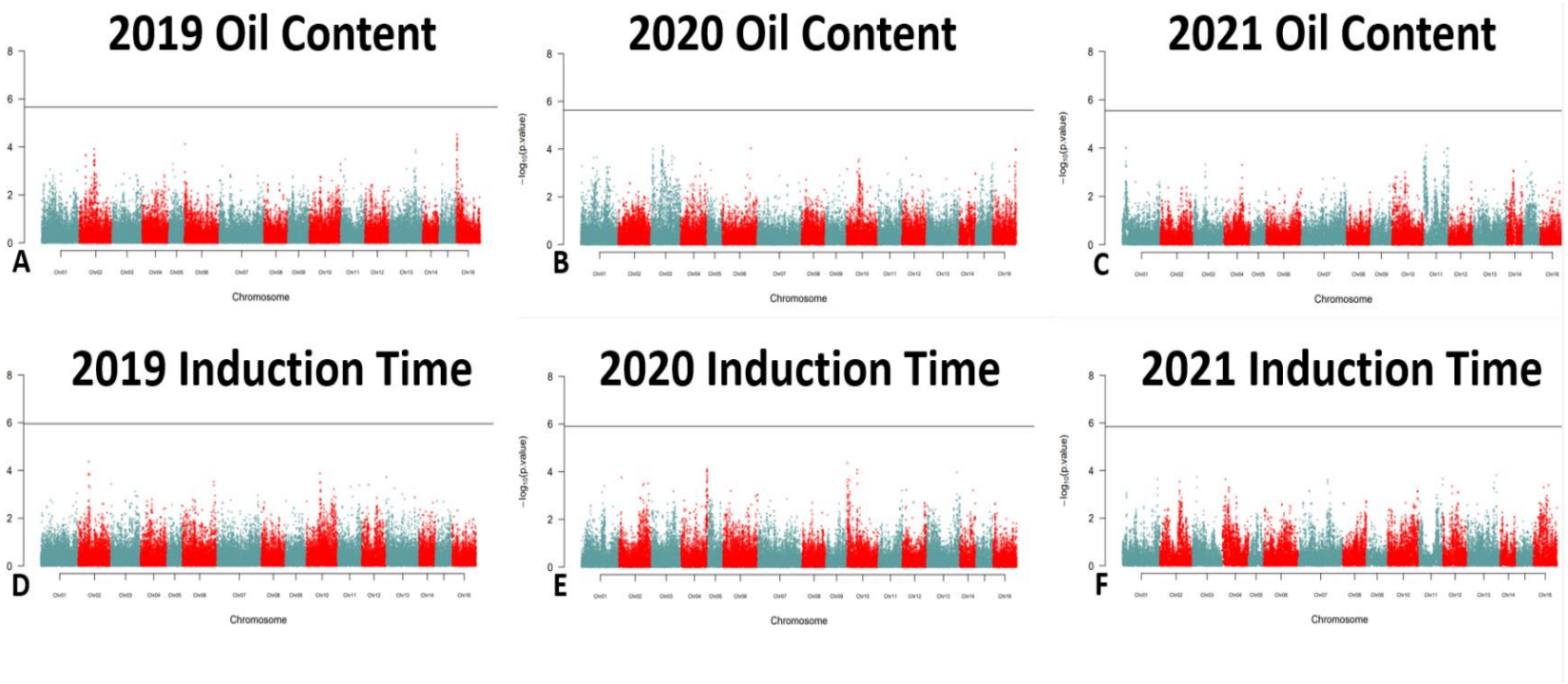

Figure 1.5: Genome-wide association for oil content (top panels) and oxidative stability (bottom panels) using single-year data from 2019, 2020, and 2021. The taxa phenotyped in each year are not identical.

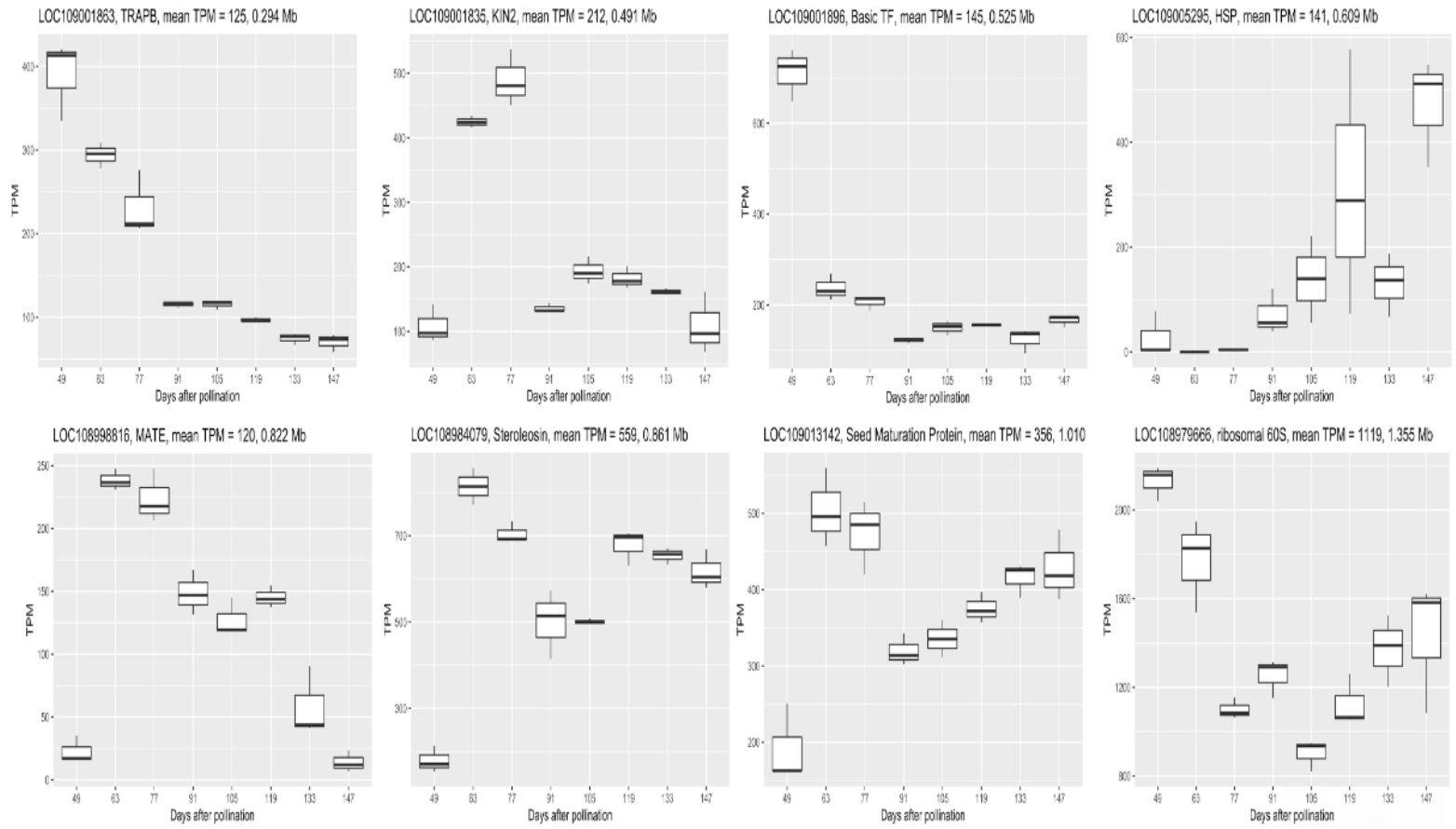

Figure 1.6: Gene expression (TPM; transcripts per million) of the eight genes most highly expressed during walnut kernel development in the 0-1.5 Mb interval on chromosome 10 in the ChandlerV2 genome assembly. Data are from Huang et al. (2021) remapped to ChandlerV2. Each of the eight time points is represented by three biological replicates.

Tables

Table 1.1: Summary of phenotypic data collected.

Year	Oxidative stability (Induction time – hours)				Oil content (%)			
	n	Taxa	Mean ± SD	**CV**	n	Taxa	Mean ± SD	**CV**
2019	348	283	6.31 ± 0.66	0.10	220	186	66.46 ± 2.13	**0.03**
2020	772	677	5.62 ± 0.85	**0.15**	**390**	358	67.75 ± 2.28	**0.03**
2021	373	325	5.96 ± 0.72	**0.12**	270	252	66.57 ± 2.59	**0.04**
Total:	1,493	875	5.86 ± 0.83	**0.14**	**880**	628	67.06 ± 2.42	**0.04**

Table 1.2: Summary of GWAS results for each trait.

Trait	chr	bp	MAF	FDR-adjusted p	Minor allele effect (+/-)
Induction Time	10	298,389	0.18	0.011	-
Induction Time	10	629,968	0.19	0.0008	-
Induction Time	10	1,016,590	0.19	0.025	-
Induction Time	10	1,052,471	0.19	0.039	-
Oil Content	10	1,052,502	0.05	0.038	-
Induction Time	10	1,417,800	0.19	0.039	-

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Chapter 2 - Chemical Composition and Oxidative Stability in Walnut

Abstract

Walnuts are a good source of tocopherols and polyunsaturated fatty acids (PUFAs), however, PUFAs are prone to oxidation leading to the development of rancid flavors. Walnut contains tocopherols in the kernel and phenols primarily in the pellicle, both of which may act as antioxidants to reduce lipid oxidation. To determine the degree to which these compounds affect walnut rancidity, 34 walnut samples were analyzed for phenols, tocopherols, and fatty acids in addition to Rancimat oxidative stability measurements. To determine the influence of pellicle phenolics on oxidative stability, 13 samples were analyzed for oxidative stability both with and without the pellicle, and the effect of the pellicle removal treatment was examined to find associations with phenol data. Additionally, gas chromatography was used to measure hexanal content in walnut before and after storage at 30°C for 3 months. Oxidative stability was only significantly correlated with oleic acid (R^2=0.24) and linolenic acid (R^2=0.27) suggesting that breeding for high-oleic, low-linolenic acid walnuts is the most effective strategy for developing shelf-stable walnut cultivars. Removal of the pellicle increased induction time by an average of 0.27 h (P<0.001) but the magnitude of this effect was not significantly associated with any phenolic measurements. Hexanal content was not correlated with Rancimat stability measurements. Given the small sample sizes and the

multitude of variables affecting rancidity, follow-up studies should be conducted to determine the extent to which these chemicals are involved with walnut rancidity.

Introduction

Walnut kernels are generally over 60% oil which contributes to their health benefits. The three major walnut fatty acids are oleic, linoleic, and linolenic acids which make up approximately 20, 56, and 12% of total fatty acids, respectively (Zwarts, 1999; Martínez and Maestri, 2008). Oleic acid is a monounsaturated fatty acid whereas linoleic and linolenic acid are polyunsaturated fatty acids (PUFAs) which have two and three degrees of unsaturation, respectively. The relative rate of oxidation increases non-linearly from one to three degrees of unsaturation. For oleic, linoleic, and linolenic acids, the ratio of oxidation rates has been determined to be 1:12:25 (DeMan, 1999). Because of walnut's high PUFA content, its shelf life is limited since lipid oxidation leads to the development of rancid odors and flavors. This also makes walnut oil less valued as a cooking oil (Zwarts, 1999). Increasing the ratio of oleic acid to PUFAs would likely reduce walnut's susceptibility to lipid oxidation. Variation in fatty acid profiles among walnut cultivars suggests that altering fatty acid ratios is achievable through selection (Greve et al., 1992; Savage et al., 1999; Martínez and Maestri, 2008). However, some of the health benefits associated with walnut consumption are associated with walnut's high PUFA content and increasing the proportion of oleic acid at the expense

of PUFAs may decrease these benefits (Kris-Etherton, 2014; O'Brien et al., 2014; Arab and Ang, 2015).

In addition to fatty acids, walnuts are a good source of tocopherols, which have vitamin E activity and have been demonstrated to have antioxidant activity in oils including walnut oil (Jung and Min, 1990; Li et al., 2007). Walnut contains α-, β-, γ-, and δ-tocopherols, with γ-tocopherol being the most prevalent (Savage et al., 1999; Li et al., 2007). Walnut pellicles are rich in phenols which may contribute significantly to the antioxidant properties of kernels (Shen et al., 2021; Haiyan et al., 2007). The pellicle, which has been observed to protect the embryo from oxidation, may derive some of its protective effect from the antioxidant properties of its phenols, particularly in unshelled walnuts and intact walnut halves (Ortiz et al., 2019; Bustamante and Mitcham, 2023).

To build upon the work described in Chapter 1, the first objective was to get walnut fatty acid, tocopherol, and phenol data of 27 English walnuts (*Juglan regia*), one fourth-generation backcross ((*Juglans regia* x *J. hindsii*) x *J. regia*), and six undomesticated walnut samples to determine the variability of these chemicals. Oxidative stability data using a Rancimat instrument was then used to collect data on 26 English walnuts, a backcross walnut, and a Northern California black walnut (*J. hindsii*) variety to determine chemical factors associated with stability. The second objective was to submit kernel samples that have chemical data to oxidative stability testing both with and

without the pellicle to identify individual phenols that may influence stability. The third objective was to assess the use of gas chromatography to measure hexanal accumulation in walnuts stored at 30°C as an alternative or supplement to Rancimat measurement.

Materials and Methods

Kernel harvest and processing

Thirty-four walnut samples consisting of 27 *J. regia* samples (representing 23 taxa), four *J. hindsii* samples, and one sample each of *J. californica*, a *J. nigra* x *J. hindsii* hybrid, and a fourth-generation backcross ((*Juglans regia* x *J. hindsii*) x *J. regia*) were selected for tocopherol, phenol, fatty acid, and moisture analysis in the lab of Selina Wang at UC Davis. Nuts were hand-harvested from trees on campus at UC Davis and at least 100 grams of kernel per sample were collected. English and backcross (EB) walnut samples were hulled but not shelled before drying whereas all other samples were not hulled before drying. EB samples were air dried for several weeks by placing them in mesh produce bags that were hung on drying racks before being moved to cold storage at 0°C. Undomesticated species were left to dry in their hulls at room temperature for at least a month.

Rancimat analysis

Rancimat data was collected on the kernel samples submitted for chemical analysis. EB walnuts were processed for Rancimat measurement as described in Chapter 1. For the *J. hindsii* samples, only four grams of kernels were collected for submission to the Rancimat due to the difficulty in extracting kernels. Rancimat data was collected in triplicate.

Compositional analysis

Walnut compositional analyses for moisture, lipids, tocopherols, and phenols were performed at the Selina Wang Lab at UC Davis. Information about the procedures can be found in Appendix 2.

Hexanal measurement

A low-temperature, heat-accelerated rancidity test was performed on 15 samples of EB walnuts with chemical data. For each sample of walnuts, the nuts were divided into two temperature treatments. Half of the nuts for a sample were stored at 30°C and the remaining nuts were stored at -20°C with oxygen absorbers to reduce lipid oxidation. After three months of storage, 6 halves from each subsample were finely chopped and sieved (USA Standard Testing Sieve #25). Gas chromatography measurements were taken following the procedure of Bustamante and Mitcham (2023).

For each measurement, one gram of the sieved nuts was put into glass screw-top vials and submitted to a gas chromatograph (model 7890B, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector, an Aglient DB-WAX column and a solid-phase microextraction (SPME) fiber. Standards were made by dissolving hexanal in triacetin to make 1, 10, 100, 500, 1000, 1500, and 2000 ppm solutions. For each standard, 10 μL was pipetted into an empty 20 mL screw-top vial. Empty vials were run by the instrument before and after each hexanal standard vial. Walnut samples were analyzed in triplicate and replicates were run in succession. A blank vial was run before and after each group of vials for a given treatment to ensure that the SPME fiber had desorbed volatiles between treatments. After loading vials into the autosampler, each vial was individually heated and shaken at 60°C for 10 minutes before solid-phase microextraction occurred for 10 minutes while heating and shaking continued. After extraction, the SPME fiber was then inserted into the GC inlet for analysis. The GC oven temperature started at 50°C and increased to 240°C over three ramps in a 28 minute period. The fiber was conditioned for 7 minutes at 200°C before and after each measurement.

Pellicle experiment

To investigate the impact of pellicle phenolics on shelf life, oxidative stability of nuts from 13 different trees representing 11 taxa was compared with and without the

pellicle. For each tree, four kernel halves, each from a different nut, were collected and flash frozen in liquid nitrogen and stored at -20°C. Afterwards, each half was cut into two quarter pieces where one quarter had the pellicle removed and the other quarter had the pellicle left intact. To remove pellicles from the embryo, a ceramic mortar was placed into a styrofoam container and was partially buried with dry ice. Four kernels quarters were then placed into the mortar after it had cooled. A pestle was used to fragment the walnut quarters into three or fewer large fragments to make pellicle removal using tweezers easier. Kernels were kept inside of the mortar while peeling the pellicle off to keep them frozen. After removing the pellicle, kernels were then stored in a -20°C freezer. For Rancimat analysis, kernels were thawed out and prepared as described in Chapter 1. Each sample was measured with three technical replicates.

Data analysis

All data analysis and plot visualization were performed using R and R Studio software. Correlations were calculated using Pearson's correlation coefficient using the corrplot and cor.mtest functions in the R package 'Corrplot' which calculated p-values and combined and visualized data into correlation plots. Paired sample T-test was used to determine both the significance of the pellicle removal treatment and hexanal measurement experiment. Data were mostly visualized with base R function and the pellicle experiment boxplots were visualized using ggplot2 functions.

Results

Walnut lipid and moisture content

Lipid profile varied substantially among the English walnut and backcross walnut samples (EB samples) (Table 2.1). Among EB samples, the total lipid content on a dry weight basis ranged from 65.55 to 75.78% with a mean of 69.54%. The three main fatty acids, linoleic acid, oleic acid, and linolenic acid, compose on average 42.79%, 12.12%, and 8.73% of nut weight in EB samples, respectively.

Induction time had significant ($P<0.05$) positive correlations with oleic acid and moisture content and a negative correlation with linolenic acid (Figure 2.1A). The R^2 values of the correlations of oleic acid and linolenic acid were 0.24 and 0.27, respectively. Some notably high-oleic acid samples including a 06-005-31 sample from 2020 grown on its own roots, 09-028-5, and Scharsch Franquette have long induction times. Conversely, UC Wolfskill, which had a low induction time, had a relatively low amount of oleic acid (Table 2.1). Samples of 06-005-31 harvested in 2021 (both from the grafted and ungrafted tree) had a lower oleic acid content than the 2020 sample of the ungrafted 06-005-31 tree and these samples had a lower induction time. High induction time accessions like 06-005-31 and Scharsch Franquette have low amounts of linolenic acid whereas low induction time accessions such as Solano, Wolfskill, and 04-003-143 had high amounts of linolenic acid.

Lipid profiles of the undomesticated walnut species differed greatly from EB samples (Table 2.2). Across all undomesticated walnut taxa, the total lipid content was lower (average of 61.70%) than any of the EB samples (average of 69.54%). The four Northern California black walnut (*Juglans hindsii*) taxa had lipid contents ranging from 59.57% to 62.95% with an average of 60.99%. The fatty acid contents of *J. hindsii* nuts had a higher monounsaturated to polyunsaturated fatty acid proportion (0.38) than EB samples (0.23) primarily due to the high oleic acid and low linoleic acid content of *J. hindsii* walnuts compared to EB samples.

Walnut tocopherol profile

The average amount of α-, β-, γ- and δ-tocopherols among EB samples were 10.14, 0.15, 258.93, and 18.91 μg/gram of kernel on a dry basis, respectively (Table 2.3). Walnuts samples for 09-002-22 and 09-028-5 had notably high amounts of δ-tocopherol. The only true outlier sample with regards to the different forms of tocopherol was for the fourth-generation backcross ((*Juglans regia* x *J. hindsii*) x *J. regia*) accession 09-028-5 which had high amounts of β-tocopherol (0.74 μg/gram kernel, dry basis). However, it made up a small fraction of the samples's total tocopherol content (0.25% of total tocopherols). Tocopherol content was not found to be significantly correlated with induction time (Figure 2.3B).

The four *J. hindsii* taxa had a different tocopherol ratio than EB samples (Table 2.4). On average *J. hindsii* had much lower α-tocopherol and δ-tocopherol compared to

EB samples and was higher in γ-tocopherol compared to EB samples. Southern California black walnut (*Juglans californica*) had a γ-tocopherol content much higher than any sample measured in this study (488.48 μg/gram, dry basis).

Walnut phenolic profile

Fifteen different phenolic compounds were detected in at least one EB sample of the 28 samples. Of these compounds, eight were found in most or all of the EB samples (gallic acid, catechin hydrate, vanillic acid, p-coumaric acid, taxifolin, (-)-epicatechin gallate, quercetin, naringenin) (Table 2.5). Catechin hydrate makes up the vast majority of the phenols found in EB samples. Of the three samples of 06-005-31 submitted for chemical analysis, the 2020 and 2021 samples from the ungrafted tree had unusually low amounts of catechin hydrate (25.49 and 35.60 μg/g on a dry basis, respectively) whereas the 06-005-31 sample harvested from the grafted tree in 2021 had a near-average amount of the phenol (102.36 μg/g, dry basis).

The phenol (-)-epicatechin was found only in low concentrations in 04-003-143 and Robert Livermore. Similarly, 3,4-dihydroxybenzoic acid was detected only in 04-003-143, Robert Livermore, and UC Wolfskill, albeit in low quantities. The 03-001-2440 sample was unusual in that it was the only EB sample to contain detectable amounts of kaempferol, luteolin, and caffeic acid (0.08, 0.02, and 0.02 μg/g on a dry basis, respectively). Robert Livermore was the only EB sample found to contain chlorogenic acid.

Some notable phenolic differences in *J. hindsii* samples compared to EB samples are that *J. hindsii* has significantly less catechin hydrate, and more vanillic acid, (-)-epicatechin, and quercetin (Table 2.6). The *J. californica* sample differed from both EB and *J. hindsii* samples; most notably, it had very low amounts of gallic acid and, along with the *J. hindsii* x *J. nigra* hybrid, was found to contain sinapic acid. Additionally, it was the only accession aside from Robert Livermore (an EB sample) found to have chlorogenic acid.

Walnut hexanal measurements

Due to the standard curve prepared for the gas chromatograph hexanal measurements being set too high, mean peak data for each sample from the instrument was analyzed and presented instead of standard-adjusted data (Figure 2.2). The difference in hexanal content between the hot and cold storage treatment within a sample (HD) varied from 10.62 to 69.74 picoamperes*second (pA*s) with a mean of 33.2 pA*s. Of the 15 samples, Chandler had a particularly high increase in hexanal after hot storage suggesting that it is the most susceptible to oxidation. Three correlations plots of HD were made for phenols, tocopherols, and fatty acid/moisture data (Figure 2.3). No tocopherol data was significantly correlated with HD, however, HD had significant negative and positive correlations with gallic acid and p-coumaric acid, respectively. HD also had significant negative correlations with total lipid content (both on a dry and

wet basis), palmitic acid, and oleic acid. HD also had a significant positive correlation with moisture. Notably, HD seemed to be independent of induction time (R^2=0.00011).

Pellicle experiment

Surprisingly, pellicle removal increased induction time (P<0.0001). For 12 out of the 13 samples, the subsample with pellicle removed had a higher induction time than its counterpart. The average difference between the pellicle and no pellicle treatments among samples is -0.27 hours and varied from a -0.7 to 0.07 hours (Figure 2.4). The difference in induction times between the two treatments for each sample was not significantly (P<0.05) associated with any particular phenolic compound or with total phenolic composition (Figure 2.5). P-coumaric acid was the only phenolic compound with a correlation to the treatment induction time difference that approached significance (P=0.08).

Discussion

Walnut lipid and moisture content

English walnut's lipid profile underlies many of its health benefits but it is also responsible for walnut's short shelf life. Studying lipid profile variation and how it relates to oxidative stability in walnut are critical for understanding how to develop more shelf-stable walnut cultivars.

Among English and backcross (EB) walnuts, induction time had significant correlations with only two fatty acids: oleic acid and linolenic acid. Oleic acid, the second most prevalent fatty acid in walnut, is a monounsaturated fatty acid which makes it much more susceptible to oxidation compared to saturated stearic acid (DeMan, 1999). Oleic acid content is also negatively correlated with linolenic and linoleic acids which are an order of magnitude more susceptible to oxidation (DeMan, 1999). Oleic acid's positive association with induction time can likely be explained by its relative oxidative stability compared to the two other major fatty acids whose presence is negatively associated with oleic acid content. Linolenic acid was found to have a significant negative correlation with induction time whereas linoleic acid did not, despite the fact that linoleic acid is both the dominant fatty acid in walnut oil and highly susceptible to oxidation. However, linolenic acid oxidizes at more than twice the rate of linolenic acid and may outcompete linoleic acid in reacting with oxidizing agents such as oxygen and free-radicals (DeMan, 1999). Further study is required to elucidate the full extent to which linoleic acid content impacts oxidative stability. The low amounts of palmitic acid, stearic acid, and other minor fatty acids in walnut likely limit their impact on walnut rancidity. The high oleic acid content of 06-005-31, 09-028-5, and Franquette could contribute to their long induction times as oleic acid is stable compared to linoleic and linolenic acids. Within three different samples of 06-005-31

from two different years, oleic acid variation helped explain variation in oxidative stability.

Since most *Juglans* species are inter-fertile, the high-oleic acid and low-linoleic acid content of undomesticated walnut species (e.g., *J. hindsii*, *J. californica*) may be of use in a hybrid walnut breeding program to develop trees that produce nuts with a fatty acid ratio aimed at a longer shelf life.

Walnut tocopherol profile

Tocopherols are a class of antioxidants that are commonly found in seed oils where they serve to reduce lipid oxidation. Because tocopherols may play a role in delaying walnut rancidity, studying tocopherol variation and how it may influence oxidative stability is important for determining how shelf-stable walnut cultivars can be developed.

EB walnut kernels contain α-, β-, γ- and δ-tocopherols with γ-tocopherol being the predominant form followed by δ-, α-, and β-tocopherol. This reflects past reporting on walnut tocopherol profiles (Savage et al., 1999; Li et al., 2007). Despite tocopherol's widely reported antioxidant properties, none of these compounds were significantly associated with oxidative stability, however, further research is needed to understand what, if any, effect these compounds have on stability. A potential issue with utilizing the Rancimat is that it is a heat-accelerated rancidity test and that the antioxidant

properties of α- and δ-tocopherol have been shown to decrease with increasing temperature. For this reason, high-temperature assays used in this study could underestimate the benefits of tocopherols for extending shelf life in walnut. However, this effect has not been studied in walnut kernels or oil. Differences in how tocopherol antioxidant activity is affected by temperature in walnut and walnut oil may also depend on potential synergistic effects among tocopherols and other antioxidants (Kamal-Eldin, 2006; Réblová, 2006). Further study is required to determine the influence tocopherol interactions have on oxidative stability.

Walnut phenols

Walnut pellicles are rich in phenols compared to the inner embryo. As a result, the pellicle may act as an antioxidant barrier that delays rancidification (Ortiz et al., 2019). Much is unknown about the influence individual phenols have and a better understanding is needed for developing walnut cultivars with a longer shelf life.

Oxidative stability was not found to have significant correlations with any phenol or the sum total of all phenols measured through liquid chromatography with tandem mass spectrometry (LC-MS/MS), however, it was associated with the total phenol estimation by spectrophotometer using the Folin-Ciocalteu method (Figure 2.1C). While the amount of absorption measured by the instrument is intended to provide an estimate of total phenols, absorption is influenced by other classes of

chemicals that have reducing properties. Another reason for the correlation with oxidative stability is that each phenol may have varying reducing capacities which could make some phenols more effective at limiting lipid oxidation (Lamuela-Raventós, 2018). Since total phenol estimate by the Folin-Ciocalteu method was the phenol-related datapoint most strongly associated with oxidative stability, utilizing this procedure as a high-throughput screening method may prove to be useful for examining a large number of walnut varieties to identify high- and low-outlier cultivars that should be submitted for LC-MS/MS screening. Due to the relatively small size of the data set along with the numerous chemical variations between walnut samples, more samples are needed to determine the influence individual phenols have on walnut oxidative stability.

Due to the variation in phenol content of samples within a variety (e.g., PI 159568 and 06-005-31 samples having greatly differing catechin hydrate contents), phenols, or at least catechin hydrate, may be heavily influenced by environmental factors. This is an important consideration if phenols were to be selected for in a breeding program.

Walnut hexanal measurements

The goal of the gas chromatograph study of hexanal accumulation in walnut was to get preliminary data on the feasibility of using this method as an alternative or supplement to Rancimat stability measurements. The difference between the raw hexanal measurements for each sample in the hot and cold storage treatment (HD) was

found to have no association on oxidative stability measurements from the Rancimat. HD was significantly positively correlated with lipids (on both a wet and dry basis), palmitic acid, and oleic acid. HD was only significantly positively associated with moisture. The correlations HD had with various chemicals were expected to be, in general, the opposite of the correlations that Rancimat stability measurements had. This was true for oleic acid, but not true for moisture and lipid content.

The Rancimat instrument exposed samples to much higher temperatures (120°C) than the hot storage temperature (30°C) which may result in different reactions that affect lipid oxidation being favored over others. Potentially, the Rancimat's high temperatures have inactivated lipoxygenase and lipase which are thermolabile enzymes. Walnut lipoxygenase activity has been observed to be lowered after heating to 55°C whereas lipase enzymes in rapeseed have been observed to be inactivated at 100°C (Ponne et al., 1996; Buranasompob et al., 2007). Since the samples in the hexanal experiment were not oven dried, these enzymes may have also had a greater effect than they would have had otherwise. Because lipase interacts with water and triacylglycerides to form free fatty acids, the role of lipase in walnut hexanal accumulation would also explain the positive correlation that moisture content has on HD (Bustamante and Mitcham, 2023). The lack of any association between HD and Rancimat stability measurements was unexpected and may be the result of the complex nature of how rancidity develops or due to the activity of lipoxygenase and lipase.

Additionally, the roles lipoxygenase and lipase may play in air-dried walnut kernel hexanal accumulation should be investigated. HD was not significantly associated with any tocopherol, however, among the 15 samples there were modest negative correlations with α-tocopherol (P=0.33, R^2=0.078), δ-tocopherol (P=0.37, R^2=0.066) and total tocopherol content (P=0.16, R^2=0.16). These tocopherol correlations are more pronounced than the correlations they have with induction time. Because only 15 pairs of samples were used in this experiment, increasing sample size may resolve the degree which, if any, tocopherol is involved with reducing hexanal accumulation during storage. If the high temperature of the Rancimat reduces tocopherol activity in walnut as has been described in a past study using pork lard mixed with tocopherols, this low-temperature, heat-accelerated experiment could serve as an improved method of measuring walnut oxidative stability that does not discount tocopherol's effect on kernel oxidative stability (Réblová, 2006).

Unexpectedly, HD was negatively correlated with gallic acid and positively correlated with p-coumaric acid. This is despite both compounds having antioxidant properties (Boo, 2019; Kahkeshani et al., 2019). If p-coumaric acid is involved in influencing hexanal accumulation, this may be through indirect means as it has the lowest presence of the eight most prevalent phenols in walnuts. Conversely, gallic acid is the second most prevalent phenol measured in this study. Its influence on HD is more likely to be direct than p-coumaric acid. Increasing sample size and increasing the time

spent in storage may be needed to determine the extent and nature of individual compounds on HD and test the viability of this alternative method of studying walnut Rancidity.

Influence of pellicle phenolics on induction time

Induction time differences between the pellicle removal and control treatments for 13 samples were not significantly(P<0.05) associated with any of the eight most prevalent phenols in walnut. Walnut phenols are concentrated within the pellicle, and as a result, the pellicle removal treatment was expected to either reduce or have no effect on induction time relative to the control. Because induction times in pellicle removal treatment were slightly yet consistently higher than the control, either the phenols or some other compounds in the pellicle contributed to the increase in lipid oxidation or the compounds in the pellicle increased the Rancimat's conductivity measurements through other means. A potential mechanism for the latter explanation could be volatilization of water-soluble pellicle compounds that increase conductivity in the Rancimat's measuring vessels independent of fatty acid oxidation. To get a better understanding of the pellicle phenolics' impact on oxidative stability, a study examining induction time differences of walnuts with and without pellicle stored at ambient temperature for an extended period of time before Rancimat evaluation could be utilized to factor in the pellicle's oxygen barrier effect.

Figures

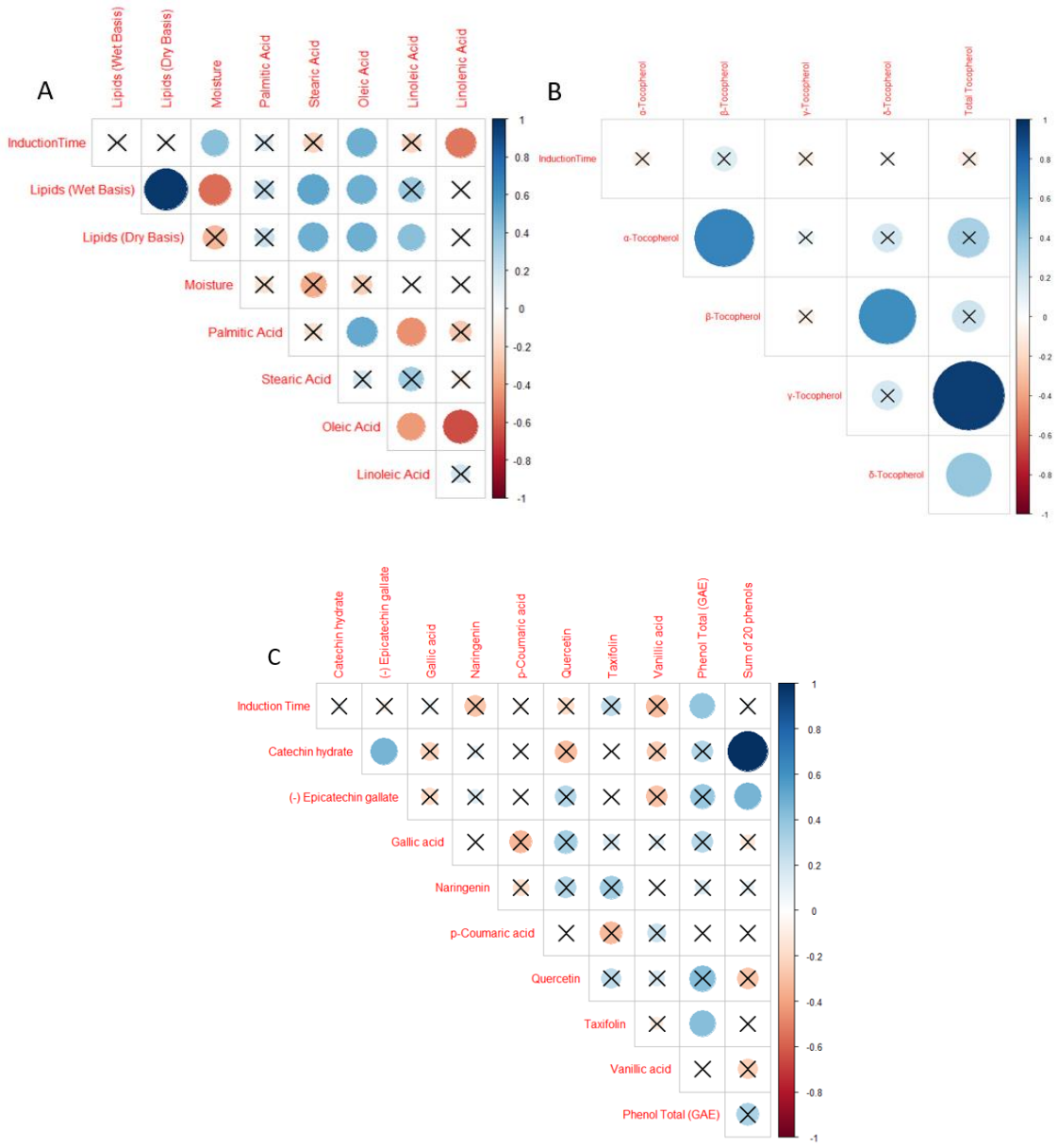

Figure 2.1: Correlation plots between induction time and lipids (A), tocopherols (B), and phenols (C). The color scale shows R^2 values, and insignificant associations (P>0.05) are crossed out.

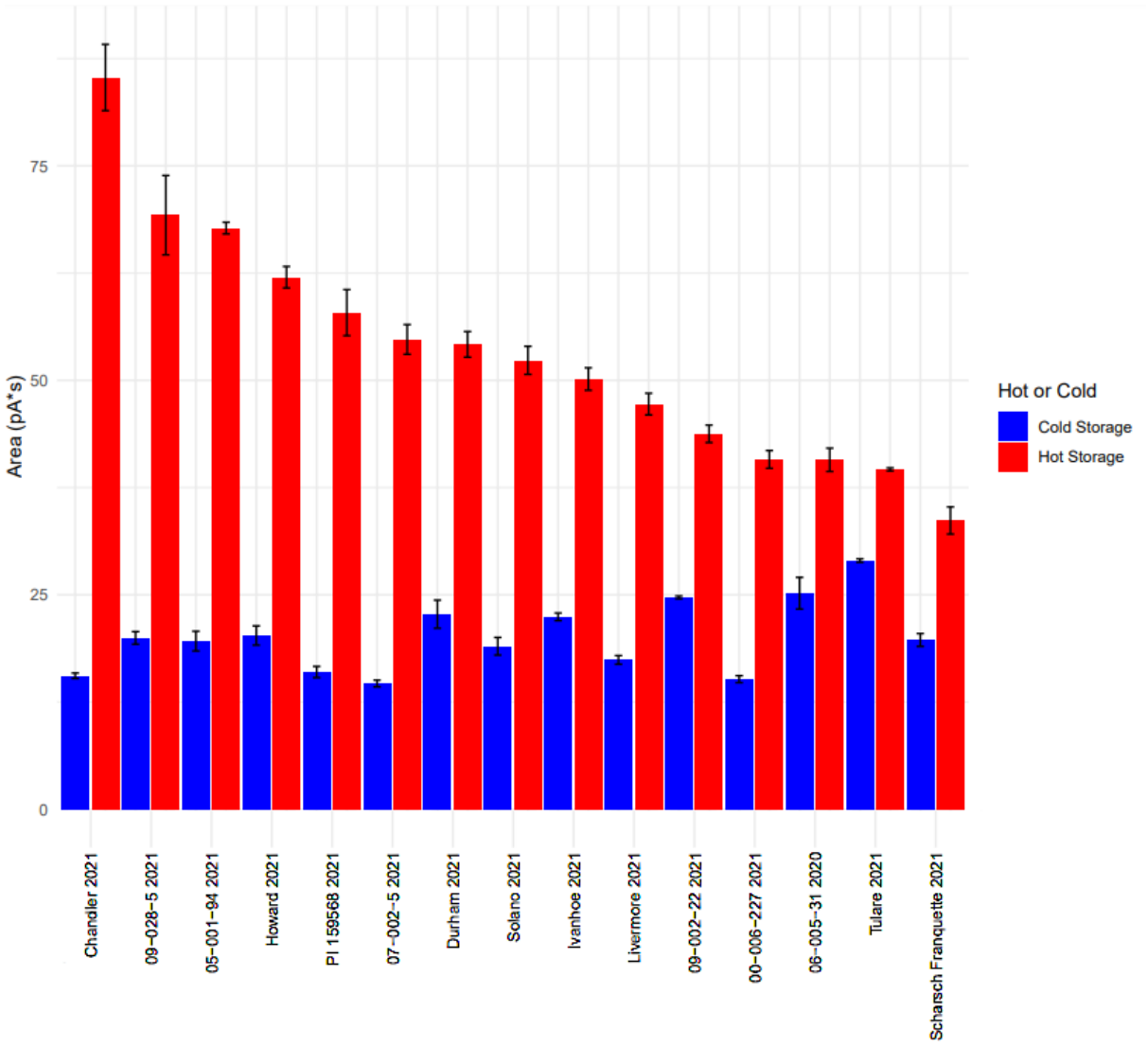

Figure 2.2: Raw hexanal data for each sample in both hot and cold storage conditions. Results are measured in peak area of gas chromatograph (pA*s). Samples are ordered from highest to lowest hot storage value.

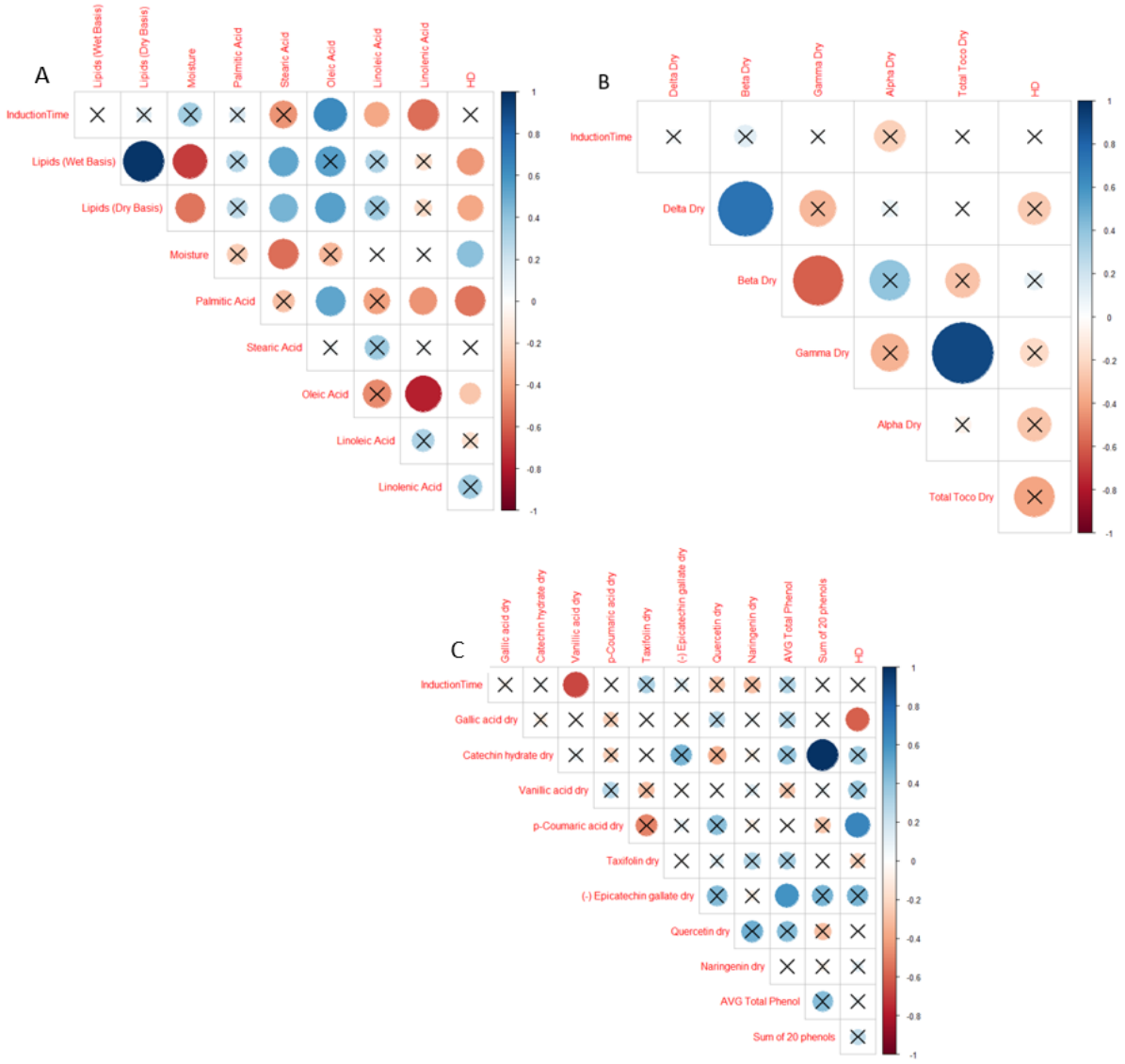

Figure 2.3: Correlations between the hexanal difference between hot and cold treatments (HD) and induction time with lipids (A), tocopherols (B), and phenolics (C). The color scale shows R^2 values and insignificant associations (P>0.05) are crossed out.

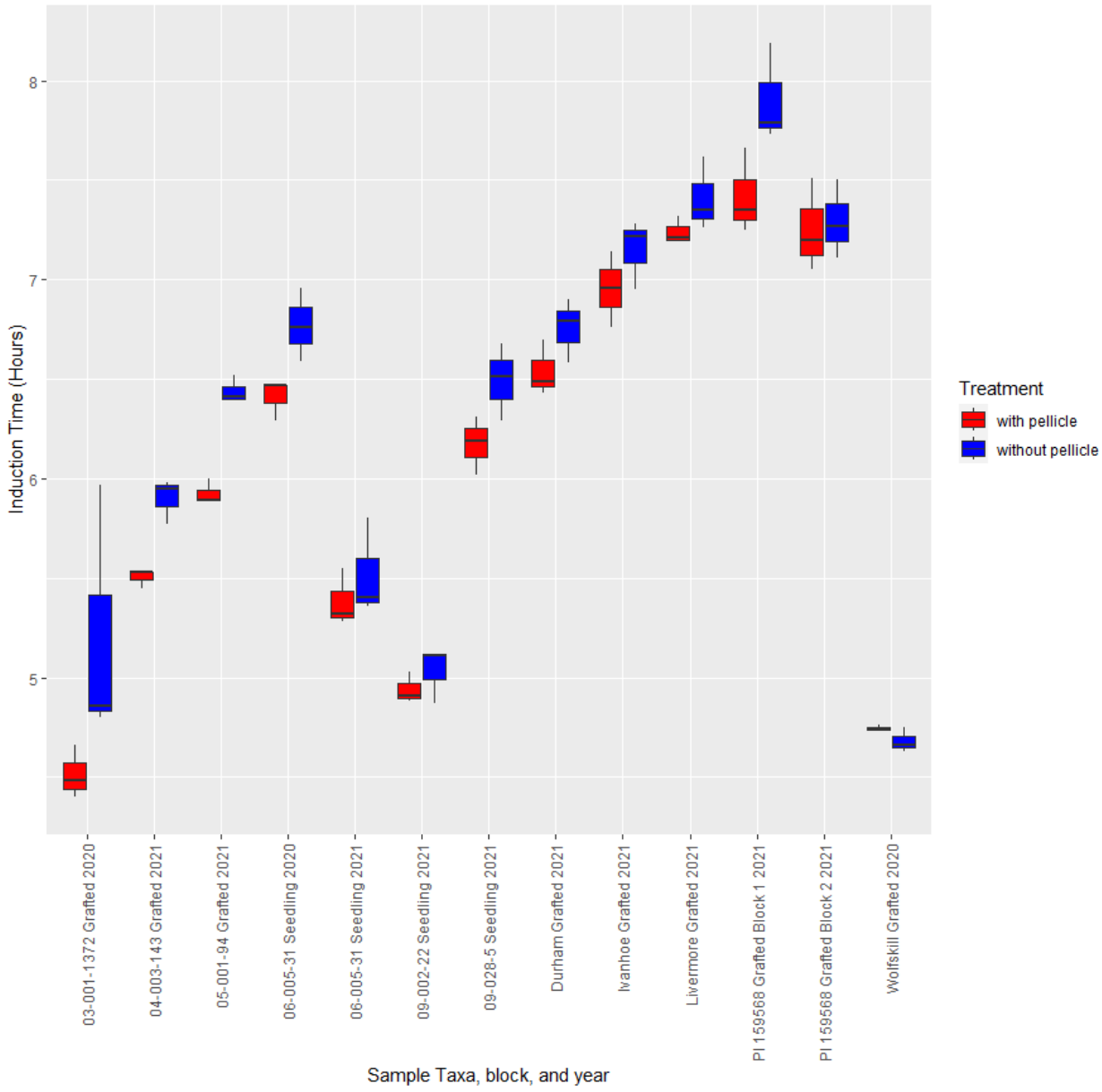

Figure 2.4: Effects of pellicle removal on induction time for 13 walnut samples representing 11 taxa. Each value is the mean of three technical replicates.

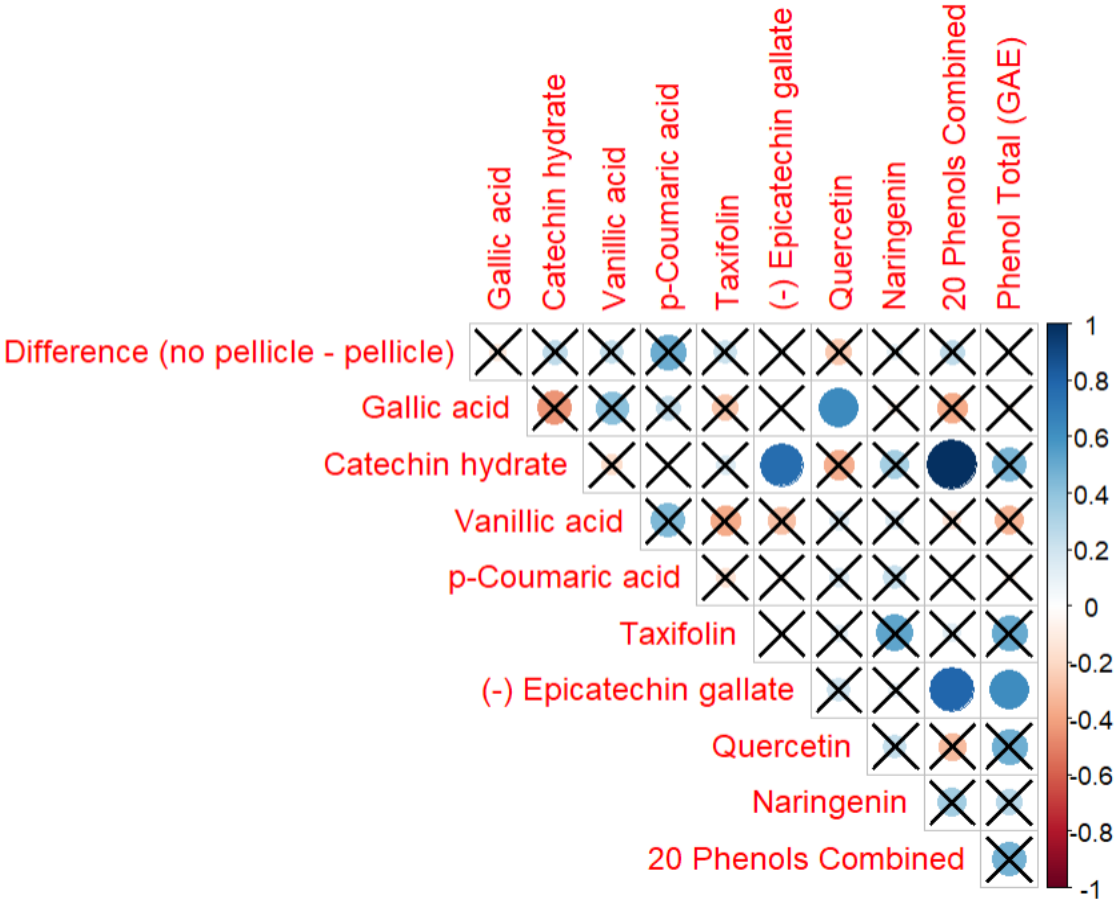

Figure 2.5: Correlations for the induction time difference between the removed pellicle treatment and the control for each sample, eight phenols, sum of phenols measured by LC-MS/MS, and total phenol estimate as gallic acid equivalents (GAE) by Folin-Ciocalteu method are illustrated. Insignificant (P>0.05) associations are crossed out.

Tables

Table 2.1: Average lipid, moisture, and fatty acid content for English walnut and backcross walnuts.

Average Moisture Content and Fatty Acid Content % (Dry Based) of English and Backcross Walnuts

Taxa	Seedling or Grafted	Species	Year Harvested	Lipid Content (Wet Based)	Lipid Content (Dry Based)	Moisture	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	Linolenic Acid
PI 159568	Grafted, Block 1	*J. regia*	2021	65.13	67.7	3.78	4.81	1.98	14.33	38.99	7.37
PI 159568	Grafted, Block 2	*J. regia*	2021	66.68	70.55	5.49	4.58	2.13	14.00	41.35	8.25
06-005-31	Seedling	*J. regia*	2021	64.85	68.56	5.42	4.98	1.86	14.46	40.76	6.29
05-001-94	Grafted	*J. regia*	2021	68.85	72.73	5.33	4.72	2.30	11.51	44.32	9.66
Durham	Grafted	*J. regia*	2021	68.09	70.79	3.82	4.51	2.29	13.84	40.92	9.02
00-006-227	Grafted	*J. regia*	2021	67.33	71.12	5.33	5.5	1.71	11.18	42.08	10.41
R. Livermore	Grafted	*J. regia*	2021	63.95	67.93	5.86	5.22	1.73	10.95	42.01	7.77
Ivanhoe	Grafted	*J. regia*	2021	66.72	70.26	5.03	5.03	1.93	11.78	42.53	8.75
09-028-5	Seedling	backcross	2021	66.67	70.09	4.88	5.43	1.89	17.60	37.41	7.52
Solano	Grafted	*J. regia*	2021	67.38	70.36	4.24	5.22	1.81	11.35	40.78	10.96
Tulare	Grafted	*J. regia*	2021	69.9	73.02	4.27	4.89	2.10	11.54	44.34	9.91
06-005-31	Grafted	*J. regia*	2021	66.61	68.95	3.39	5.02	2.04	13.30	41.44	6.93
04-003-143	Grafted	*J. regia*	2021	68.39	71.14	3.86	4.33	1.78	11.43	43.18	10.19
07-002-5	Grafted	*J. regia*	2021	66.04	69.25	4.64	5.23	1.73	12.93	40.23	8.91
07-002-5	Seedling	*J. regia*	2021	64.38	67.17	4.16	5.11	1.48	11.90	39.08	9.38
03-001-977	Grafted	*J. regia*	2021	67.78	70.64	4.04	4.26	1.88	11.47	45.11	7.69
09-002-22	Seedling	*J. regia*	2021	66.46	69.43	4.28	4.76	2.02	11.38	43.15	7.90
Wolfskill	Grafted	*J. regia*	2020	65.02	67.49	3.65	4.93	1.75	9.25	41.04	10.31

03-001-1372	Grafted	*J. regia*	2020	66.38	69.73	4.81	4.96	1.78	10.98	41.82	9.96
03-001-2440	Grafted	*J. regia*	2020	68.17	71.26	4.33	4.31	2.09	11.25	44.78	8.59
06-005-31	Seedling	*J. regia*	2020	72.45	75.78	4.39	5.23	1.85	18.85	42.52	7.06
Franquette	Grafted	*J. regia*	2021	64.75	69.06	6.24	5.40	1.49	15.40	39.97	6.57
03-001-1938	Grafted	*J. regia*	2021	61.5	65.55	6.18	4.17	1.65	9.41	40.99	9.13
91-090-41	Grafted	*J. regia*	2021	62.95	66.68	5.59	4.46	1.81	12.15	40.75	7.32
Chandler	Grafted	*J. regia*	2021	64.12	68.52	6.42	3.88	1.71	9.71	42.89	10.13
Howard	Grafted	*J. regia*	2021	63.29	67.36	6.03	4.20	1.70	7.87	41.61	11.78
Vina	Grafted	*J. regia*	2021	64.23	68	5.55	4.40	1.70	9.54	43.50	8.67
Hartley	Grafted	*J. regia*	2021	64.07	67.95	5.71	4.97	1.96	9.73	43.18	7.92
			AVERAGE	66.15	69.54	4.88	4.80	1.86	12.11	41.81	8.73
			SD	2.33	2.17	0.88	0.43	0.21	2.46	1.82	1.41

Table 2.2: Average lipid, moisture, and fatty acid content for undomesticated walnut species.

Moisture Content and Fatty Acid Content % (Dry Based) of Undomesticated *Juglans* Species											
Taxa	Seedling or Grafted	Species	Year Harvested	Lipid Content (Wet Based)	Lipid Content (Dry Based)	Moisture	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	Linolenic Acid
Hindsii											
W17	Grafted	*J. hindsii*	2021	55.73	59.57	6.44	4.87	1.31	16.18	30.92	6.08
Rawlins	Grafted	*J. hindsii*	2021	56.33	60.16	6.36	4.87	1.44	15.54	31.18	6.93
Leonard											
James	Grafted	*J. hindsii*	2021	58.25	62.95	7.47	5.20	1.34	16.71	32.95	6.54
Hagus #8	Grafted	*J. hindsii*	2021	57.8	61.28	5.67	5.07	1.21	12.64	32.39	9.75
Burbank		*J. nigra* x *J.*									
Hybrid	Grafted	*hindsii*	2021	58.56	62.26	5.94	3.72	1.64	19.92	31.48	5.29
HQ		*J.*									
Californica	Grafted	*californica*	2021	61.05	63.97	4.56	4.96	1.44	13.43	36.48	7.41
			Average	57.95	61.70	6.07	4.78	1.40	15.73	32.57	7.00
			SD	1.88	1.68	0.96	0.53	0.15	2.59	2.06	1.53

Table 2.3: Average tocopherol content data for English and backcross walnut species.

Average Tocopherol Content (μg/g Kernel, Dry Based)

Taxa	Seedling or Grafted	Species	Year Harvested	Delta	Beta	Gamma	Alpha	Total Tocopherol
PI 159568	Grafted, Block 1	*J. regia*	2021	16.96	0	242.72	5.71	265.38
PI 159568	Grafted, Block 2	*J. regia*	2021	21.68	0	274.59	6.64	302.92
06-005-31	Seedling	*J. regia*	2021	16.61	0.26	269.44	15.41	301.71
05-001-94	Grafted	*J. regia*	2021	22.68	0.4	245.16	14.58	282.83
Durham	Grafted	*J. regia*	2021	19.78	0.07	293.73	9.73	323.32
00-006-227	Grafted	*J. regia*	2021	20.93	0.27	270.12	10.38	301.7
R. Livermore	Grafted	*J. regia*	2021	16.96	0.18	261	17.73	295.87
Ivanhoe	Grafted	*J. regia*	2021	16.49	0.08	294.73	14.33	325.64
09-028-5	Seedling	Backcross	2021	29.88	0.74	250.67	10.29	291.58
Solano	Grafted	*J. regia*	2021	14.8	0	267.43	9.78	292.02
Tulare	Grafted	*J. regia*	2021	25.61	0.14	265.48	9.56	300.79
06-005-31	Grafted	*J. regia*	2021	16.65	0.35	251.11	17.18	285.29
04-003-143	Grafted	*J. regia*	2021	21.19	0.36	217.74	19.66	258.94
07-002-5	Grafted	*J. regia*	2021	17.45	0.22	235.52	10.53	263.73
07-002-5	Seedling	*J. regia*	2021	19.97	0	260.4	7.75	288.11
03-001-977	Grafted	*J. regia*	2021	20.61	0.49	285.46	23.53	330.09
09-002-22	Seedling	*J. regia*	2021	29.19	0.36	238.95	11.24	279.74
Wolfskill	Grafted	*J. regia*	2020	16.82	0	262.34	4.24	283.4
03-001-1372	Grafted	*J. regia*	2020	23.99	0.06	288.47	5.42	317.95
03-001-2440	Grafted	*J. regia*	2020	15.74	0.07	281.06	10.35	307.21
06-005-31	Seedling	*J. regia*	2020	16.11	0.09	270.3	15.03	301.52
Franquette	Grafted	*J. regia*	2021	22.24	0.08	277.92	5.09	305.33
03-001-1938	Grafted	*J. regia*	2021	11.42	0	236.16	6.92	254.49
91-090-41	Grafted	*J. regia*	2021	12.9	0	201.21	3.48	217.59
Chandler	Grafted	*J. regia*	2021	14.22	0	263.9	4.42	282.53
Howard	Grafted	*J. regia*	2021	16.79	0	272.43	3.22	292.44
Vina	Grafted	*J. regia*	2021	13.78	0	240.14	6.93	260.85
Hartley	Grafted	*J. regia*	2021	17.96	0	231.92	4.73	254.61
			Average	18.91	0.15	258.93	10.14	288.13
			SD	4.53	0.19	22.79	5.317	25.16

Table 2.4: Average tocopherol content data for undomesticated walnut species.

Average Tocopherol Content in Undomesticated *Juglans* (μg/g Kernel, Dry Based)

Taxa	Seedling or Grafted	Species	Year Harvested	Delta	Beta	Gamma	Alpha	Total Tocopherol
Hindsii W17	Grafted	*J. hindsii*	2021	18.88	0	324.38	1.51	344.77
Rawlins	Grafted	*J. hindsii*	2021	29.94	0	310.7	1.98	342.62
Leonard James	Grafted	*J. hindsii*	2021	21.88	0	282.35	0.49	304.72
Hagus #8	Grafted	*J. hindsii*	2021	9.14	0	311.06	0.22	320.41
Burbank Hybrid	Grafted	*J. nigra* x *J. hindsii*	2021	5.88	0	299.83	0.11	305.83
HQ Californica	Grafted	*J. californica*	2021	21.47	0	488.48	0.91	510.86
			Average	17.87	0	336.13	0.87	354.87
			SD	8.90	0	75.94	0.75	78.35

Table 2.5: Average phenol content data for English and backcross walnut species including total phenol estimate by the Folin-Ciocalteu method.

Average Phenol Content in English Walnut (μg/g Kernel, Dry Based)

Taxa	Grafted	Species	Year Harvested	Gallic acid	Catechin hydrate	Vanillic acid	p-Coumaric acid	Taxifolin	(-)-Epicatechin gallate	Quercetin	Naringenin	Total Phenols
PI 159568	Grafted, Block 1	*J. regia*	2021	18.05	168.89	0.12	0.02	0.22	2.63	0.11	0.22	22.39
PI 159568	Grafted, Block 2	*J. regia*	2021	15.49	85.35	0.13	0.02	0.18	1.42	0.12	0.21	18.73
06-005-31	Seedling	*J. regia*	2021	19.25	35.6	0.18	0.01	0.06	1.34	NA	0.03	16.15
05-001-94	Grafted	*J. regia*	2021	14.38	120.09	0.17	0.01	0.16	2.09	0.1	0.07	16.17
Durham	Grafted	*J. regia*	2021	26.5	109.82	0.15	0.02	0.13	2.78	0.12	0.05	19.92
00-006-227	Grafted	*J. regia*	2021	18.13	87.08	0.2	0.02	0.19	1.33	0.11	0.03	19.48
R. Livermore	Grafted	*J. regia*	2021	26.48	65.04	0.14	0.03	0.15	3.1	0.16	0.12	25.49
Ivanhoe	Grafted	*J. regia*	2021	25.97	91.18	0.14	0.02	0.07	2.43	0.11	0.04	16.47
09-028-5	Seedling	Backcross	2021	11.51	188.47	0.07	0.01	0.17	3.26	0.11	0.03	26.55
Solano	Grafted	*J. regia*	2021	24.13	105	0.18	0.03	0.07	1.87	0.1	0.07	18.67
Tulare	Grafted	*J. regia*	2021	24.98	82.69	0.11	0.02	0.06	1.05	0.11	0.04	15.72
06-005-31	Grafted	*J. regia*	2021	16.54	102.36	0.11	0.01	0.14	1.2	NA	0.03	18.31
04-003-143	Grafted	*J. regia*	2021	11.99	253.18	0.12	0.03	0.09	3.63	0.1	0.12	20.43
07-002-5	Grafted	*J. regia*	2021	20.5	118.41	0.15	0.03	0.08	2.57	NA	0.03	24.06
07-002-5	Seedling	*J. regia*	2021	22.38	101.82	0.09	0.02	0.1	1.79	0.1	0.06	15.92
03-001-977	Grafted	*J. regia*	2021	11.89	277.8	0.06	0.08	0.1	2.06	0.1	0.03	17.94
09-002-22	Seedling	*J. regia*	2021	13.82	56.65	0.09	0.01	0.16	1.57	0.1	0.03	19.59
Wolfskill	Grafted	*J. regia*	2020	17.24	98.61	0.11	0.02	0.1	2.51	0.11	0.06	15.27
03-001-1372	Grafted	*J. regia*	2020	20.13	58.93	0.18	0.07	0.12	1.45	0.11	0.08	16.49
03-001-2440	Grafted	*J. regia*	2020	11.46	98	0.11	0.04	0.07	2.51	NA	0.04	15.91
06-005-31	Seedling	*J. regia*	2020	17.98	25.49	0.06	0.01	0.15	1.92	NA	0.02	16.41

Franquette	Grafted	*J. regia*	2021	33.8	114.54	0.08	0.01	0.19	1.82	0.1	0.05	25.32
03-001-1938	Grafted	*J. regia*	2021	19.01	193.02	0.1	0.04	0.05	2.45	NA	0.04	20.3
91-090-41	Grafted	*J. regia*	2021	13.58	71.04	0.13	0.02	0.06	1.46	0.1	0.14	17.73
Chandler	Grafted	*J. regia*	2021	12.91	60.92	0.15	0.14	0.07	2.25	NA	0.03	19.36
Howard	Grafted	*J. regia*	2021	13.31	145.22	0.16	0.03	0.08	2.5	0.1	0.03	16.84
Vina	Grafted	*J. regia*	2021	12.48	86.42	0.19	0.13	0.11	1.52	NA	0.03	21.3
			Average	18.55	113.12	0.13	0.03	0.12	2.06	0.11	0.06	19.27
			SD	5.84	59.86	0.04	0.03	0.05	0.69	0.01	0.05	3.25

Table 2.6: Average phenol content data for undomesticated walnut species including the total phenol estimate by the Colin-Ciocalteu method.

Average Phenol Content in Undomesticated Juglans Species (µg/g Kernel, Dry Based)

Taxa	Seedling or Grafted	Species	Year Harvested	Gallic acid	3,4-dihydroxybenzoic acid	Catechin hydrate	Vanillic acid	(-)-Epicatechin	p-Coumaric acid	Taxifolin	(-)-Epicatechin gallate	Quercetin	Naringenin	Total Phenol
Hindsii W17	Grafted	*J. hindsii*	2021	31.34	0.04	1.54	0.52	0.2	0.02	0.32	4.37	0.13	0.03	6.86
Rawlins	Grafted	*J. hindsii*	2021	23.98	0.04	1.49	0.22	NA	0.03	0.62	1.69	0.16	0.13	4.46
Leonard James	Grafted	*J. hindsii*	2021	4.76	0.04	NA	0.59	0.26	0.01	0.1	0.43	0.16	0.06	1.95
Hagus #8	Grafted	*J. hindsii*	2021	22.76	0.04	NA	0.22	0.19	0.01	0.53	1.51	0.17	0.38	4.3
Burbank Hybrid	Grafted	*J. nigra* x *J. hindsii*	2021	17.53	0.04	NA	0.25	0.15	0.01	0.1	0.6	0.11	0.08	5.15
HQ Californica	Grafted	*J. californica*	2021	3.95	0.12	NA	0.9	0.47	0.02	0.05	0.23	0.12	0.01	1.18
			Average *Hindsii*	20.71	0.04	1.52	0.39	0.22	0.02	0.39	2.00	0.16	0.15	4.39
			SD *Hindsii*	11.29	0.00	0.04	0.20	0.04	0.01	0.23	1.68	0.02	0.16	2.01
			Average	17.39	0.05	1.52	0.45	0.25	0.02	0.29	1.47	0.14	0.12	3.98
			SD	11.02	0.03	0.04	0.27	0.13	0.01	0.24	1.54	0.02	0.14	2.10

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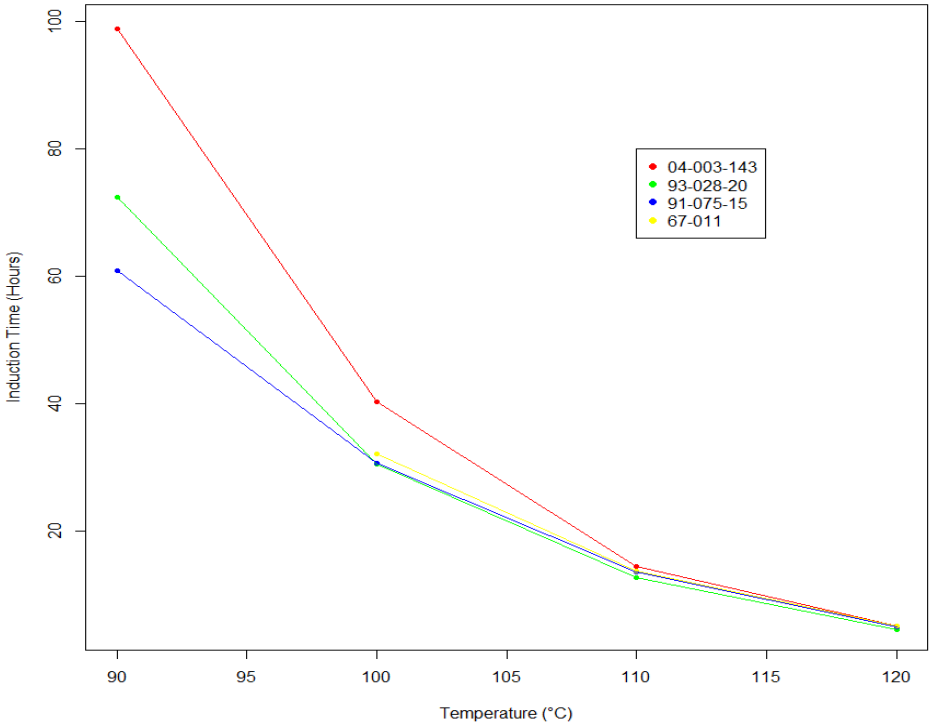
Appendix 1



Walnut samples from four different accessions were processed and submitted to the Rancimat as described in Chapter 1, however, samples were either heated to 90, 100, 110, or 120°C. Each accession was measured once at each temperature except for 67-011 (Tulare) which had no measurement for the 90°C treatment. Of the three taxa that were subjected to the 90°C treatment, the induction time for 04-003-143 was 26.5 hours higher than the induction time for 93-028-20 which was 11.49 hours higher than for 91-075-15. This large disparity began to vanish in the lower temperature treatments. Among the samples within the 120°C treatment there was only a 0.57-hour induction time difference between the highest and lowest taxa. There seems to be some degree of

temperature-taxa interaction as different samples decrease at different rates. The data suggests that for increased oxidative stability measurement resolution, lower temperatures could be used. A potential issue with lower temperatures is that water in the measuring vessels evaporates significantly over the course of a few days. It is unknown to what extent this affects the Rancimat's accuracy and reproducibility.

Appendix 2

Compositional Analysis

Phenols were analyzed through liquid chromatography with tandem mass spectrometry (LC- MS/MS) using an Agilent LC 1260 Infinity II (Agilent Technologies, Santa Clara, CA, USA) coupled to an Aglient 6470 triple quadrupole mass spectrometer with an Agilent Jet Stream electrospray ionization as described by Shen et al. with modifications (Shen et al., 2021). For each sample, three grams of ground walnuts were weighed and placed in a 50 mL falcon tube. Twenty-five mL of hexane was added to the ground nuts and the tube was vortexed for five seconds followed by sonication for five minutes using a Raytech Turbo Sonic 6000 (Raytech, Phoenix, AZ, USA). The sample was then centrifuged at 2000 g for five minutes with the Sorvall Legend X1 Centrifuge (Thermo Scientific, Waltham, MA, USA). The resulting supernatant was discarded leaving behind a pellet. Then 15 mL of hexane was added to the pellet, and the sample was vortexed, sonicated, and centrifuged in the same manner as before. The

supernatant was then discarded and 30 mL of an acetone-water solution (7:3, v/v) was added to the pellet and vortexed for two minutes followed by 30 seconds of sonication. The mixture was then centrifuged at 10,000 g for five minutes and the supernatant was collected. A final 20 mL addition of the acetone-water solution was added to the pellet and the same vortexing, sonicating, and centrifuging procedure was repeated. The supernatant was collected and combined with the previous supernatant.

For phenols of high abundance (gallic acid and catechin hydrate), 1.5 mL of the supernatant was collected for injection into an LC-MS/MS system. For LC-MS/MS analysis of other phenols, the solution was prepared by evaporating the remaining supernatant with a Rotavapor R-300 (Büchi Corporation, Flawil, Sankt Gallen, CHE) set to heat at 40°C. Afterwards, a 7:3 acetone:water solution was added to the supernatant so that the total volume was 1.5 mL. This solution was then filtered through a 0.45 μm PTFE filter before 5 μL were injected into an LC-MS/MS system. A Poroshell 120 EC-C18 column (100 x 2.1 mm, 1.9 μm) heated at 40°C was used to separate the sample. Each sample was measured in triplicate.

Measurement of total phenol content was performed using the Folin-Ciocalteu method. For each sample, 0.1 mL of supernatant was saved for spectrophotometer analysis. The supernatant was then mixed with five mL of water, 0.5 mL of Folin-Ciocalteu reagent, and 3 mL of a 17.5% Na_2CO_3 aqueous solution. DI water was then

added until the total volume of the solution was 10 mL. The solution was incubated for 1 hour in darkness before measuring the absorbance of the solution at 725 nm with a Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Using solutions of gallic acid in a 7:3 acetone:water solution, a concentration curve was prepared using 0.05, 0.1, 0.5, 1, 1.5, 2, and 2.5 mg/L solutions. The concentration curve was then used to calculate total phenol content and the resulting data was reported as gallic acid equivalents.

For oil extraction, walnuts were sprinkled with water and stored in a vacuum bag held at 5°C to reach 3% (w/w) moisture content. Oil pressing was conducted using a KK Oil Prince F Universal oil press (Oil press GmbH & Co. KG, Reut, Bavaria, DEU) with an electrical resistance-heating ring attached to the press barrel set to heat at 27°C. The machine was run for 10 minutes to reach extraction temperature before feeding it kernels.

Tocopherol analysis was performed following methods by Górnaś with modifications (Górnaś, 2015). For each replicate, about 0.2 gram of walnut oil was put into a centrifuge tube followed by 0.9 mL of isopropanol and 0.1 mL of alpha-tocopherol acetate as an internal standard. The mixture was vortexed for 3 minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and filtered with a 0.45 μm nylon filter before measurement using high-performance liquid

chromatography with diode-array detection (HPLC-DAD) with a reverse phase pentafluorophenyl column (3 μm, 150 mm x 4.6 mm), an isocratic elution with a methanol:water (93:7, v/v) mobile phase, and a flow rate of 1 mL per minute. The DAD signal was recorded at 290 nm. Each sample was measured in triplicate.

For fatty acid profiling, the fatty acids were converted to their methyl ester derivatives for gas chromatography/mass spectrometry following a protocol developed for walnut lipid profiling (Tapia et al., 2013). After oil extraction, 0.1 gram of each oil sample was mixed with 1 mL of n-hexane for 10 seconds. This was followed by the addition of 0.1 mL of a 2 N KOH-methanol solution and was vortexed for 2 minutes. Afterwards, the solution turned clear and 500 μL of the top layer containing the fatty acid methyl esters was decanted. This liquid was then diluted with n-hexane to a total volume of 1 mL.

Samples were analyzed with a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) within 12 hours of preparation. The gas chromatograph had a flame ionization detector and a split injector (30:1 split ratio) which were held at 260°C and 250°C, respectively. The ZB-23 capillary column (20 m, 180 μm, 0.2 μm) was set at 80°C for 30 seconds, raised to 175°C at a rate of 65°C per minute, and then to 230°C at a rate of 7°C per minute with each stage being held at constant temperature for 0, 0.5, and 5 minutes, respectively. For each replicate, 1 μL was injected into the gas

chromatograph. A 37 Component fatty acid methyl esters standard (Supelco, Bellefonte, PA, USA) was used to assist FAME identification and quantification of walnut fatty acids was performed using Agilent open Lab ChemStation. Each sample was measured in triplicate.

For each moisture content replicate, three grams of walnut kernels were ground with a Cuisinart Mini-Prep Plus Food Processor model DLC-2A (East Windsor, NJ, USA). Afterwards, the ground kernels were placed in a 600 mL beaker and oven dried at 105°C until weight loss was no longer measurable. The samples were then placed in a desiccator and left to cool to room temperature. The samples were then weighed to determine how much water was lost. Each sample was analyzed in triplicate.