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Exploration of Genes Controlling Grain Yield Heterosis in Hybrid Wheat (*Triticum aestivum* L.) Utilizing 3′ RNA Sequencing

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EXPLORATION OF GENES CONTROLLING GRAIN YIELD HETEROSIS IN HYBRID WHEAT (*Triticum aestivum* L.) UTILIZING 3' RNA SEQUENCING

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EXPLORATION OF GENES CONTROLLING GRAIN YIELD HETEROSIS IN HYBRID WHEAT (*Triticum aestivum* L.) UTILIZING 3' RNA SEQUENCING

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The implementation and future success of hybrid wheat (*Triticum aestivum* L.) is impacted by breeders' inability to create consistent high yielding, high heterosis hybrids. This research addresses this problem by conducting an exploration of transcriptomes from hybrids and parent lines to determine what genes are active in heterotic or non-heterotic hybrids and how their level of expression can explain the phenotype of grain yield heterosis. Using hybrids that showed positive mid-parent heterosis (MPH), classified as heterotic in our study, and negative or no difference MPH hybrids, classified as non-heterotic, differentially expressed genes (DEGs) potentially related to heterosis and hybrid yield response can be identified. Differential gene expression analysis found that more genes are differentially expressed in the non-heterotic hybrid to parent comparisons than in the heterotic hybrid to parent comparisons. Another important aspect of conducting a transcriptome study is adequately preserving the RNA for extraction and sequencing. Previous work has used liquid nitrogen to preserve samples taken out in the field, but this is dangerous and cumbersome. RNA*later*® has been used as an alternative to liquid nitrogen but is not as consistent at preservation compared to liquid nitrogen. Another study to investigate this problem was conducted by sampling leaf and immature kernels from wheat, storing the samples at two temperatures for up to six months, extracting the RNA, and testing the quality parameters associated with using RNA for

sequencing. The results showed that the lower storage temperature had a negative impact on the parameters while storage time only negatively affected the purity. Both studies can be applied to research conducted on the transcriptome of wheat and allow for differences to be detected to explain heterosis.

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CHAPTER 1: EXPLORATION OF GENES CONTROLLING GRAIN YIELD HETEROSIS IN HYBRID WHEAT (*Triticum aestivum* L.) UTILIZING 3' RNA SEQUENCING

Abbreviations: HRWW: hard red winter wheat; MPH: mid-parent heterosis; CHA: chemical hybridizing agent; DAF: days after flowering; DEG: differentially expressed genes

1. ABSTRACT

Pure-line wheat cultivar development has not kept pace with increasing consumer demand. Hybrid wheat could meet the growing demand due to higher yields, but more research is needed to allow breeders to take advantage of the heterosis (hybrid vigor) seen in other hybrid crops. The goal of this research was to conduct a comprehensive transcriptomic study on hybrids and their corresponding parents to identify mechanisms that impact heterosis through differentially expressed genes (DEGs). Immature kernel and flag leaf tissue from ten triads (a hybrid and its parents) were sampled from two years. Five triads were identified as heterotic with positive mid-parent heterosis (MPH) and five were identified as non-heterotic with negative MPH. Biological replicates were sequenced using 3' RNA-Seq, transcript sequences were mapped to the IWGSC RefSeq 2.0 reference genome, and DEG identification was done using *DESeq2*. The results found that gene expression was related to the year it was sampled as well as what tissue was sampled. However, we identified an average of 13 DEGs in the heterotic/non-heterotic hybrid comparisons of both leaf and seed tissue. We also found that more genes were differentially expressed in the non-heterotic hybrids when compared to the heterotic

hybrids. 600 and 402 genes were found to be differentially expressed across the ten hybrids for the seed and leaf tissues respectively. The results suggest that there are unique genes expressed in specific hybrids that could explain the differences in heterosis. Therefore, researchers should identify what role these genes have in the phenotypic response to heterosis.

2. INTRODUCTION

Bread wheat, *Triticum aestivum* L., is an important cereal crop in the United States for both human consumption and producers' income. In 2020, U.S. bread wheat production at 49,690,680 tons (Food and Agriculture Organization of the United Nations, 2020) was the third largest grain crop behind maize (*Zea mays* subsp. *mays* L.) and soybeans (*Glycine max* L. Merrill). Hard red winter wheat (HRWW) grown in the Great Plains region accounts for 40% of the United States wheat production and is used to make bread flour, a staple in human diets (*USDA ERS - Wheat Sector at a Glance*, n.d.). Graybosch & Peterson (2012) found that, between 1987 and 2010, the genetic potential for grain yield for wheat varieties increased at a rate of ~1% every year in the Great Plains region. During that time, demand for HRWW had grown by 1.3% annually and has continued to grow by 1.4% in 2021 (USDA-ERS, 2021). While production has kept up with the current growth in demand, wheat acreage and overall production of wheat has decreased from the previous years (Food and Agriculture Organization of the United Nations, 2020). Decreasing wheat production trends are incompatible with increasing world population and caloric demands (Vespa et al., n.d.).

Yield genetic gain must increase for wheat production to increase to meet consumer demand. Yield could be improved more rapidly in hybrid wheat than in inbred cultivars

due to heterosis. Heterosis can be classified in three ways: (1) hybrid compared to the average of the parents, defined as mid-parent heterosis (MPH), (2) hybrid compared to the best parent, known as high parent heterosis, and (3) hybrid compared to the best commercial check, known as commercial heterosis (Bernardo, 2010). These estimates can be classified as either positive heterosis, > 0%, no change, heterosis = 0, or negative heterosis, < 0% based on the calculated performance. Previous estimates of grain yield increase due to heterosis in wheat ranged from 3.5 to 15% (Longin et al., 2012). In addition to heterosis, hybrid wheat benefits producers through yield stability, especially in marginal environments, with increased grain weight and tillering, improved biomass production and rooting depth, and increased biotic and abiotic stress tolerance (Longin et al., 2012; Tadesse et al., 2019; Tester & Langridge, 2010).

However, it is difficult to create wheat hybrids due to floral structure that generates inbreeding and challenges related to creating a cost-effective and easy to use hybrid fertility system (Whitford et al., 2013). Within a breeding program, a hybrid's heterosis expression could be decreased due to the genetic similarity between parental lines. Maize breeders observed that genetic similarity can decrease yield heterosis. The breeders found that by crossing two parents with optimized genetic differences, hybrids yielded more than hybrids with parents having less or more extreme genetic differences (Koekemoer et al., 2011; Melchinger, 2015). To increase hybrid wheat yield potential, the genetic mechanisms that impact heterosis should be investigated and divergent populations of inbred parents that maximize heterosis need to be created (Whitford et al., 2013).

The genetic differences that lead to heterosis could also lead to gene expression differences that help explain heterosis. Differentially expressed genes (DEGs) can be

categorized into different expression gene groups. The expression gene groups are defined as follows: over high parent, where there are more gene transcripts present in the hybrid compared to the parent with the highest expression level for that gene; high parent, where the gene expression of the hybrid is equal to the parent with the highest expression level of the specific gene; between parent, which is less than the high parent but more than the low parent; low parent, where a gene's expression level in the hybrid is equal to the parent with the lowest expression level; or under low parent, where the gene expression present in the hybrid is lower than the parent with the lowest expression level (Yang et al., 2018). Researchers found up to 82% of DEGs expressed in maize F_1 hybrids derived from crossing B73 and MO17, two maize lines that are known to produce hybrids with high positive heterosis, were classified as between parents. However, classification of expression differs across species and even within species (Stupar & Springer, 2006). For example, Yang et al., (2018) showed different gene action classifications in Brazilian rubber tree, (*Hevea brasiliensis* (Willd. Ex A. Juss.) Müll. Arg.) seedlings. Three rubber tree hybrids were created from two phenotypically distinct, heterozygous parents, and DEGs were identified and classified using the above gene expression groups. The researchers found that for the high performing, high heterosis hybrids, many DEGs were grouped as having expression over the high parent or under the low parent. In contrast, the low performing, low heterosis, hybrid showed minimal DEGs categorized into under the low parent or over high parent while 78% of the DEGs were classified as greater than the high parent or equal to the low parent's expression (Yang et al., 2018).

The gene expression groupings discussed in Yang et al., (2018) were used to introduce three common genetic models to explain the phenotypic response to heterosis

mechanisms on the allelic level: dominance, overdominance, and epistasis. The dominance model describes heterosis as the function of complementing action of marginally deleterious recessive alleles. An example of the allelic interaction would be when the gene contains the two dominant alleles, AA, the phenotypic response is equal to the heterozygote, Aa, but both produce a phenotype that is better than the recessive alleles, aa. The overdominance model explains how a favorable allelic interaction at a heterozygous locus is responsible for heterosis, for example the phenotypic performance is higher when the gene has heterozygotic loci Aa compared to AA or aa. Epistasis models describe heterosis resulting from gene-by-gene interactions at different loci. For example, if a hybrid contains the alleles A_B_ for two different loci, the presence of both dominant alleles produces a better phenotypic response than when there is only one dominant allele at one locus, A_bb (Birchler et al., 2006). In rubber trees, traits such as improved hybrid yield, seedling growth, and vigor, found in the high-yielding hybrids, are modeled by overdominance, while the phenotypic trait of decreased hybrid yield found in the low-yielding hybrids is explained by the dominance genetic model. The presence of heterozygote alleles at loci made the high heterosis hybrids perform better than hybrids with loci containing both dominant alleles. In both the high yielding and low yielding rubber tree hybrids, it was concluded that DEGs can be used to explain observed heterosis (Yang et al., 2018). It is apparent that gene action classification differences are present between and within species, so DEGs should be studied for each crop species.

Because gene expression can be used to explain heterosis mechanisms in a hybrid, RNA-Seq, a method for quantifying gene expression, can be used to explore the genetic basis of heterosis. RNA-Seq uses mRNAs extracted from tissues to create cDNAs that are

then sequenced, producing transcript information to identify DEGs that are used to explain the phenotypic differences between samples. The mRNA is sheared at random locations using enzymes, the fragments are converted to cDNA libraries which are then sequenced, and the total number of reads produced for a certain transcript is proportional to the expression level of the corresponding gene. However, the proportion can be biased towards longer transcripts. This bias can be partially corrected by knowing the size of the transcript though transcript size can be inaccurate in non-model (less characterized) species. 3' RNA-Seq was introduced to correct for the bias in standard RNA-Seq. 3' RNA-Seq only sequences the transcript fragment from the 3' end. Transcript size does not affect fragment sequencing. This method produces the number of transcripts directly related to the expression level of the gene (Tandonnet & Torres, 2017). Another study conducted by Lohman et al., (2016) compared RNA-Seq and 3' RNA-Seq and concluded that the main difference was the expense of the two methods but both methods identify similar DEGs. RNA-Seq is more expensive than 3' RNA-Seq due to sequencing the full lengths of the transcripts and requiring a high depth of coverage. ENCODE Consortium suggests the best practice for an RNA-Seq study is to have ~30 million raw reads per sample (The ENCODE Consortium, 2011), which limits the level of multiplexing. The high cost of creating cDNA libraries and sequencing make RNA-Seq unaffordable for researchers to use this method on a large scale. However, small sample numbers negatively impact the statistical power of the research design and run the risk of missing nuanced ways that heterosis can be quantified. 3' RNA-Seq libraries cost less to generate and do not require as many raw reads to be produced for each sample. Therefore, more samples can be run per lane. Drawbacks to the 3' method include the inability to

distinguish alternatively spliced transcripts from a locus or identify polymorphisms or allele-specific expression (Lohman et al., 2016). However, 3' RNA-Seq was able to increase sample size, increase experimental power to improve the ability to detect differences in DEGs, and identify low abundance transcripts more accurately than RNA-Seq (Lohman et al., 2016; Tandonnet & Torres, 2017).

While yield heterosis exists in hybrid wheat, it has not been studied to the same extent as it has been in maize, rice (Oryza *sativa* L.), or even rubber trees. While in the early stages of developing a hybrid wheat breeding program, the University of Nebraska-Lincoln small grains breeding program has shown that yield heterosis varies over year and environment (Easterly et al., 2020). Therefore, we decided to investigate the genes that were expressed in positive heterosis (e.g., heterotic) hybrids and genes that were expressed in negative heterosis, (e.g., non-heterotic or decreased yield) hybrids. We hope to determine what controls hybrid performance and how those hybrids differ from their parents to understand the genetic mechanisms that impact observed yield heterosis.

To explore the gene expression of heterosis, our study was designed to explore the following objectives: 1) determine if the identified DEGs are expressed in novel groupings that may help explain the observed phenotypic differences among parents and the hybrid, 2) determine how gene expression can be classified in relation to the parents' gene expression by taking hybrid purity into account and 3) to determine if the environment impacted heterosis expression through DEG analysis between the two sampling years.

3. MATERIALS AND METHODS

3.1 Plant Material

Every year, ~350 wheat hybrids are created in the UNL small grains hybrid research project, in collaboration with TAMU, using Nebraska- and Texas-developed inbred lines that are crossed in a field crossing block. The female lines were sterilized using Croisor 100®, (active ingredient sintofen; 1-(4- chlorophenyl)-5-(2-methoxyethoxy)-4-oxo-1,4-dihydrocinnoline-3-carboxylic acid) (Asur Plant Breeding, Estrées-Saint-Denis, France), a chemical hybridizing agent. The resulting seed was harvested and the F_1 progeny is grown in three sites across the state of Nebraska, Lincoln (Havelock Research Farm, UNL), North Platte (West Central Research and Extension Center, UNL), and near Alliance (collaborator's field site) to evaluate yield and agronomically important traits. The F_1 trial at Lincoln served as the source material for this study. An average of 17 unique triads (defined as an F_1 hybrid and both parent genotypes) were collected each year for two years, 2019, Year 1, and 2021, Year 2, and to find a representative five heterotic triads and five non-heterotic triads.

In Year 1, the hybrids were grown in an augmented design and the parents were grown in a randomized complete block design (RCBD). Triads were selected for tissue sampling based on positive and negative MPH calculated with grain yield data from the previous year's hybrid yield trial (MPH equation shown below). MPH was used in this research instead of high parent heterosis or commercial heterosis because it is the standard in genetic research studies. Twenty triads were identified to sample.

$$\%\text{MPH} = \left[\frac{\left(\text{Yield}_{\text{Hybrid}} - \left(\frac{\text{Yield}_{\text{Female Parent}} + \text{Yield}_{\text{Male Parent}}}{2} \right) \right)}{\left(\frac{\text{Yield}_{\text{Female Parent}} + \text{Yield}_{\text{Male Parent}}}{2} \right)} \right] \text{x } 100$$

The selected genotypes were scored for flowering date (BBCH stage 65, (German Federal Biological Research Centre for Agriculture and Forestry, 2001)), on a plot basis. Eight single stems were randomly tagged within the plot two to four days after flowering (DAF) representing the biological replicates to be sampled. Flag leaf and immature kernels were sampled from each replicate. Samples were collected on either May 31 or June 5 depending on the plot's flowering date as well as staging the stems that were previously tagged. All tissues of a triad were collected on the same day. Sampling began around 11:00 a.m. and ended by 1:00 p.m. to minimize circadian rhythm effects on gene expression. Seed tissue age ranged between five and 12 DAF with an average of nine DAF, falling within the 69-71 stages on the BBCH scale. Differences in seed age can be seen in Figure 1.1. From each stem, 10 immature seeds were collected followed by cutting off and storing half of the flag leaf. The samples were flash frozen in liquid nitrogen in the field and moved to a -80℃ freezer for storage.

In Year 2, the hybrid trial with a subset of parents was grown in an alpha lattice field design with each genotype replicated three times. 16 triads were selected for sampling based on high parent heterosis calculations from previous yield trials and flowering dates were taken (high parent heterosis equation shown below). High parent heterosis was used to pick triads for sampling because we found it increased the probability of identifying triads with the desired heterosis levels compared to MPH. All

final triad selections were made using MPH based on the yield of the actual hybrid and parent plots sampled.

$$\%\text{High Parent Heterosis} = \left[\frac{\left(\text{Yield}_{\text{Hybrid}} - \text{Yield}_{\text{Highest Yielding Parent}}\right)}{\text{Yield}_{\text{Highest Yielding Parent}}}\right] \text{x } 100$$

Six individual stems in each plot were tagged to mark the biological replicates two to four DAF. Sampling was done on May 29 and June 1 based on plot flowering dates and individual stem age. Sampling was conducted using the same method used in 2019 with the exception that RNA*later*® (Thermo Fisher Scientific, Waltham, Massachusetts), an RNase inactivator and RNA stabilizer for tissues and cells collected in the field, was used to preserve the samples. The sampled tissues were stored at 4℃ overnight, and then moved to -80℃ freezer for longer storage following the manufacturer's protocol. Seed tissue age ranged between 6 and 14 DAF with an average of 9 DAF, again falling within the 69-71 stages of the BBCH scale (Figure 1.1).

The2019 and 2021 trials were harvested for grain yield and the raw values were adjusted for spatial variation using the ASReml-R v4.1.0.160 to produce the associated BLUPs (Butler et al., 2007). The statistical model of the yield response variable, Y_{ijk}, of the i^{th} number of treatments, j^{th} number of times each treatment is replicated, and k^{th} number of blocks found in each replicate, was analyzed as:

$$Y_{ijk} = \mu + \tau_i + \gamma_j + \rho_{k(j)} + \epsilon_{ijk}$$

Where μ is the grand mean for the entire experiment, τ_i is the effect of the i^{th} treatment, γ_j is the effect for the j^{th} replicate, $\rho_{k(j)}$ is the block within replicate effect, which is a nested variable in this model, and ϵ_{ijk} is the error term. This model produced the BLUPs for each genotype in the trial. The adjusted BLUPs were used to calculate MPH for each

sampled triad. In Year 1, four triads were selected for RNA extraction, two heterotic and two non-heterotic. In Year 2, six triads were selected for RNA extraction, with three as heterotic and three as non-heterotic.

3.2 RNA Extractions

After identifying the appropriate triads, total RNA was extracted from five biological replicates from the flag leaf and immature seed tissue using the Qiagen RNeasy® Plant Mini Kit (Qiagen®, Hilden, Germany) and its protocol with a few modifications. The remaining three replicates were stored at -80℃ for future testing if needed. The provided RLT buffer was used for the leaf tissue and RLC buffer was used for the seed tissue as it was superior for extracting RNA from starchy immature seed compared to the RLT buffer, as determined by optimization testing competed before RNA extractions (unpublished results). Occasionally, when the ground seed tissue was added to the RLC buffer, it would congeal and between 200-400 µL RLC buffer was added to liquify the solution. We performed the optional on-column DNase digestion step on the kit-provided spin column using the Qiagen® RNase-free DNase Kit (Qiagen®, Hilden, Germany) following the manufacturer's protocol. Leaf tissue samples required one to two additional washings with buffer RPE to remove excess chlorophyll from the column as determined by the greenish extract color (Figure 1.2). Finally, the spin column was washed with 20 µL RNase-free water and stored in a 1.5 mL collection tube, and then washed again and stored in a 1.5 mL collection tube to remove any remaining RNA for a backup sample.

3.3 RNA Sequencing and Read Processing

After extraction, the RNA samples were sent to the University of Nebraska Medical Center Genomics Core Facility in Omaha, NE. There, the samples were evaluated on an Advanced Analytical Technologies, Inc. Fragment Analyzer™ (recently acquired by Agilent Technologies, Inc., Santa Clara, California) for quality, producing RQN values and sample concentrations for both years. Using the RQN quality scores and RNA concentrations received from the Genomics Core, the three best biological replicates per genotype were selected for sequencing resulting in 168 samples (ten triads x two tissues x three biological replicates x three genotypes per triad minus the common parents). All samples were diluted to 50 ng/µL and submitted to the Genomics Core for RNA sequencing. Libraries were prepared using the Lexogen® QuantSeq™ 3' mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria). Library quality was checked using a Qubit® 3.0 fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) and a BioAnalyzer 2100 (Agilent Technologies, Inc., Santa Clara, California). Sequencing was completed on an Illumina® NextSeq550 using 75SR High Output Flow Cell kits (Illumina®, San Diego, California) and produced 75 bp, single-end reads. In Year 1 and Year 2, we randomly assigned samples to a flowcell at 33-multiplexed and 30-multiplexed respectively.

The raw reads were investigated using *FastQC* v0.11 to assess the quality of the reads (Andrews et al., 2015). The results showed the first 12 bp contained high variation in per base sequence content and 10 bp that had low per base sequence quality at the tail end. *Trimmomatic* v0.38 was used to trim off the poor-quality bases and any other contaminants found in the reads using a head crop of 12 bp, a crop length of 60 bp, as well as a contaminants file that contained the most common Illumina contaminants

associated with 3’ RNA sequencing (Bolger et al., 2014). After rerunning the trimmed reads through *FastQC*, the overall quality of the reads improved and could be mapped to the reference genome using the *STAR* v2.7 read aligner package and the IWGSC RefSeq v2.1 genome assembly and annotations (Dobin et al., 2013; T. Zhu et al., 2021). Annotation files with gff3 extension were converted to gtf files using *Cufflinks* v2.2 (Trapnell et al., 2010). Gene level counts were created using the *htseq -count* command in the *HTSeq* v0.9 program and then exported into a csv file (Anders et al., 2015).

3.4 Gene Expression Analysis

The R program *DESeq2* v1.32.0 was used to identify major sources of variation in the dataset, normalize and filter gene counts, and create contrasts to compare DEGs (Love et al., 2014). Variation was investigated using the built in “plotPCA” command in the *DeSeq2* program as well as *ggplot2* v3.3.5 (Wickham, 2009). Gene normalization and filtering was accomplished using the built-in programing of the “DESeq” command on the data set. Results were created with the “results” function using the pairwise comparisons defined in

418 Table 1.4, Table 1.5, Table 1.6,

Table 1.7, and Table 1.8, and an alpha of 0.05. The results' log fold change was shrunk using "lfcShrink" command with the "apeglm" option in *DeSeq2* (Anders & Huber, 2010; A. Zhu et al., 2019). The shrunken results were then exported after filtering the DEGs for an adjusted p-value less than 0.05 and an absolute log fold change of 2-fold or greater. The filtered DEGs from each parent to hybrid comparison were combined and evaluated for shared gene expression across triad hybrid/parent comparisons. All hybrids were then evaluated for differential expression trends across triads, for example Log Fold Change = Triad 1 Female Parent Expression Score – Triad 1 Hybrid Expression Score. Genes were considered up-regulated, or expressed more in the hybrid, if the log fold expression change was positive. Genes were considered downregulated, or expressed more in the parent, if the log fold change was negative.

4. RESULTS

4.1 Plant Material

Trial yields were used to calculate MPH for each triad as shown in Table 1.1 with trial yields, flowering dates, and sampling dates included for year and triad comparison. Year 1 MPH calculations identified two triads with positive MPH, triads 1 and 2, and two with negative MPH, triads 6 and 7. There was one shared parent, "Panhandle", found in the two negative MPH triads. In Year 2, we used an additional six unique triads not sampled in 2019, three with positive MPH and three with negative MPH, triads 3, 4, and 5 and 8, 9, and 10 respectively. In Year 2, there was one shared parent, "SD10W153", found in a positive MPH triad and a negative MPH triad. The average yield and percent MPH were calculated for both hybrid yield trials in Year 1 and Year 2 as well as the time of year the trials flowered. There were differences observed between the two trials. In

Year 2, flowering occurred earlier than in Year 1. There average yield for Year 2 was higher than Year 1, related to the positive MPH observed in Year 2. Lower yields and negative MPH were observed in Year 1. Triad 1, sampled in Year 1, had the best MPH of 35.67%, meaning the hybrid yielded 36% more than the average of the parents. The lowest MPH was observed in Year 1 as well in Triad 6, where the hybrid yielded 33% less than the average of the parents.

4.2 RNA Extraction

After extracting the RNA from the selected triads and evaluating their quality, RQN and concentration was compared to select the best three biological replicates/genotype. Year 1 immature seed samples had an average RQN of 9.1 and concentration of 150 ng/µL while the leaf samples had an average RQN of 6.6 and concentration of 1,449 ng/µL. Year 2 immature seed samples had an average of 5.7 RQN and 337.42 ng/µL and leaf samples had an average of 6.1 RQN and 1,856.81 ng/µL (Table 1.2). By selecting the replicates with high RQN values and concentrations above 50 ng/µL, the sequenced samples' quality improved to 9.4 and 6.8 for Year 1 seed and leaf samples respectively and 6.4 and 6.2 for the Year 2 leaf and seed respectively (Table 1.2).

4.3 RNA Sequencing and Read Processing

Sequencing produced an average of 14,660,092 sequences. *FastQC* reports found no sequencing barcode or adaptor contamination and no sequences flagged as inadequate quality in any of the 168 samples. Two issues were identified with the reads: specifically, per base sequence quality decreased (quality score less than 20) on the 5' end of the read and had poor per base sequence content on the 3' end of the reads. *Trimmomatic* kept an

average of 12,888,106 reads per sample, keeping 87.95% and dropping 12.05% of reads. The *STAR* aligner mapped reads back to the reference genome at an average rate of 52% uniquely mapped. However, we observed a major difference in the rate of leaf and seed samples mapping to the genome, 45% to 60% uniquely mapping, respectively (Table 1.3). The program mapped read lengths within two bp of the input read lengths.

4.4 Gene Expression Analysis

The two main components for variation within the dataset were identified as the sample tissue (leaf vs. seed) component, representing 86% of the variation, and a secondary unidentified component in the seed tissue, which represented 6% of the overall variation (Figure 1.3). The secondary component was explored by looking at the interaction of triad and type which did not provide an explanation for the second component of variation (Figure 1.4). The age of the sample when it was collected was then investigated and showed that younger samples, seven to eight DAF, clustered together and explained the second source of variation (Figure 1.5).

To identify any genes that were differentially expressed between the heterotic and non-heterotic hybrids, all of the heterotic hybrids and non-heterotic hybrids pairwise comparison showed that in the leaf tissue hybrids, 9 more genes were over-expressed in the heterotic hybrids when compared to non-heterotic hybrids. Conversely, 9 more genes were expressed in the non-heterotic hybrids than the heterotic hybrids in the seed tissue (

Table 1.4). ~2,000 more genes were expressed in leaf tissue than in seed tissue for both heterotic and non-heterotic triads (Table 1.5). The pairwise comparison between the 2019 and 2021 samples found that for both leaf and seed tissues, ~100 and 600 unique genes were expressed in 2019 compared to 2021 respectively (Table 1.6). Additional analysis on how the hybrids' gene expression compares to the parents' gene expression will be completed in the future.

We then conducted pairwise comparisons between each parent to the hybrid to identify DEGs. Comparisons done on the leaf samples (shown in

Table 1.7) found a total of 1,189 genes differentially expressed in the heterotic hybrids compared to their parents, while 1,707 genes were differentially expressed in the non-heterotic hybrid/parent comparisons. On average, 64 genes were identified with higher expression in the heterotic hybrids for each hybrid/parent comparisons while 98 genes were identified with higher expression in the non-heterotic hybrids for the hybrid/parent comparisons. The results of the hybrid/parent comparisons were compared and 402 shared DEGs across the 20 comparisons were identified and depicted in Figure 1.6. This figure shows the expression pattern of these DEGs and depict how the two heterotic classes share genes with opposite log fold change values. Expression analysis of the seed tissue found that 2,615 and 3,130 genes were differentially expressed in the heterotic and non-heterotic hybrid/parent comparisons respectively (Table 1.8). As was found in the leaf tissues, in the immature seed tissues more genes were over-expressed in the non-heterotic hybrids. When evaluating the DEGs across the 20 hybrid/parent combinations, the comparisons found 600 genes differentially expressed in the 20 comparisons and are shown in Figure 1.7. In the DEG analysis for hybrid/parent comparisons, we identified four triads in both seed and leaf tissue whose gene expression patterns depicted the possibility of self-pollination. Triads 5, 8, 9, and 10 are potentially self-pollinated due to the higher number of DEGs found in the male/hybrid comparisons than in the female/hybrid comparisons. This observation could indicate the hybrids are more like the female parents than the male parents because the larger number of DEGs present in the male/hybrid indicate a difference in gene expression that is not shared in the female/hybrid comparison.

5. DISCUSSION

3’ RNA-Seq has proved to be a valuable tool for investigating genes being expressed in different tissues as well as different performance groups. In this study, we used 3’RNA-seq to characterize gene expression in ten triads that showed positive or negative levels of mid-parent heterosis. The triads had to be sampled over two years as we could not accurately predict if a hybrid would show positive or negative heterosis and complete the sampling in the same year, thus removing most environmental effects on the number of DEGs identified. We were able to extract quality RNA from both leaf and immature kernel tissue. Sequencing the tissues produced an average of almost 15 million sequences per sample, providing enough coverage to identify differentially expressed genes. However, with the observed variability in creating hybrids with high mid-parent heterosis in the UNL program, it was still unknown if we would find any genes that were differentially expressed (Easterly et al., 2020). While using 3’ RNA-Seq reduced our mapping rate to an average of 50% of transcripts mapped to unique locations in the genome, less than 1 bp was removed from the input reads indicating the trimming removed them majority of unmappable base pairs. Also, despite reducing the mapping rate, 3’ RNA-Seq still allowed us to identify DEGs and was more cost effective than traditional RNA sequencing.

Before identifying DEGs, we had to understand if and what kind of variation present in the dataset. While most of the variation is due to tissue type, there was a slight amount of variation found in the seed samples that could not be explained by the other obvious categories, heterotic classification (not shown), triad, or the type of line (hybrid, male, or female) (Figure 1.4). However, it was concluded that the age of the kernels when

they were collected explained the spread of variation in the seed samples. Figure 1.5 shows that the blue dots, representing seven and eight DAF, have the greatest variation, but there was also considerable variation between the blue and red dots, 11 and 12 DAF. This observation makes sense biologically because as a kernel develops, it may have different genes expressed compared to younger kernels. The variation in sample age highlights that sampling time was very important and why we tried to sample the triads as close to the same age as possible. The variation found between the leaf and seed tissue groups suggested that the analysis needed to be done separately as they would have a confounding effect on any observed DEGs. The tissue effect was also seen in Table 1.5 where there were always more genes expressed in the leaf tissue compared to the seed tissue, possibly due to the kernels being immature and still developing or the leaves are more complex organisms.

Although the hybrids were grown in two different years, we were able to identify DEGs associated with heterotic and non-heterotic hybrids pairwise comparison. While gene expression in the leaf and seed tissue differed significantly; more DEGs were identified in the heterotic hybrids for the leaf samples while more DEGs were found in the non-heterotic hybrids for the seed samples. We did not expect this result because the number of DEGs should be similar in both tissues when sampled from the same plant. If more DEGs were found in the heterotic hybrid leaf samples, we expected there should be more DEGs present in the heterotic hybrid seed samples. It will warrant further investigation to determine why this is occurring and its biological meaning.

The pairwise comparisons conducted on the parent/hybrid contrast of each triad showed that in both leaf and seed samples, more DEGs between the parents and hybrids

were found in the non-heterotic hybrids. We could hypothesize that the genes that show higher expression in the non-heterotic hybrids could be controlling a metabolic pathway which impacts the yield produced. However, this hypothesis needs to be explored further using pathway annotations to understand what role these genes have in expression. These gene pathways could show why the heterotic hybrids perform better than the non-heterotic group. The metabolic pathway hypothesis can be visualized in Figure 1.6 and 1.7 as there are genes that are shared across the two groups while some are unique to a specific group.

The pairwise comparisons in both leaf and seed tissues also introduced concern that some of the hybrids were self-pollinations instead of crosses. There were large numbers of genes found to be differentially expressed in the male/hybrid comparison compared to the female/hybrid comparison, indicating that more of the hybrid's alleles are from the female. The similarity could indicate that the genes that are differentially expressed in the female/hybrid comparison are due to environmental factors and not to the hybrid being a mixture of the female's and male's alleles. This observation is also shown in Figure 1.8, showing the variation present in the three parts of Triad 5. The hybrids and female parents are clustered together while there is more gene count variation between the hybrids/females to the male genotypes for both leaf and seed tissue. While we have not been able to identify if a self-pollination occurred with this dataset, future hybrid studies should take this into consideration and conduct DNA sequencing to identify self-pollinations.

Because samples had to be collected in two different years, we investigated how that impacted the number of DEGs and found that more genes were expressed in 2019

compared to 2021 for both tissues. The two environments had significant differences between temperature and precipitation. In calendar year 2019, the yearly average overall temperature was 10.5℃, the average maximum temperature was 27.5℃, and the average minimum temperature was -5.6℃. Total precipitation that year was 917.19 mm (National Weather Service, 2020). On the other hand, in 2021 the average temperature was 12℃, average maximum temperature was 18.8℃, and the average minimum was 5.3℃ with total precipitation totaling 682.24 mm (National Weather Service, 2022). We believe that more genes were expressed in 2019 as it had more extreme temperatures while 2021 was not as stressful of an environment.

6. CONCLUSIONS

The results presented in this study indicate the validity of exploring the transcriptome in hybrid wheat to identify reasons why heterotic hybrids perform better than non-heterotic hybrids. There are significant differences between the transcriptomes of the two classes of hybrids that indicate the importance of identifying the roles that these DEGs play in metabolic pathways which would allow us to identify why a heterotic hybrid is phenotypically different than a non-heterotic. Metabolic pathway identification will be conducted in future work on this project. The differences between the hybrids and their parents also imply the need to determine gene expression classifications for these triads to further tease out how the expressed genes impact the positive and negative heterosis expressed in the hybrids compared to the parents. When conducting these parent/hybrid comparisons, we will need to distinguish and remove possible self-pollinated hybrids before doing the DEG analyses. This research is the corner stone for future work done within the UNL small grains group to continue to understand heterosis in hybrid wheat.

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711

Table 1.1: Grain yield data from hybrid and parental line (triads) replicated trials grown at Lincoln, NE in 2019 or 2021.

Hybrids are in bold followed by the female then male parent. Triads 1, 2, 6, and 7 were sampled in 2019. Triads 3, 4, 5, 8, 9, and 10 were sampled in 2021.

Category	Genotype	Flowering Date (Julian)	Yield (kg/ha)	MPH (%)
Trial Mean	Year 1	145	5,462.71	-3.04
	Year 2	141	5,895.82	2.74
Triad 1	**NXB17-5214**	144	6,437.24	35.67
	TX12A001638	147	3,701.60	
	NE14663	145	5,788.08	
Triad 2	**NXB17-5769**	148	5,630.69	4.52
	NE14419	147	5,241.73	
	NI14729	147	5,532.55	
Triad 3	**NXB17-5818**	142	6,188.58	12.72
	TX14M7088	142	5,997.23	
	TX12A001420	139	4,983.05	
Triad 4	**NXB17-5716**	141	6,416.46	6.87
	TAM204	142	6,353.81	
	SD10W153	140	5,654.38	
Triad 5	**NXB17-5226**	140	5,944.51	6.45
	TAM305	139	5,655.67	
	NI15713	144	5,513.25	
Triad 6	**NXB17-5393**	145	3,639.42	-32.58
	TX14M7051	145	4,937.54	
	Panhandle	146	5,858.03	
Triad 7	**NXB17-5394**	145	4,209.29	-26.04
	Sturdy2K	145	5,525.05	
	Panhandle	146	5,858.03	
Triad 8	**NXB17-5375**	142	5,685.82	-0.28
	TX14M7290	142	5,351.59	
	TX12V7220	142	6,052.15	
Triad 9	**NXB17-5713**	142	5,376.82	-1.46
	TX12A001638	142	5,258.65	
	SD10W153	140	5,654.38	
Triad 10	**NXB17-5861**	141	5,383.60	-3.49
	TX14M7051	142	5,461.38	
	TX14A001336	141	5,694.74	

Table 1.2: Comparison of RNA Quality Parameters

Comparison of the average RQN quality, 28S/18S integrity ratio, and concentration of both leaf and seed samples for Year 1 triads and Year 2 triads. Within a year, the rows show how the sample quality improved from the original five reps extracted to the three reps that were selected for sequencing.

	Sample	RQN	28S/ 18S	Concentration (ng/µL)
Year 1	Leaf Original 5 reps	6.6	1.3	1,448.87
	Leaf Selected 3 reps	6.8	1.3	1,668.04
	Seed Original 5 reps	9.1	1.7	150.54
	Seed Selected 3 reps	9.4	1.8	167.49
Year 2	Leaf Original 5 reps	6.1	1.2	1,856.81
	Leaf Selected 3 reps	6.4	1.3	2,276.84
	Seed Original 5 reps	5.9	1.6	337.42
	Seed Selected 3 reps	6.2	1.6	347.76

Table 1.3: *STAR* Aligner Mapping Results

Comparison between the average of all samples when mapped using *STAR* and how the tissue being mapped impacted the mapping result. All samples mapped the entire input read length minus 1 bp, indicating good input quality. Percentage uniquely mapped was higher for the seed samples than the leaf samples.

Sample	# of Input Reads	Input Read Length (bp)	Mapped Read Length (bp)	Uniquely Mapped (%)	Mapped to Multiple Loci (%)	Unmapped Reads (%)
Sample Avg	12,976,532	60	58.98	52	36	12
Leaf Avg	13,110,935	60	59.02	45	45	10
Seed Avg	12,854,238	60	58.95	60	27	13

Table 1.4: DEG Analysis-Heterotic vs Non-Heterotic Pairwise Comparison

DEG pairwise analysis to determine the number of genes differentially expressed in the heterotic and non-heterotic group. Similar number of DEGs were found, but more genes were expressed in the heterotic hybrids for the leaf tissue while more genes were expressed in the non-heterotic seed hybrids.

Pairwise Comparison	# of Genes Higher Expression in Heterotic Hybrids	# of Genes Higher Expression in Non-Heterotic Hybrids	Total # of DEGs
Leaf Tissue: Non-Heterotic vs Heterotic	10	1	11
Seed Tissue: Non-Heterotic vs Heterotic	3	12	15

Table 1.5: DEG Analysis-Leaf vs Seed Pairwise Comparison

DEG pairwise analysis to determine the number of genes differentially expressed in the leaf and seed tissue groups. Similar number of DEGs were found, but more genes were expressed in the leaf tissue for both heterotic groups.

Pairwise Comparison	# of Genes Higher Expression in Leaf Tissue	# of Genes Higher Expression in Seed Tissue	Total # of DEGs
Heterotic Tissue: Seed vs Leaf	8,499	6,532	15,031
Non-Heterotic Tissue: Seed vs Leaf	8,392	6,744	15,136

Table 1.6: DEG Analysis-2019 vs 2021 Pairwise Comparison

DEG pairwise analysis to determine the number of genes differentially expressed in the two years samples were taken. Similar number of DEGs were found, but more genes were expressed in 2019.

Pairwise Comparison	# of Genes Higher Expression in 2019	# of Genes Higher Expression in 2021	Total # of DEGs
Leaf Samples: 2021 vs 2019	682	509	1,191
Seed Samples: 2021 vs 2019	1,020	432	1,452

Table 1.7: DEG Analysis-Leaf Triad Pairwise Comparisons

DEG pairwise analyses for the parental lines to the hybrid of each triad for the leaf tissue. More DEGs were identified in the non-heterotic hybrid comparisons than the heterotic hybrid comparisons.

Pairwise Comparison	# of Genes Up-Regulated in Hybrid	# of Genes Down-Regulated in Hybrid	Total # of DEGs
Triad 1: Female vs Hybrid	53	46	99
Triad 1: Male vs Hybrid	5	13	18
Triad 2: Female vs Hybrid	3	13	16
Triad 2: Male vs Hybrid	67	34	101
Triad 3: Female vs Hybrid	5	100	105
Triad 3: Male vs Hybrid	49	71	120
Triad 4: Female vs Hybrid	133	39	172
Triad 4: Male vs Hybrid	30	31	61
Triad 5: Female vs Hybrid	2	16	18
Triad 5: Male vs Hybrid	292	187	479
Triad 6: Female vs Hybrid	0	20	20
Triad 6: Male vs Hybrid	0	40	40
Triad 7: Female vs Hybrid	0	6	6
Triad 7: Male vs Hybrid	1	37	38
Triad 8: Female vs Hybrid	1	2	3
Triad 8: Male vs Hybrid	141	44	185
Triad 9: Female vs Hybrid	8	17	25
Triad 9: Male vs Hybrid	476	149	625
Triad 10: Female vs Hybrid	5	39	44
Triad 10: Male vs Hybrid	350	362	721

Table 1.8: DEG Analysis-Seed Triad Pairwise Comparisons

DEG pairwise analyses for the parental lines to the hybrid of each triad for seed tissue. More DEGs were identified in the non-heterotic hybrid comparisons than the heterotic hybrid comparisons.

Pairwise Comparison	# of Genes Up-Regulated in Hybrid	# of Genes Down-Regulated in Hybrid	Total # of DEGs
Triad 1: Female vs Hybrid	0	3	3
Triad 1: Male vs Hybrid	1	6	7
Triad 2: Female vs Hybrid	0	3	0
Triad 2: Male vs Hybrid	0	0	0
Triad 3: Female vs Hybrid	5	6	11
Triad 3: Male vs Hybrid	88	200	288
Triad 4: Female vs Hybrid	0	0	0
Triad 4: Male vs Hybrid	241	83	324
Triad 5: Female vs Hybrid	20	47	18
Triad 5: Male vs Hybrid	1,254	710	1,964
Triad 6: Female vs Hybrid	1	15	16
Triad 6: Male vs Hybrid	1	15	16
Triad 7: Female vs Hybrid	0	1	1
Triad 7: Male vs Hybrid	2	14	16
Triad 8: Female vs Hybrid	58	165	223
Triad 8: Male vs Hybrid	218	62	280
Triad 9: Female vs Hybrid	0	1	1
Triad 9: Male vs Hybrid	77	27	104
Triad 10: Female vs Hybrid	41	14	55
Triad 10: Male vs Hybrid	1,643	775	2,418

Figure 1.1: Kernel Age Comparison.
Differences in kernel size and development at the six time periods they were sampled from the plots; (a) 7 DAF; (b) 8 DAF; (c) 9 DAF; (d) 10 DAF; (e) 11 DAF; (f) 12 DAF

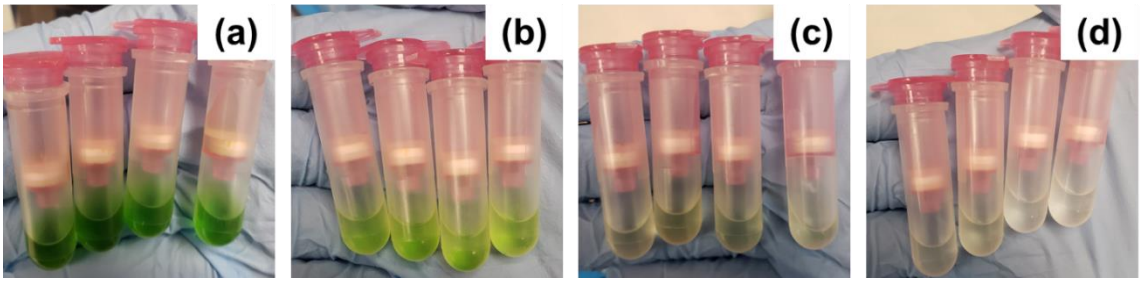


Figure 1.2: Buffer RPE Leaf Tissue Washing Stages.
Visual representation of the washing step of leaf extractions where the chlorophyll was removed from the sample and how four washes were needed to remove the chlorophyll; (a) Wash 1; (b) Wash 2; (c) Wash 3; (d) Wash 4

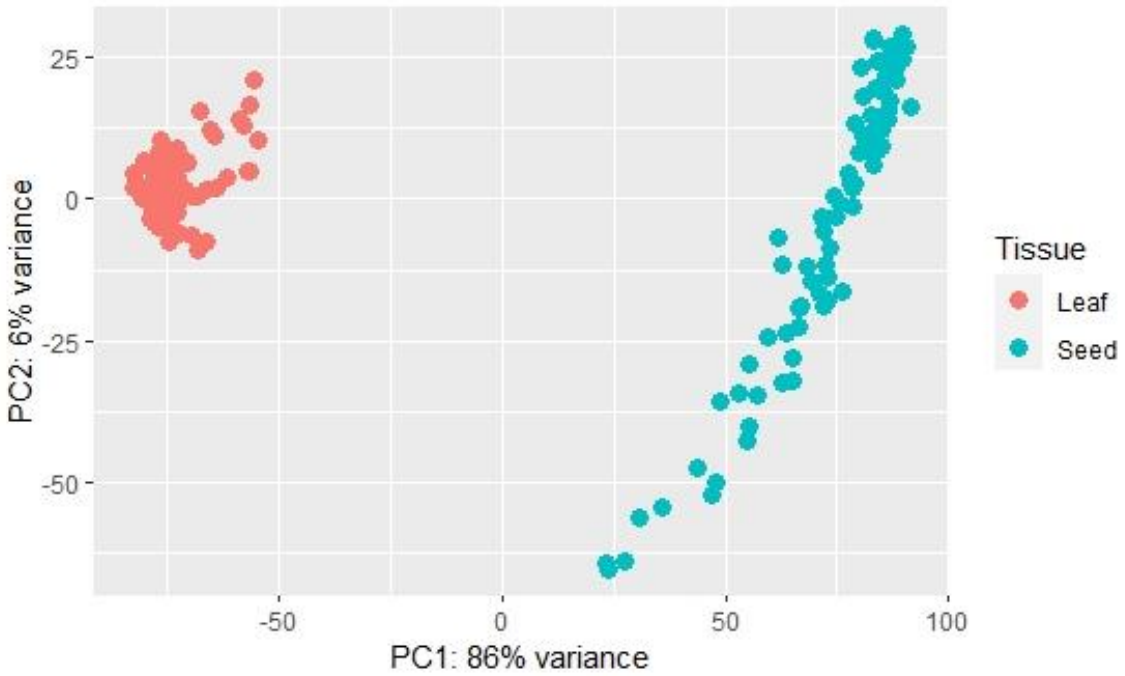


Figure 1.3: PCA Analysis of Sources of Variation in Dataset

Relationship between the two tissues sampled as they make up the majority (86%) of the variation in the dataset.

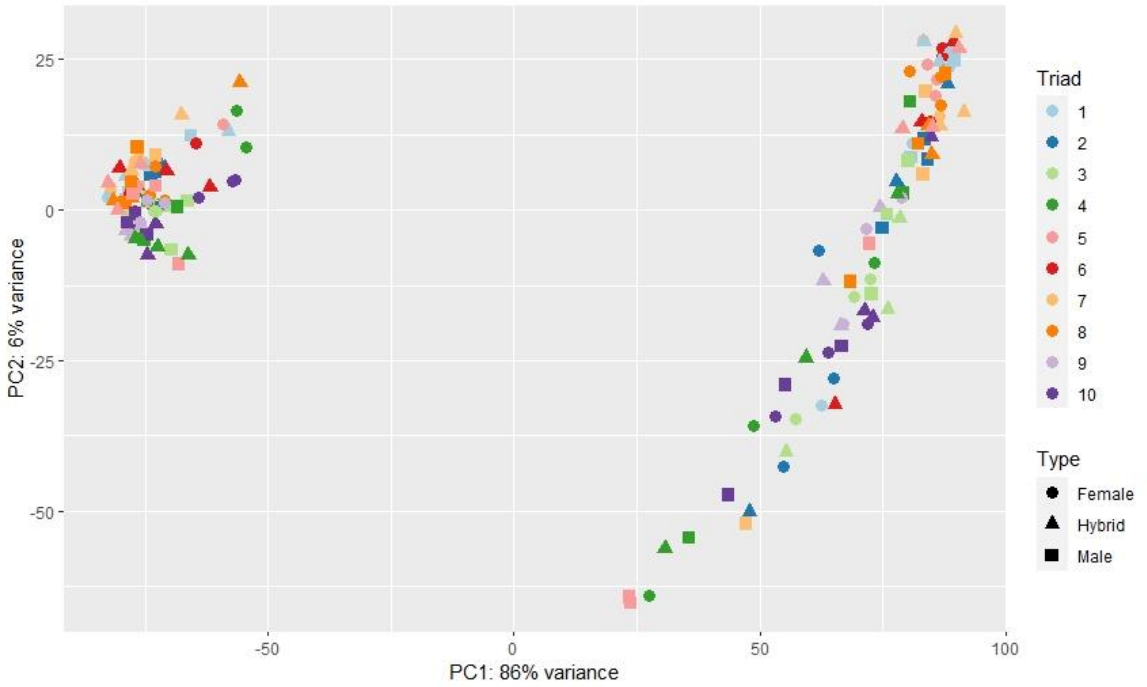
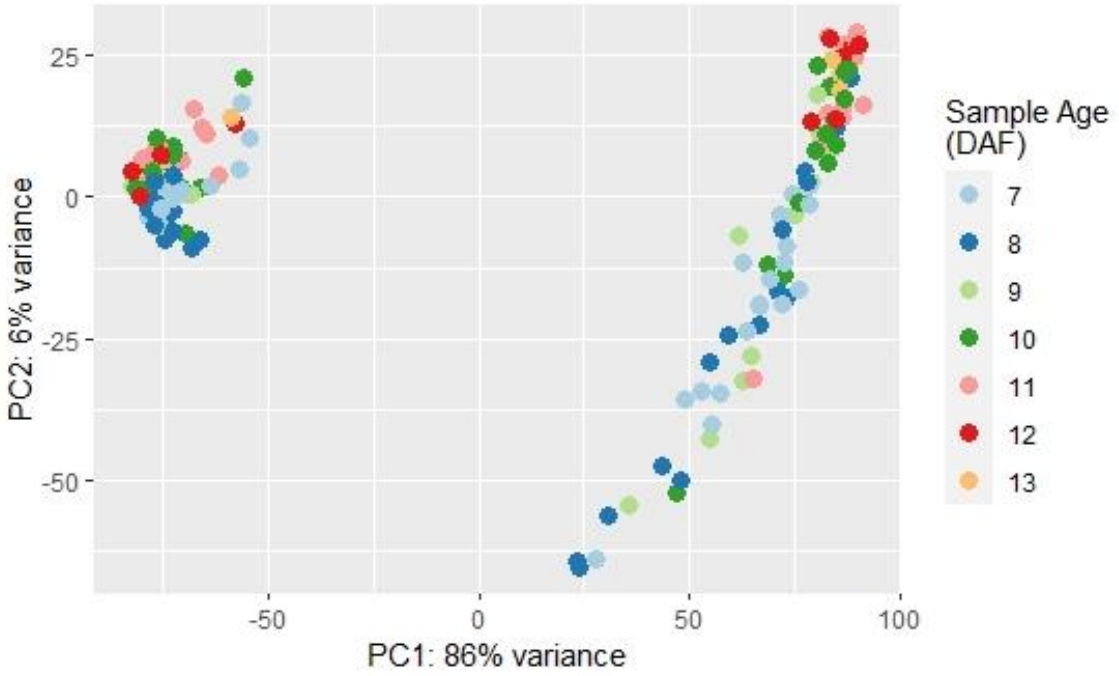


Figure 1.4: PCA Analysis of Triad x Type Interaction as Source of Variation

Relationship between the interaction of triad and type of triad on the secondary source of variation in the dataset. It does not explain the presence of variation in the seed samples on the right side of the figure.



778

779 **Figure 1.5: PCA Analysis of Sample Age as Source of Variation**
780 Relationship of the sample's age when it was collected from the plot to the secondary
781 source of variation in the dataset. Kernel age is the secondary component of variation in
782 the dataset and proves the importance of sampling at the same tissue age.

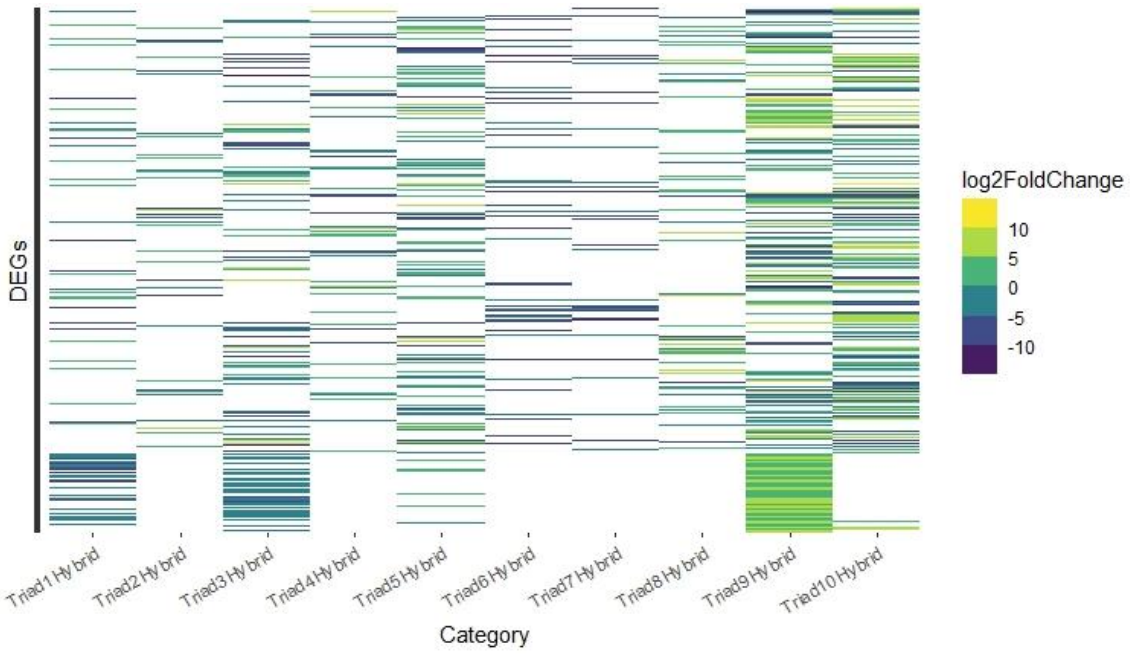


Figure 1.6: Shared DEGs Across Hybrids-Leaf

Comparison of the identified DEGs found in the leaf tissue hybrid/parent comparisons for both heterotic and non-heterotic triads, Triads 1-5 and 6-10 respectively. A positive log fold change indicates the gene is more expressed in that hybrid compared to the parents. A negative log fold change indicates the gene has higher expression in the parent than in the hybrid. Genes shared within triads in a heterotic group and among groups can also be identified.

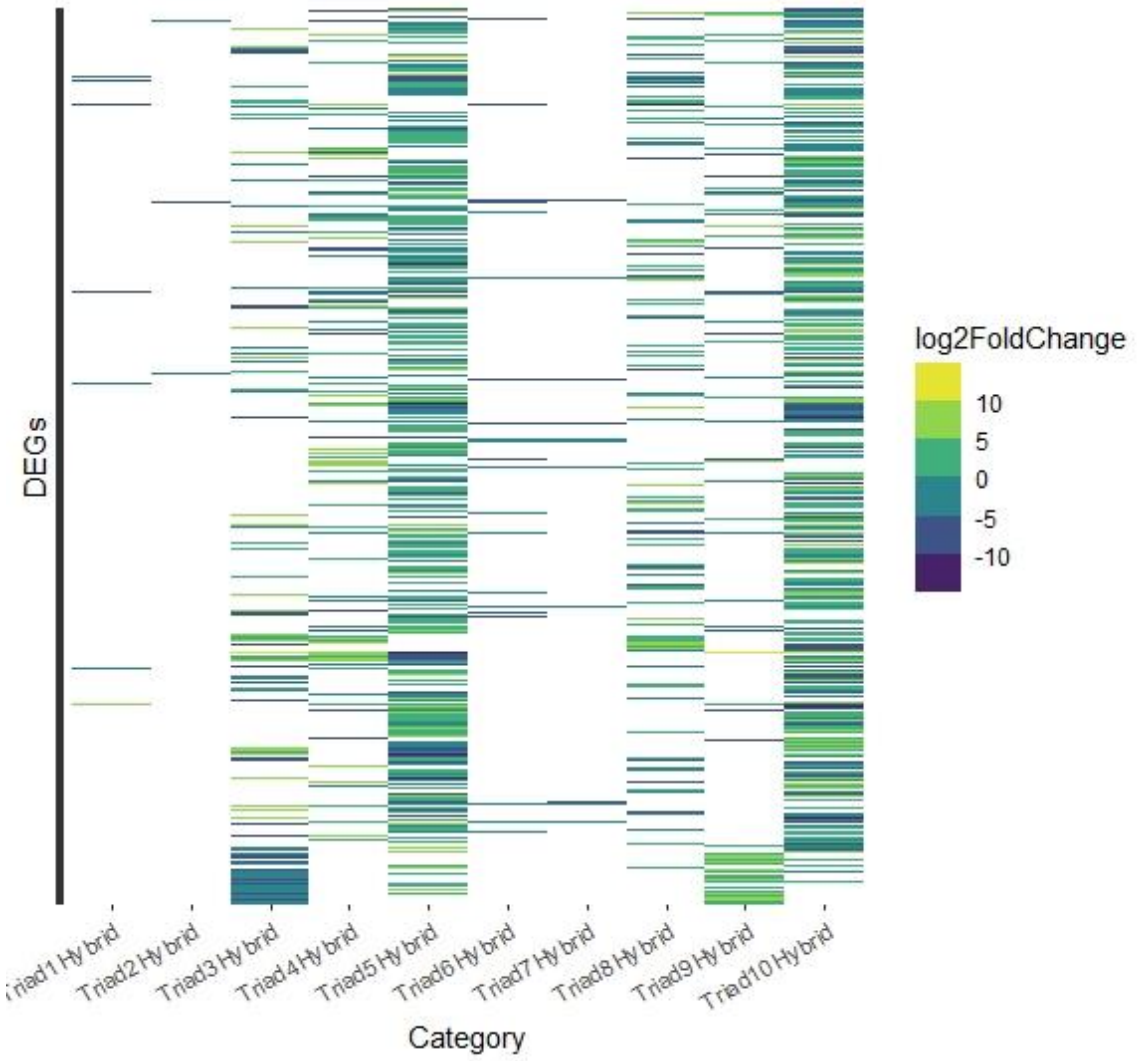


Figure 1.7: Shared DEGs Across Hybrids-Seed

Comparison of the identified DEGs found in the seed tissue hybrid/parent comparisons for both heterotic and non-heterotic triads, Triads 1-5 and 6-10 respectively. A positive log fold change indicates the gene is more expressed in that hybrid compared to the parents. A negative log fold change indicates the gene has higher expression in the parent than in the hybrid. Genes shared within triads in a heterotic group and among groups can also be identified.

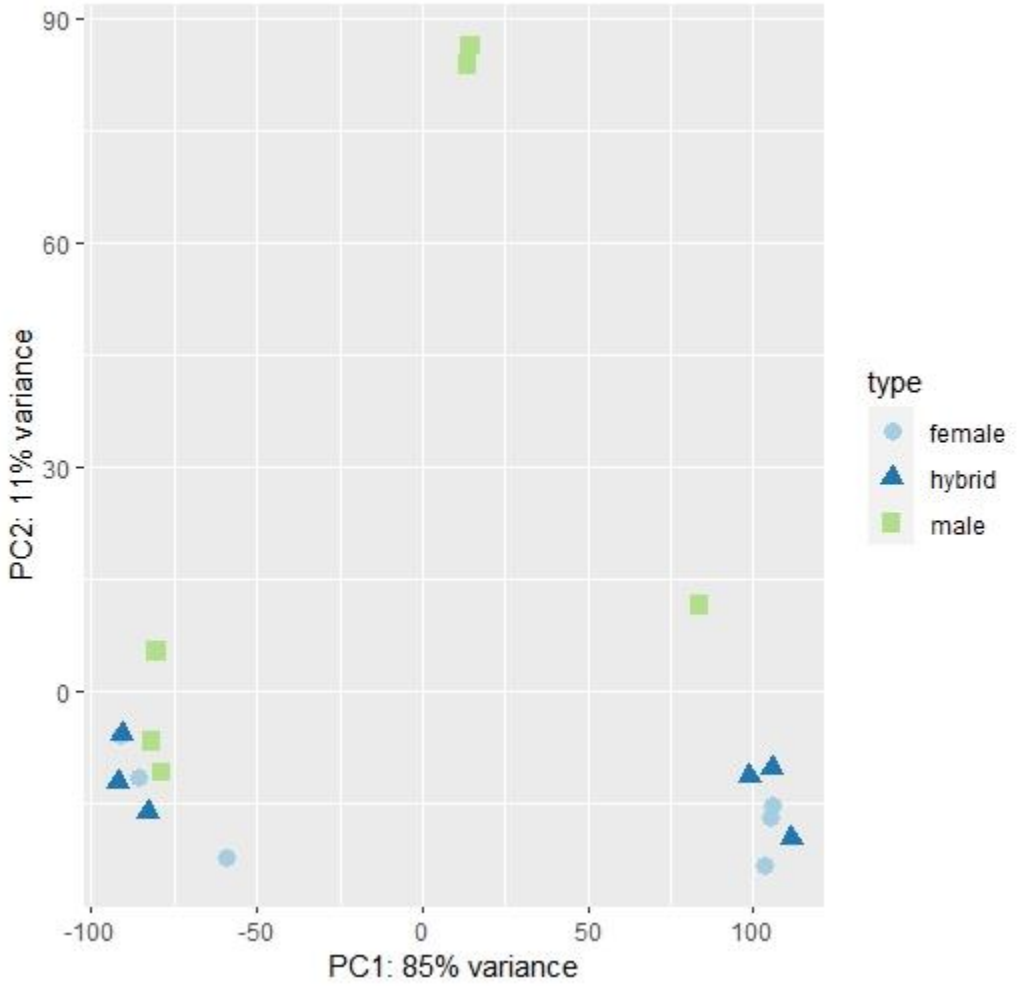


Figure 1.8: PCA Analysis of Triad 5 to Evaluate Selfing

Evaluation of selfing possibly present in Triad 5. With the clustering of a female and hybrid replicate and the male replicates spread out away from the cluster, it indicates the hybrid shares more genes with the female line than the male line and could potentially be a selfed female genotype instead of a unique combination of the two parents. This pattern is present in both the leaf samples on the left and the seed samples on the right.

CHAPTER 2: OPTIMIZING SAMPLE PRESERVATION METHODS FOR SEQUENCING USING RNA*later*® IN WHEAT

1. ABSTRACT

When conducting RNA sequencing studies, researchers must ensure the RNA is intact and pure to create cDNA libraries that produce high quality sequences for transcriptome analysis. Preservation is the important step to creating high quality transcripts and the preservation standard has been using liquid nitrogen to flash freeze tissue and then keep the samples at -80℃. However, liquid nitrogen is dangerous and difficult to handle. RNA*later*® has been created to replace liquid nitrogen as a safe to handle solution to preserve RNA, but the methods described in the protocol have produced inconsistent results in terms of sample purity and integrity, especially for long-term storage. To determine if there is a better storage temperature to keep the samples in and how much degradation occurs as the samples are stored for longer amounts of time, a study was conducted to sample leaf and immature kernel tissues from wheat plants and then place them in storage for one to six months at -20℃ and -80℃. RNA was extracted from each treatment combination and was tested for the quality, quantity, and purity of the RNA. The response variables were analyzed using an analysis of variance. The results showed an improved response when samples were stored in -80℃ but the RNA decreased from month one through month six. However, there was no significant decrease in sample quality and quantity as they were kept in storage. The results suggest that storing high quality samples for up to six months at -80℃ was possible.

2. INTRODUCTION

Gene expression analysis is an important tool for characterizing the genes that control phenotypes in crop species. RNA sequencing is used to characterize gene expression through the analysis of RNA transcripts. However, it is still a relatively new high-throughput sequencing method with improvements being made to reduce the cost and read-depth requirements. These methods have allowed for faster sequencing and sequencing to be completed on more samples and transcripts simultaneously when compared to Sanger sequencing or quantitative real-time PCR (Tandonnet & Torres, 2017; Torres et al., 2008). As more research projects use RNA sequencing, sample collection methods must be considered and evaluated to identify how to preserve the RNA in tissue samples during the period between collection and RNA extraction (García-Baldenegro et al., 2015).

High-quality sample preservation is key for ensuring RNA integrity for sequencing and transcript analyses. It is necessary to have high quality total RNA, quality score greater than four (RQN value), to make good cDNA libraries for sequencing (Sangha et al., 2010). For a sample to be considered for sequencing, it must meet a quality threshold RQN value, also known as the RIN value. RQN values are on a 1-10 scale where 10 is the intact, high quality RNA sample and a 1 is totally degraded. Each project must determine a cutoff RQN value aiming for as high as a score as possible (Schroeder et al., 2006). RNA purity is measured with $A_{260/280}$ and $A_{260/230}$ ratios, which measure the amount of protein contamination, and polyphenol and polysaccharide contamination respectively (de Wever et al., 2020). $A_{260/280}$ is a ratio of the absorbance molecules in solutions at 260 nm and 280 nm on a spectrophotometer. A pure RNA sample has an

$A_{260/280}$ ratio ~2.0 and an $A_{260/230}$ ratio between 2.0 and 2.2 (Thermo Fisher, 2017). In addition to sample purity, sample integrity must be evaluated. The most common method of estimating RNA integrity is to measure 28S/18S ratio which is derived from the 28S and 18S molecules of ribosomal RNA and compare that ratio to other bands on an agarose gel. A ratio close to 2.0 is desired (Schroeder et al., 2006). Total RNA concentration present in the sample will also help the researcher determine if the sample is usable. For example, the protocol for 3' RNA-Seq library preparation using Lexogen® QuantSeq™ 3' mRNA-Seq Library Prep Kit (Lexogen®, Vienna, Austria) and sequencing completed on Illumina® NextSeq550 using 75SR High Output Flow Cell kits (Illumina®, San Diego, California) requires a total RNA concentration of 50 ng/µL. Therefore, any samples with concentrations less than 50 ng/µL would not produce acceptable sequencing results.

Previous RNA studies in wheat have used leaf tissue as the source of RNA and used liquid nitrogen to flash freeze and preserve the sampled tissue (Amirbakhtiar et al., 2021; Chu et al., 2021; Poretti et al., 2021). Tissue preservation in liquid nitrogen after the sample is taken is considered the "gold standard" in RNA preservation (Burden, 2008; García-Baldenegro et al., 2015). However, liquid nitrogen is dangerous to handle and cumbersome to work with, especially in a field versus lab setting. To work around the complications of sampling, a tissue preservation solution, RNA*later*® (Thermo Fisher Scientific, Waltham, Massachusetts), had been introduced that can inactivate RNases and stabilize the RNA for storage before RNA extractions. This preservation method has been used in different plants like annatto (*Bixa orellana* L.), cacao trees (*Theobroma cacao* L.), Arabidopsis (*Arabidopsis thaliana* L.), and wheat (*Triticum aestivum* L.), but each

crop required some modifications for optimal storage conditions (de Wever et al., 2020; Paul et al., 2005; Rodrigues et al., 2007).

Due to the logistical challenges of using liquid nitrogen in the field, the UNL small grains breeding program transitioned to using RNA*later*® to preserve tissue collected from field trials for RNA extraction. A short test was conducted to determine the quality of RNA*later*® preserved samples. Leaf and seed tissues were sampled from greenhouse-grown plants and treated with RNA*later*® based on the manufacturer's protocol. Samples were stored at -20℃, and extractions were completed within a month of sampling. Quality testing showed that the RNA had been preserved adequately for down-stream sequencing with an average RQN of 6.0, 28S/18S ratio of 1.1, concentration of 522 ng/µL, $A_{260/280}$ value of 2.13, and $A_{260/230}$ value of 1.78. Therefore, RNA*later*® was used for a large field-based gene expression study in 2020 and samples were stored at -20℃. However, RNA extraction was delayed until six months after sampling due to COVID-19 restrictions. When the samples were subjected to quality control analysis, the RQN values averaged 3.0-4.0 for both leaf and kernel samples, and 28S/18S ratios were also close to zero for most samples. These values were not acceptable for use in sequencing, and the study was delayed for an additional year to collect new samples.

This experience highlighted the need to evaluate storage temperature and storage longevity for up to six months for RNA*later*® treated samples. Our objectives for this study were 1) to evaluate the longevity of samples preserved in RNA*later*® up to six months and if or when major degradation occurs; and 2) to determine what storage temperature will preserve samples better. Our results are important for designing studies

where RNA cannot be extracted from tissue immediately due to sampling location or the need to collect additional data before selecting samples for extraction.

3. MATERIALS AND METHODS

3.1 Plant Material and Treatment Structure

Sample tissues were collected from the UNL developed wheat cultivar, “Ruth”, which is a commonly used check in the breeding program and a widely used commercial cultivar in the state (Baenziger et al., 2020). Samples were collected from six check plots in a 2021 wheat yield trial located at the Havelock Research Farm in Lincoln, NE. Four individual stems were sampled from each plot, taking both the kernels and flag leaf biological replicates from the same stem, and were immediately submerged in a 2.0 mL microcentrifuge tube filled with in RNA*later*®. Sampling was done between 10:00 am and 12:00 pm on June 6, 2021, and tissue age was 15 days after flowering. Once the samples were collected, they were transported to the wet lab on dry ice. Following the manufacturer’s protocol, the tubes with tissue stored inside were moved to 4℃ overnight. The next day, the RNA*later*® supernatant was removed from the samples and the tissues were placed in their randomly assigned storage temperature, 24 samples in -20℃ and 24 samples in -80℃. Extractions were completed on four samples per tissue per storage temperature once a month for six months. The six storage lengths ranged from ideal storage length of one month to six months. This storage protocol resulted in 12 storage length by storage temperature combinations. The two storage temperatures were coded as “A_” for -20℃ and “B_” for -80℃ with the corresponding number referring to the storage length in months (Table 2.1).

3.2 RNA Extraction

Every month, RNA was extracted from the 4 biological replicates of each tissue for both storage temperatures, -20℃ and -80℃ on the same day. Extractions were completed using the Qiagen RNeasy® Plant Mini Kit (Qiagen®, Hilden, Germany) protocol with a few adjustments for tissue optimization. The kit provided buffer RLC was used for the seed samples as through previous testing, it was found to work better on the starchy immature kernels than the other kit buffer RLT did. RLT worked well on the leaf samples and was used for that process of extracting RNA from the ground tissue. With the additional starches found in older kernels, the initial solution of ground kernel tissue and RLC buffer would create a congealed solution in the microcentrifuge tube. To fix this problem, additional RLC buffer, 200-400 µL was added to re-liquify the solution. The on-column DNase digestion step was performed using the Qiagen® RNase-free DNase Kit (Qiagen®, Hilden, Germany). Due to the large amount of chlorophyll found in leaf tissue, those leaf samples required one to two additional washing steps using the RPE buffer on the spin column (Figure 2.1). The last adjustment to the protocol was using 20 µL RNase-free water and a second 1.5 mL collection tube to complete an additional washing to remove any additional RNA found in the column and to create a backup sample. Once the extractions were completed, samples were stored at -80℃ until quality testing could take place.

3.3 RNA Quantity, Quality, and Integrity Testing

After RNA samples were extracted, they were submitted to the University of Nebraska Medical Center Genomics Core in Omaha, NE for quality control analysis. The Core evaluated samples for RNA quality, quantity, and integrity using an Advanced

Analytical Technologies, Inc. Fragment Analyzer™ (recently acquired by Agilent Technologies, Inc., Santa Clara, California) as well as a ThermoScientific NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). The standard for sequencing is to use the results produced by the fragment analyzer. However, not every research lab can access a fragment analyzer and a NanoDrop™ is more affordable, even though its data are variable. The core returned the concentrations, RQN scores, and 28S/18S ratios for each sample produced from the fragment analyzer. They also returned concentrations, $A_{260/280}$ ratios, and $A_{260/230}$ ratios from the NanoDrop.

3.4 Statistical Analysis

Standard deviations for RQN values were calculated for each tissue and any sample whose result was two standard deviations positive or negative was considered as an outlier and removed from the dataset. Furthermore, samples whose concentrations were less than 50 ng/µL were also removed. These quality control measures were done to match how RNA samples would be handled when submitting them for sequencing. The equipment protocol requires a concentration of 50 ng/µL to ensure quality sequencing is completed, therefore any samples below that concentration need to be discarded while samples above that concentration were diluted to 50 ng/µL.

The experiment was designed in a complete randomized design using a 2x6 factorial treatment design. The statistical model of the response variable Y_{ijk}, referring to either concentration, quality, or integrity, of the i^{th} level of storage temperature, j^{th} level of storage time treatment, and k^{th} experimental unit, was analyzed as:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \epsilon_{ijk},$$

Where μ is the overall mean, α_i is the main effect of the i^{th} level of storage temperature treatment, β_j is the main effect for the j^{th} level of storage time treatment, $\alpha\beta_{ij}$ is the interaction term between the i^{th} temperature and j^{th} time, and ϵ_{ijk} is the error term. Tissues were analyzed separately as the leaf and seed sample's concentrations are drastically different.

A mixed linear model analysis of variance using the PROC MIXED procedure was used with the Type 3 fixed effects tests applied to the procedure in SAS 9.4 (SAS Institute, Cary, North Carolina) to evaluate the effects of the temperature and storage length treatments on concentration, quality, purity, and integrity. Least square means, LS-Means, were used to detail how the significant results in the ANOVAs were reflected biologically. An alpha level of $\alpha < 0.1$ was used to more liberally identify differences and those differences identified can be used by researchers to improve their sample preservation. The estimates and confidence intervals created by the LS-Means test were exported to Excel files and used to create line graphs using the R program *ggplot2* v3.3.5 (Wickham, 2009).

4. RESULTS

After removing outliers for leaf samples, 46 leaf tissue samples remained for the RQN, concentrations, and 28S/18S variables produced by the fragment analyzer and 47 leaf tissue samples remained for the NanoDrop™ concentrations, $A_{260/280}$, and $A_{260/230}$ variables. The seed samples had more outliers removed and resulted in 30 observations used in the fragment analyzer RQN, concentrations, and 28S/18S variables and 34 in the NanoDrop™ concentrations, $A_{260/280}$, and $A_{260/230}$ variables analysis. However, four of

these removed samples were from month three samples stored at -20℃ and analyzed by the NanoDrop™. No comparisons could be made for that month.

The analysis of variance on the leaf samples (Table 2.2) found the storage temperature was significant for the leaf samples' RQN, 28S/18S ratio, concentration produced from the NanoDrop™, and the $A_{260/280}$ ratio. Storage time was significant for the fragment analyzer concentration, NanoDrop™ concentration, and $A_{260/280}$ ratio. The interaction of the two factors, temperature and storage length, was significant for both concentrations and purity ratios.

Further investigation using the LS-Means comparisons illustrate how each response variable is affected by the treatments. RQN values for leaf samples were not significant for each month except for month two compared to three through six. The-80℃ storage temperature also produces a higher RQN score for every month except month five (Figure 2.2). An unexpected spike response was also seen in the concentrations produced from the fragment analyzer for month five for kernel tissues, but overall follows the same temperature pattern of leaf tissues of higher concentrations in the -80℃ treatment. Concentrations are significantly lower for the values from months one and two to the other four months (Figure 2.3). The 28S/18S ratio response shows higher ratios in the -80℃ samples but no differences in the storage length treatment (Figure 2.4). The concentrations produced from the NanoDrop™ show a major decrease in concentration as the samples were stored for longer lengths of time. The figure also shows that storing the samples for one month at -20℃ produced higher concentrations than storing them for six months at -80℃ (Figure 2.5). Figure 2.6 shows a decrease in $A_{260/280}$ values as the samples are kept in -80℃. There was no difference in response to storage temperature

except for a large decrease in the $A_{260/280}$ ratio value at month five for the -80℃ samples. The $A_{260/230}$ ratio was improved in the first month when stored at -20℃ compared to -80℃ (Figure 2.7).

The analysis of variance for the seed samples showed significant response for the 28S/18S ratio to storage temperature, storage time, and their interaction. The NanoDrop™ concentration and the $A_{260/230}$ ratio was significant for the storage time and temperature treatments. Only storage time was significant for the $A_{260/280}$ ratio (Table 2.3).

The LS-Means comparison results for the RQN values of the seed samples found no difference among storage length treatments, but a slight increase in the score when the samples were stored in -80℃ (Figure 2.8) compared to -20℃. In Figure 2.9, the concentrations from the fragment analyzer were slightly higher in samples stored at -80℃ and concentrations improved the longer they were stored. 28S/18S ratios were higher in the samples stored in -80℃ as well (Figure 2.10). The figure also shows that the ratio increased during the six months as the samples were stored in -20℃ but overall remained constant in -80℃ (Figure 2.10). The response of the NanoDrop™ concentrations showed that for almost every month, they were higher in the -80℃ samples than for the -20℃ (Figure 2.11). The $A_{260/280}$ ratio was affected by the length of storage and the ratio decreased as the length of storage increased. While the ratio is not significant, there was an observed increase in the ratios when stored in -80℃ (Figure 2.12). Finally, in Figure 2.13, samples had higher $A_{260/230}$ higher ratios when stored in -80℃ and the ratios trended down in the later months, but it was not significant at the alpha level.

5. DISCUSSION

To extract high quality RNA from plant tissue, the samples need to be preserved at the appropriate temperature, and a researcher needs to know how long the tissue can be kept before the samples degrade below usable limits. Before conducting the analysis on this study, many outliers had to be removed in both the fragment analyzer and NanoDrop™ results, indicating variation present in both datasets. The nature of these samples means that a researcher will have a lot of variation and more reps could have been helpful to identify significant differences.

Based on the results of this study, RQN values remained constant as they were kept in storage for both leaf and seed tissues. The values ranged between 5 and 6 meaning they could be used in sequencing. This result contradicted what was seen in our previous experience with RNA*later*® in 2020 and we cannot explain this difference. However, we can conclude that the less time kept in storage, the RQN values were better. Sample concentration ensures the researcher has enough RNA present in the sample to produce adequate sequencing, but our research showed opposite trends for the two methods of measuring concentrations. While the concentrations reported from the fragment analyzer showed no significant difference in response to storage time for leaf tissue after month three, there was an unexplained increase in concentration between months two and three. This observation is probably due to sample-to-sample variation. The NanoDrop™ concentrations for leaf tissue show a significant decrease between months one and two at -20°C with a non-significant decreasing trend after that for both temperatures. These differences could be due to many samples being removed with concentrations less than 50 ng/µL, indicating the variability in the NanoDrop™ dataset.

Sample integrity remained constant during the six months of storage for both leaf and seed samples, indicating ribosomal RNA remained present in the samples and was not degraded. The purity measurement $A_{260/280}$ ratio saw the greatest response to storage time. The ratio decreased as the number of months in storage increased, indicating that purity suffers the longer it is stored and polyphenol contamination increases. This response was seen in both leaf and seed tissues. The $A_{260/230}$ ratio was not significantly impacted by storage time, meaning polysaccharide contamination is not affected by length of storage. For the temperature treatment, the -80°C treatment produced higher RQN, concentrations, and integrity and purity ratios on average. While it was not significant across every response variable, there was an improvement observed when samples were stored at the -80°C, which would help researchers ensure they are getting the highest possible values for the response variables.

6. CONCLUSIONS

Identifying the appropriate storage conditions for tissue samples is imperative for RNA sequencing as RNA degrades once the tissue is taken from the host. While there was considerable variation in the data collected for this study, important responses were identified and can be applied to sample collection in the future. Leaf and kernel tissues have significant differences when it comes to the value of the RQN, concentrations, 28S/18S ratio, $A_{260/280}$ ratio, and $A_{260/230}$ ratio variables; however, there are shared similarities in their overall response to the treatments that a common storage treatment could be applied to both leaf and kernel tissues and produce high quality RNA for sequencing. Based on these results, we conclude that storing samples at -80°C will improve the preservation of samples compared stored in a -20°C freezer. While we did

not identify a point in time that samples are too degraded to be used, the overall quality is nearly constant for up to six months and is above the minimum requirements for sequencing. -80℃ always produced higher response values, indicating it is the better choice to ensure the highest quality is produced. It is our recommendation that RNA*later*® can be used as a viable replacement to liquid nitrogen. Researchers should use -80℃ as their storage temperature and then extract the samples as soon as reasonably possible to ensure quality is not diminished.

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Table 2.1: Treatment Structure

The table depicts the design structure of the treatments applied to the tissue samples. The structure was applied to both seed and leaf tissues. Samples were randomly assigned to the month they were extracted.

Storage Temperature	Storage Length					
	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months
-20℃	A1	A1	A3	A4	A5	A6
-80℃	B1	B2	B3	B4	B5	B6

Table 2.2: Leaf ANOVA

Leaf sample ANOVA indicating the response of the variables to the treatments. ** is significant at $p < 0.1$.

	Fragment Analyzer Results				NanoDrop Results			
		RQN	Concentration (ng/µL)	28S/18S Ratio		Concentration (ng/µL)	$A_{260/280}$ Ratio	$A_{260/230}$ Ratio
Source of Variation	Degrees of Freedom	Pr > F	Pr > F	Pr > F	Degrees of Freedom	Pr > F	Pr > F	Pr > F
Storage Temperature Treatment	1	0.0281**	0.1192	<0.0001**	1	0.0220**	<0.0001**	0.2733
Storage Time Treatment	5	0.1300	0.0019**	0.5929	5	0.0003**	<0.0001**	0.4014
Storage Temperature Treatment x Storage Time Treatment	5	0.5027	0.0005**	0.3483	5	<0.0001**	<0.0001**	0.0212**
Error	36	-	-	-	36	-	-	-

Table 2.3: Seed ANOVA

Seed sample ANOVA indicating the response of the variables to the treatments. ** is significant at $p < 0.1$.

	Fragment Analyzer Results				NanoDrop Results			
		RQN	Concentration (ng/µL)	28S/18S Ratio		Concentration (ng/µL)	$A_{260/280}$ Ratio	$A_{260/230}$ Ratio
Source of Variation	Degrees of Freedom	Pr > F	Pr > F	Pr > F	Degrees of Freedom	Pr > F	Pr > F	Pr > F
Storage Temperature Treatment	1	0.3250	0.4061	0.0001**	1	0.0949**	0.1823	0.0675**
Storage Time Treatment	5	0.6769	0.6245	0.0181**	5	0.0193**	<0.0001**	0.0283**
Storage Temperature Treatment x Storage Time Treatment	5	0.1806	0.9705	0.0024**	5	0.5110	0.2892	0.5368
Error	36	-	-	-	36	-	-	-

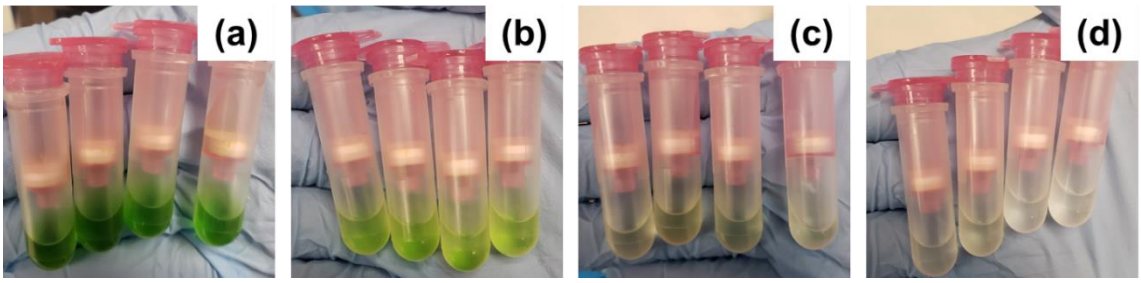

Figure 2.1 Buffer RPE Leaf Tissue Washing.

Visual representation of the washing step of leaf extractions where the chlorophyll was removed from the sample and how four washes were needed to remove the chlorophyll; (a) Wash 1; (b) Wash 2; (c) Wash 3; (d) Wash 4

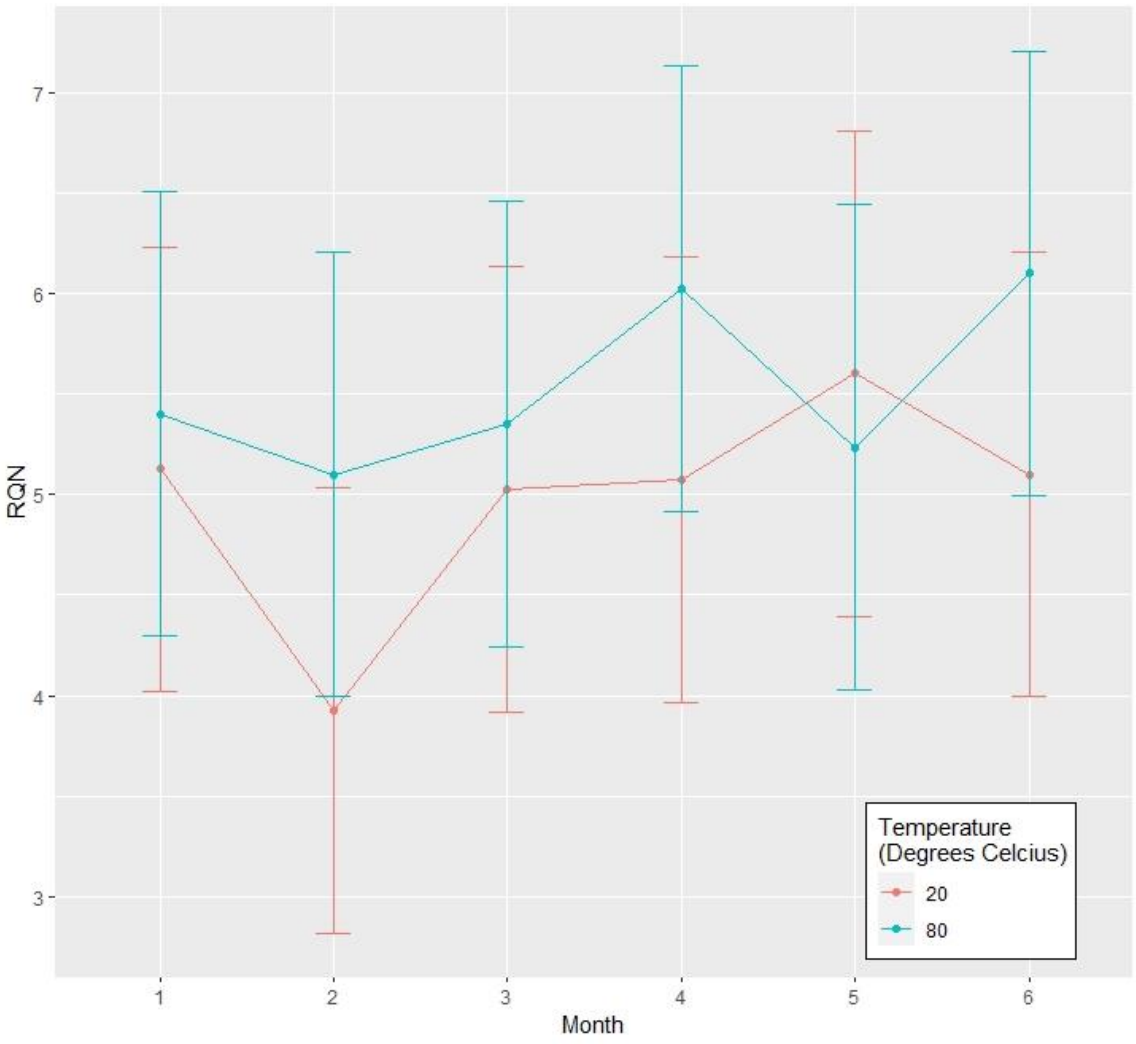

Figure 2.2: LS-Means for RQN Response to Treatments-Leaf

Comparison of the leaf tissues' RQN response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. No significant differences were identified, but samples stored in -80℃ produced higher RQN values, > 5, for all six months.

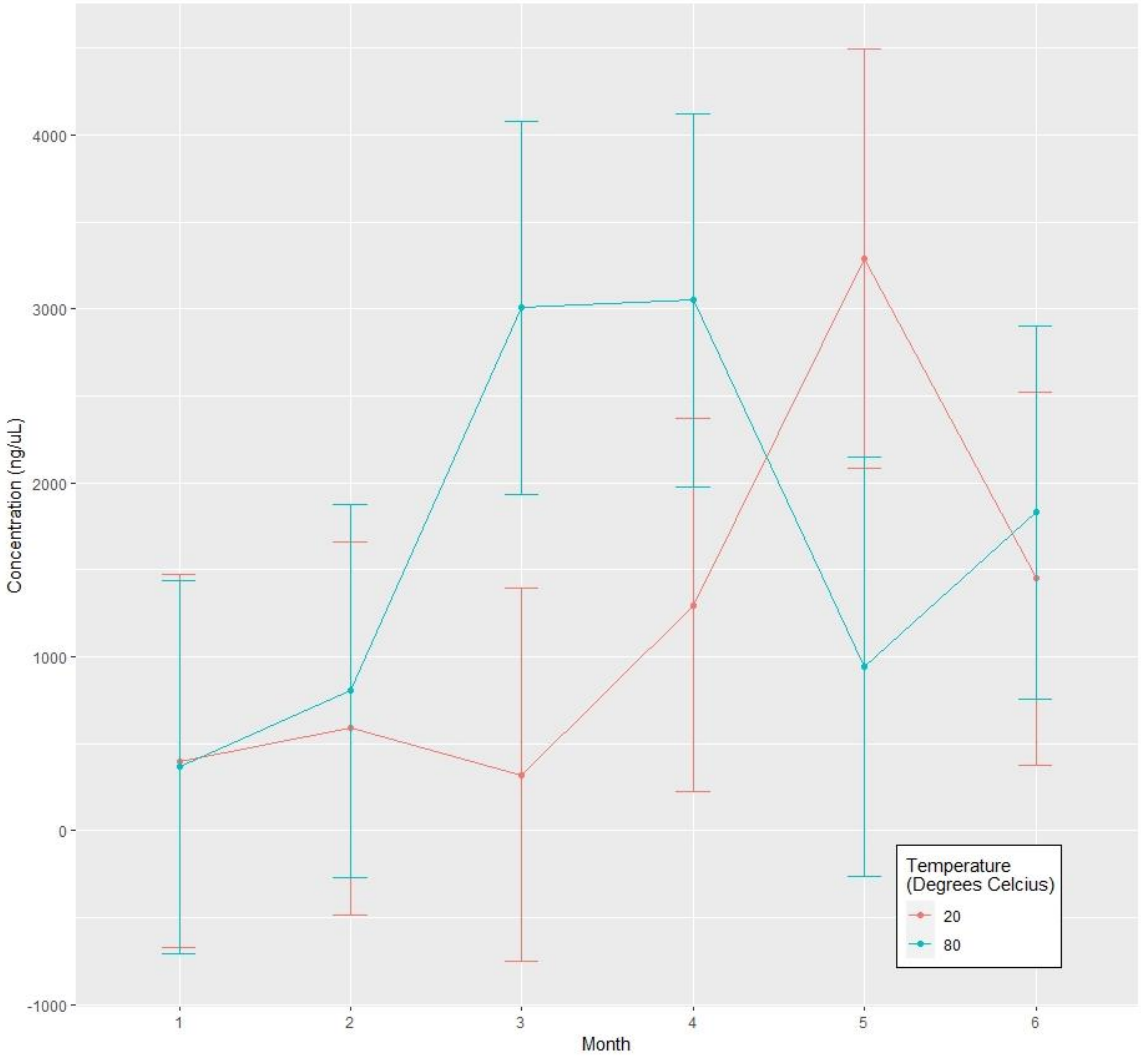

Figure 2.3: LS-Means for Fragment Analyzer Concentration Response to Treatments-Leaf

Comparison of the leaf tissues' concentration response (reported from the fragment analyzer) to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. A significantly higher concentration was produced from the samples stored in -80℃ compared to those in -20℃. No other significant differences were identified, but samples stored in -80℃ produced higher concentrations, > 500 ng/µL, on average for all six months.

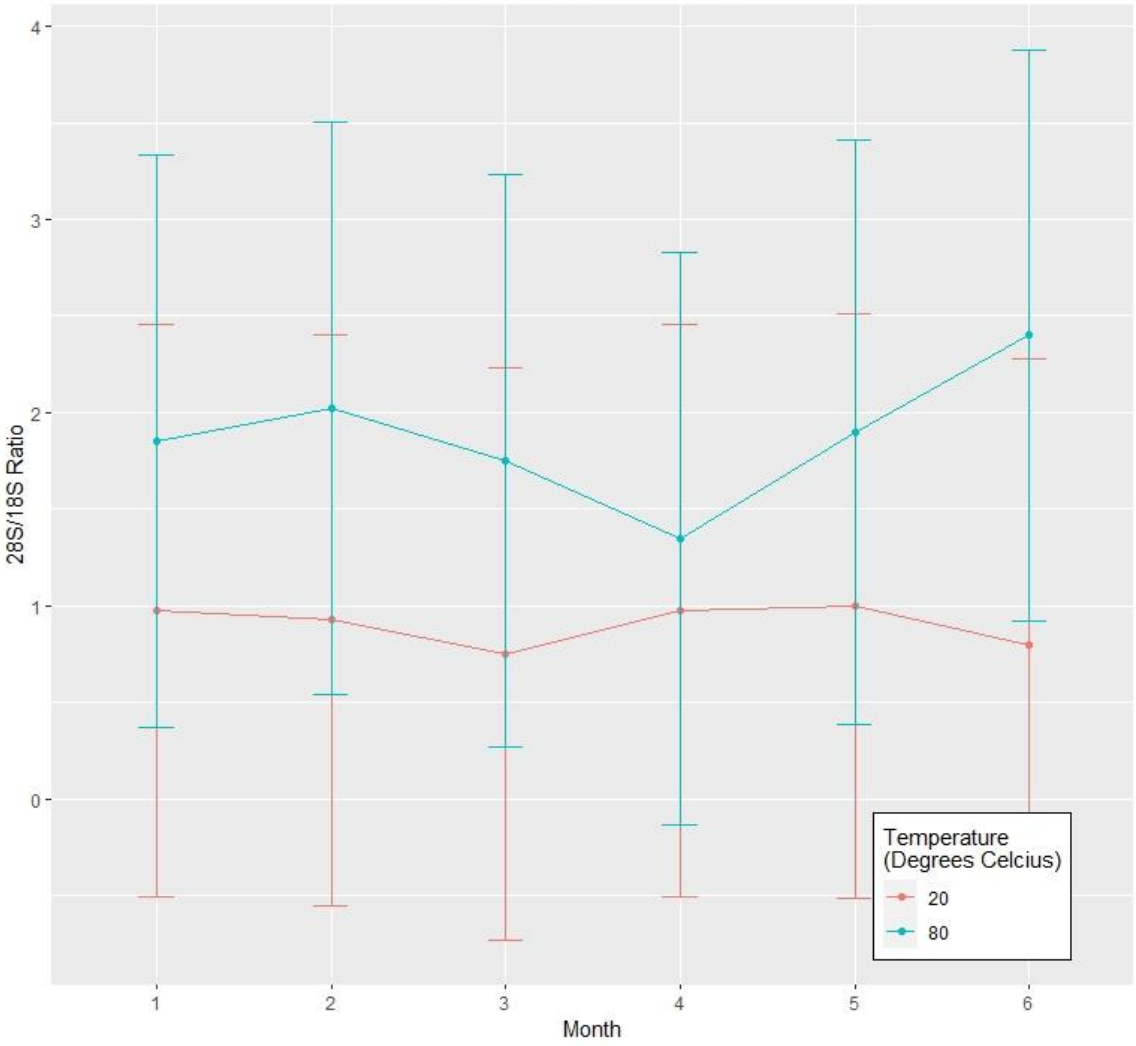

Figure 2.4: LS-Means for 28S/18S Ratio Response to Treatments-Leaf
Comparison of the leaf tissues' 28S/18S response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. No significant differences were identified, but samples stored in -80℃ produced ratios closer to 2 for all six months.

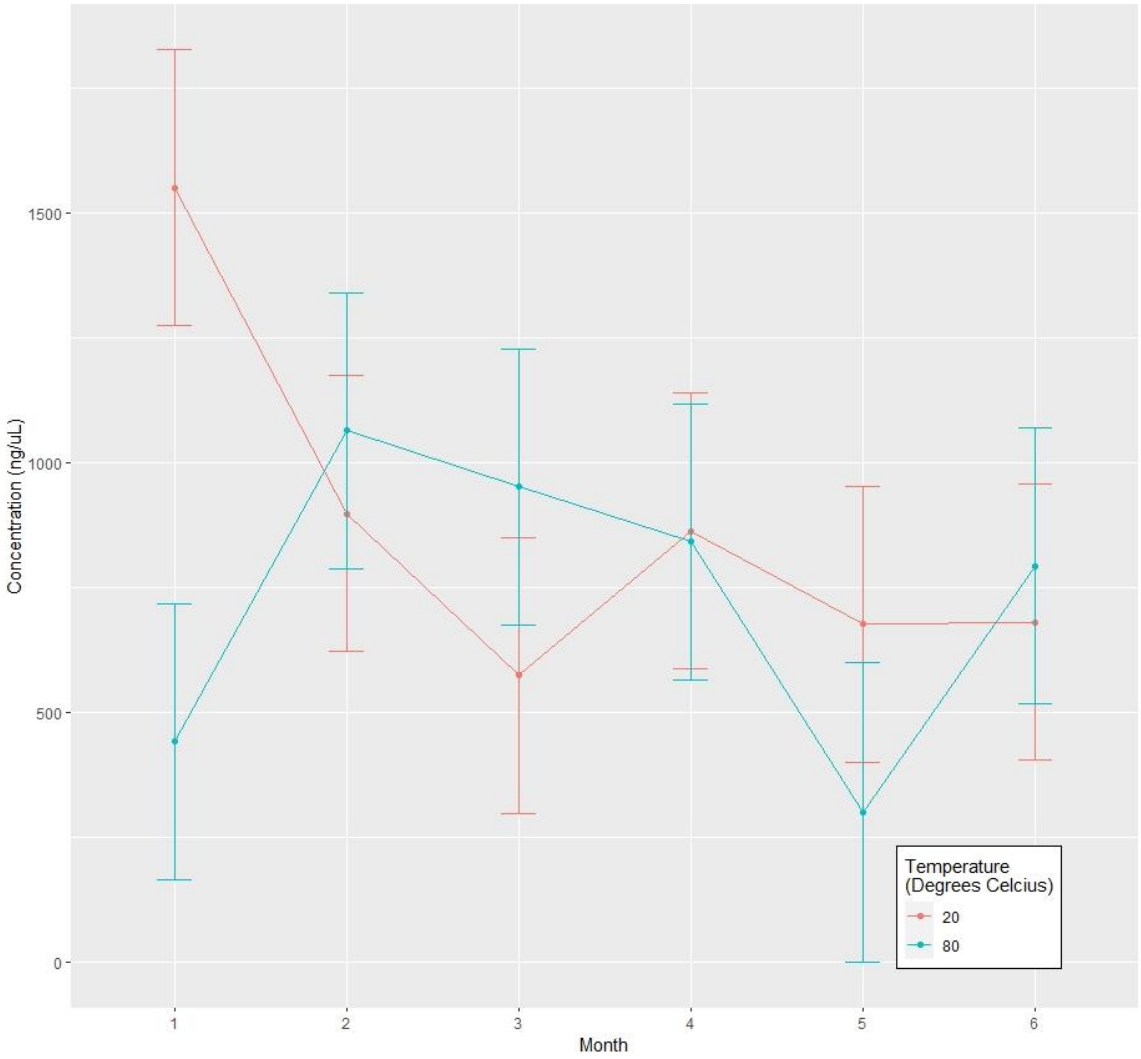

Figure 2.5: LS-Means for NanoDrop Concentration Response to Treatments-Leaf Comparison of the leaf tissues' concentration response (reported from the NanoDrop™) to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. A significantly higher concentration was produced from the samples stored in -20℃ compared to those in -80℃ in month one. No other significant differences were identified, but samples stored in -80℃ produced higher concentrations, > 500 ng/µL, on average for the other five months.

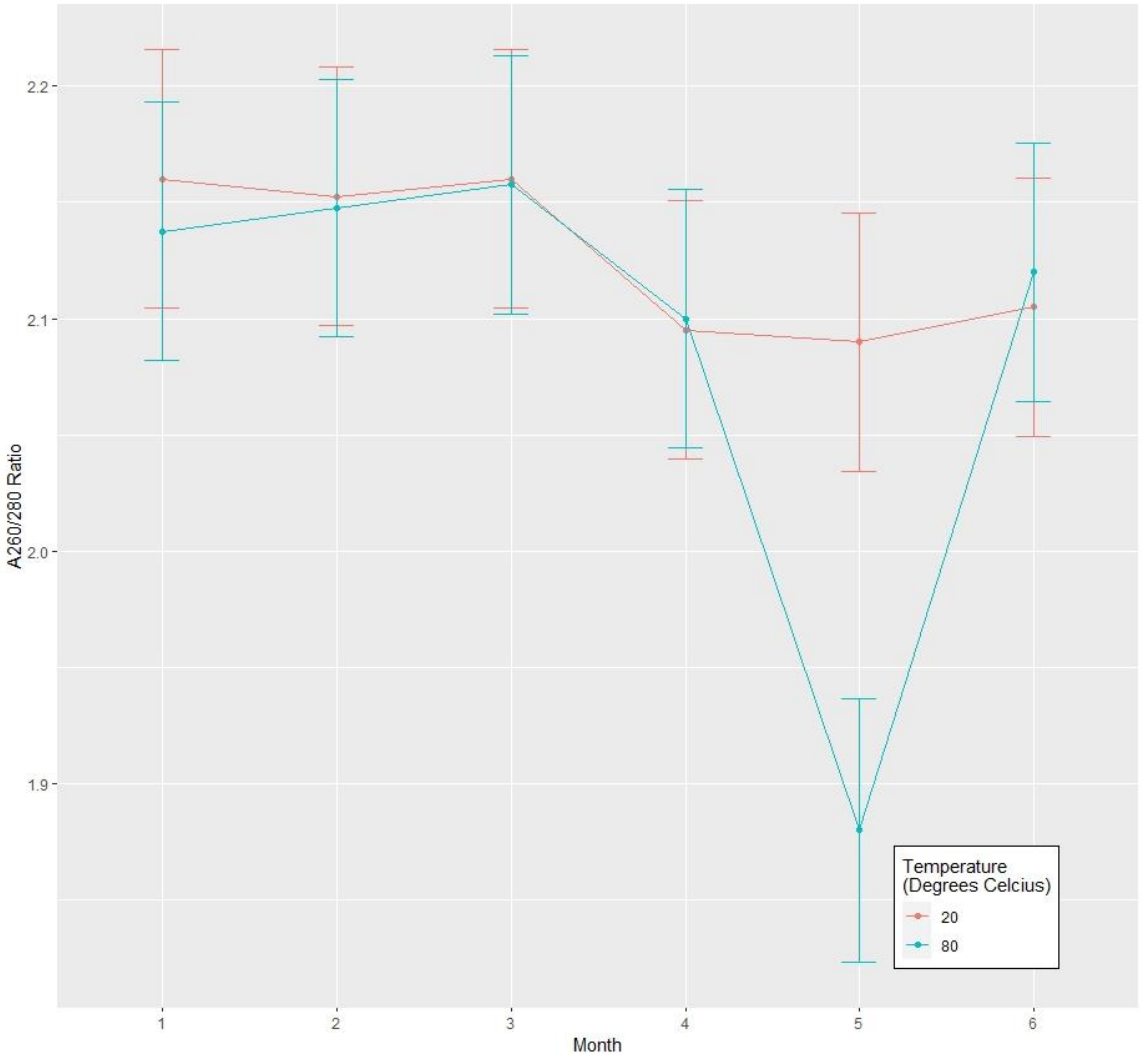

Figure 2.6: LS-Means for $A_{260/280}$ Ratio Response to Treatments-Leaf

Comparison of the leaf tissues' $A_{260/280}$ response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. A significant difference was seen in month five where samples stored in -20℃ produced higher ratio response than those in -80℃. There was no other difference between the storage month or temperature.

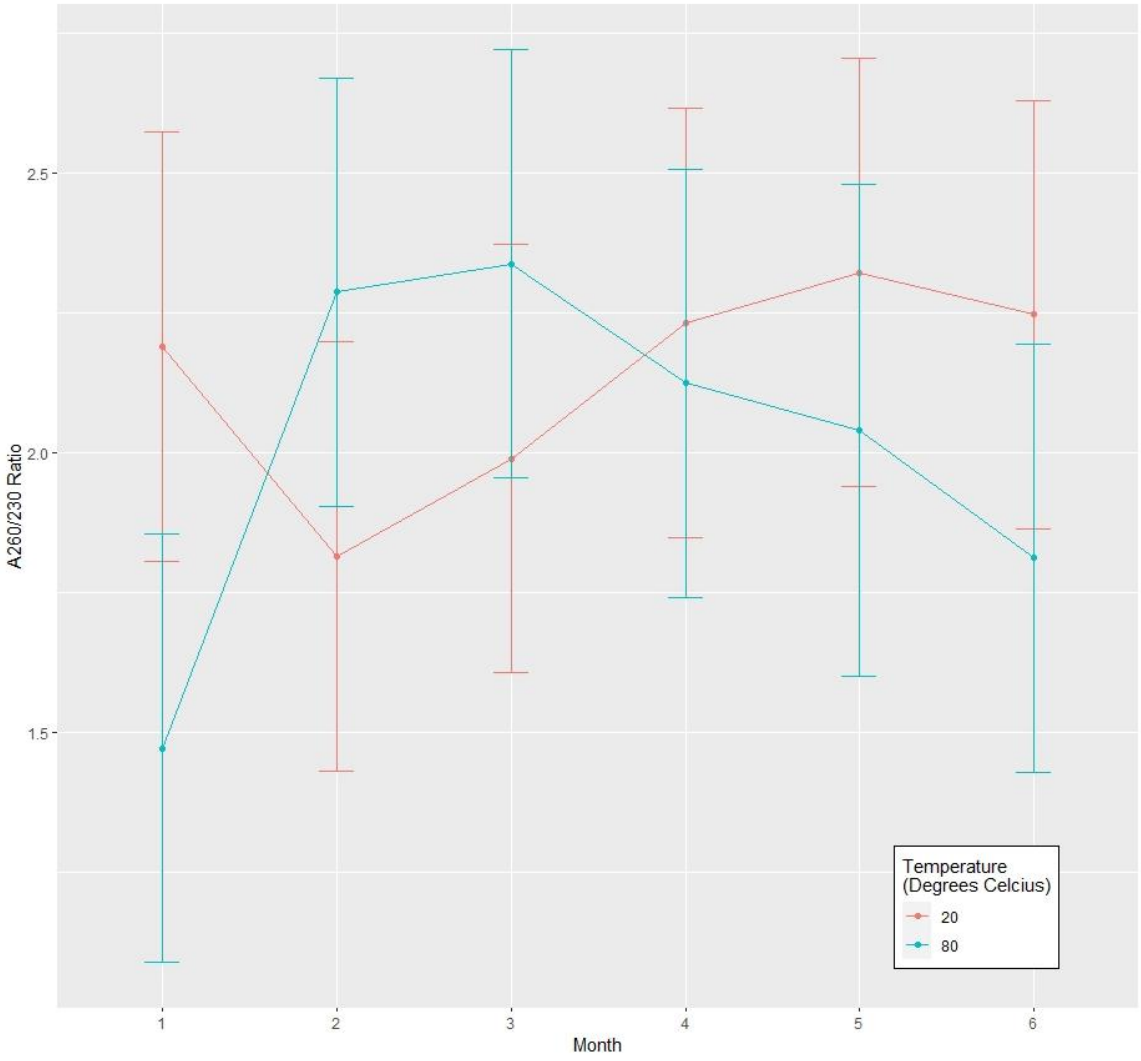

Figure 2.7: LS-Means for $A_{260/230}$ Ratio Response to Treatments-Leaf

Comparison of the leaf tissues' $A_{260/230}$ response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. There was no difference between the storage month or temperature and unusual variation present.

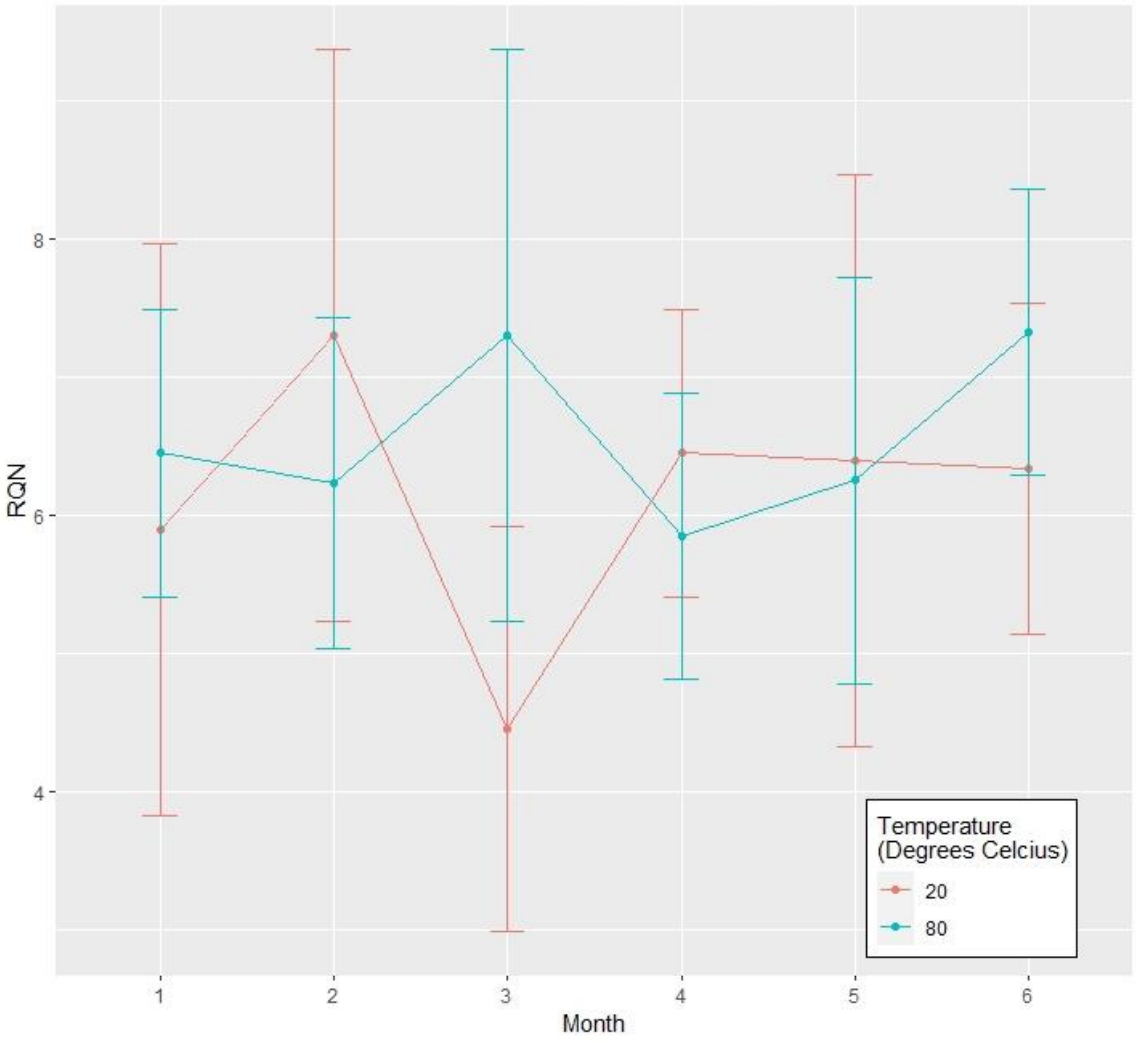

Figure 2.8: LS-Means RQN Response to Treatments-Seed Samples

Comparison of the seed tissues' RQN response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. There was no difference between the storage month or temperature and unusual variation present, but most samples had an RQN > 5.

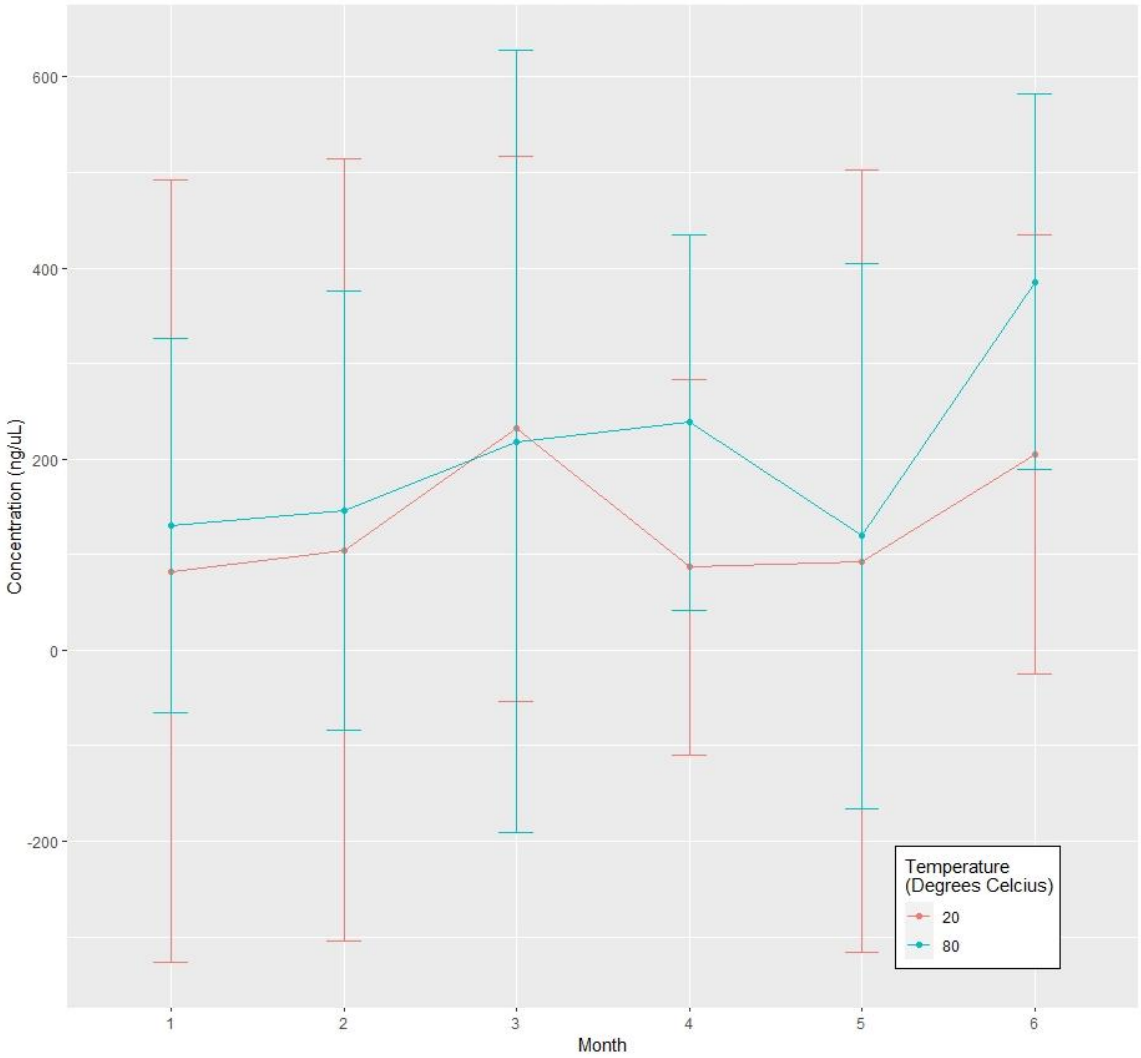

Figure 2.9: LS-Means for Fragment Analyzer Concentration Response to Treatments-Seed

Comparison of the seed tissues' concentration response (reported from the fragment analyzer) to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. No significant differences were identified, but samples stored in -80℃ produced higher concentrations, > 100 ng/µL, for all six months.

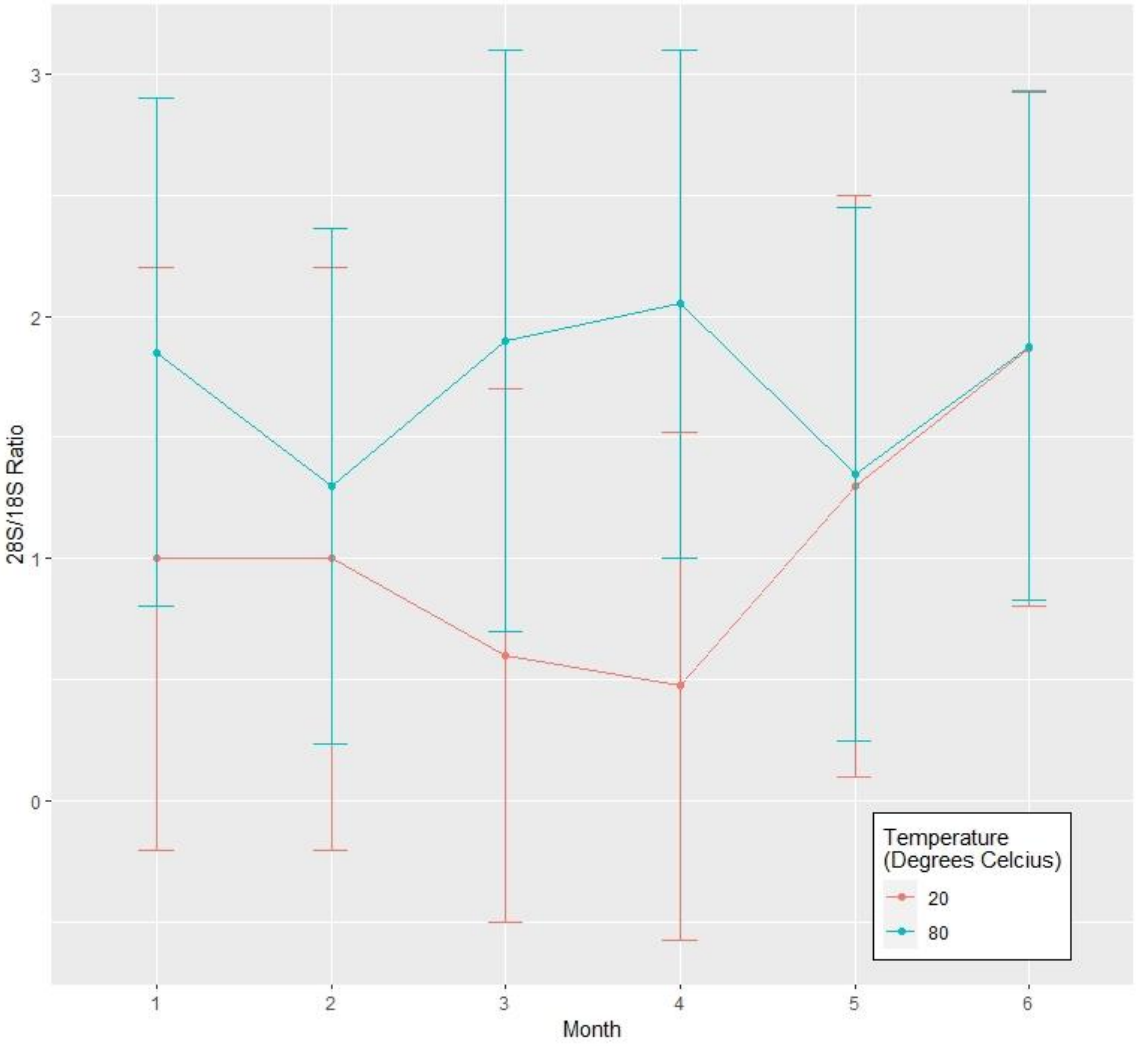

Figure 2.10: LS-Means for 28S/18S Ratio Response to Treatments-Seed
Comparison of the seed tissues' 28S/18S response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. No significant differences were identified, but samples stored in -80℃ produced ratios closer to 2 for all six months.

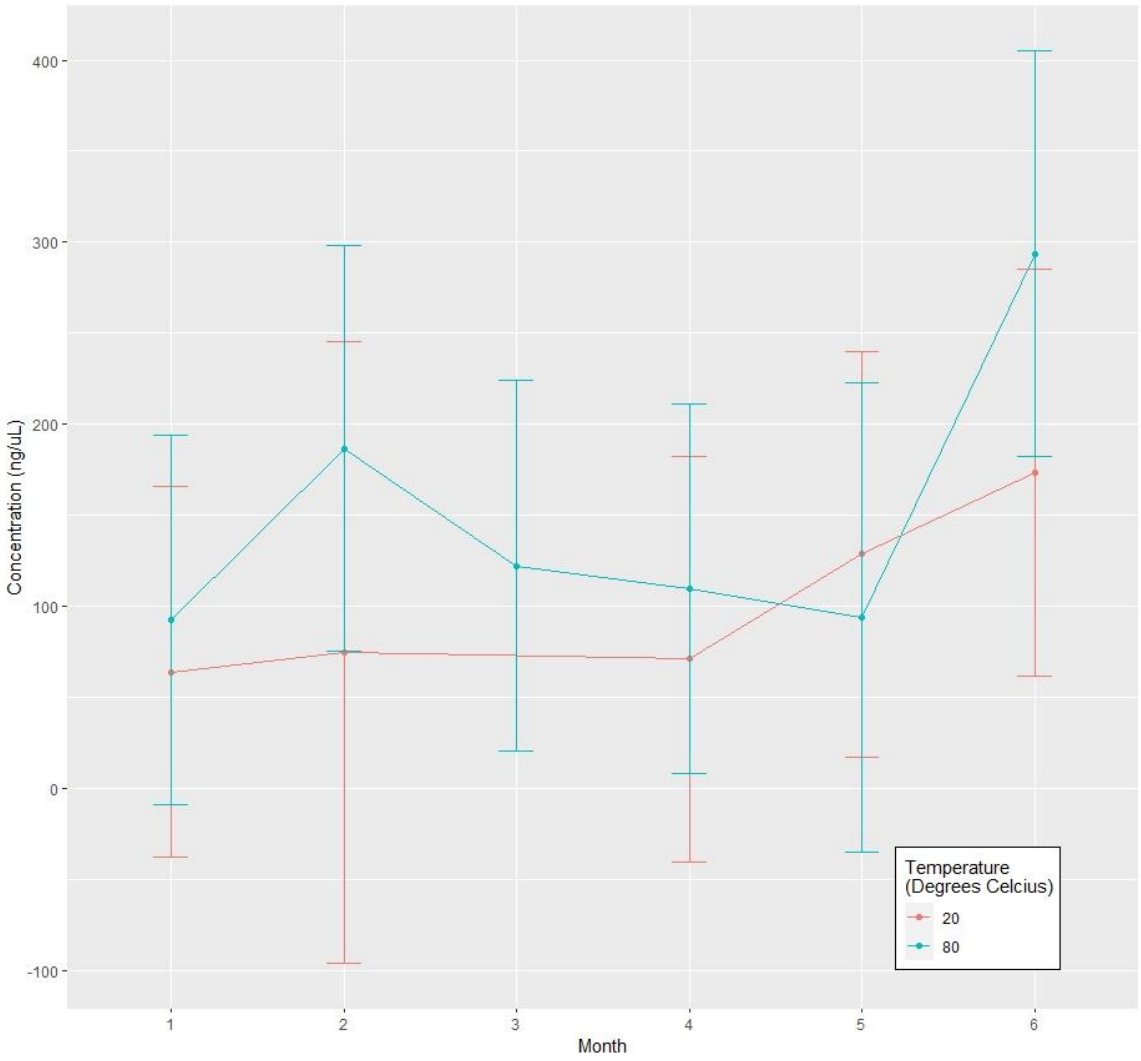

Figure 2.11: LS-Means for NanoDrop Concentration Response to Treatments-Seed Comparison of the seed tissues' concentration response (reported from the NanoDrop™) to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. No significant differences were identified, but samples stored in -80℃ produced higher concentrations, > 100 ng/µL, on average for the six months.

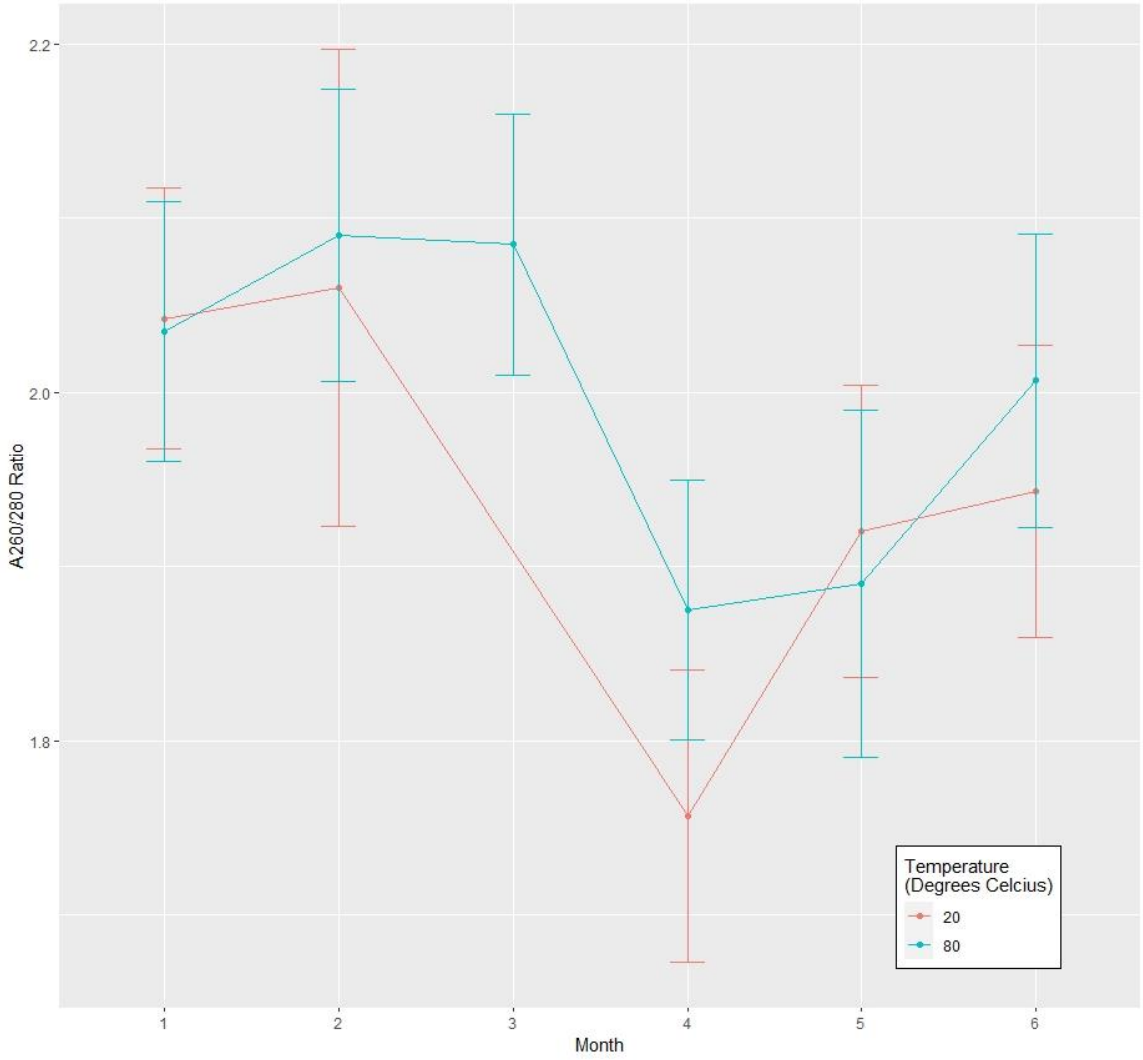

Figure 2.12: LS-Means for $A_{260/280}$ Ratio Response to Treatments-Seed

Comparison of the seed tissues' $A_{260/280}$ response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. No significant differences were seen for storage length and temperature, but the ratio decreased from month two through six. Samples stored in -80℃ produced higher ratios, > 1.8, across the six months which means the samples were better preserved at that temperature.

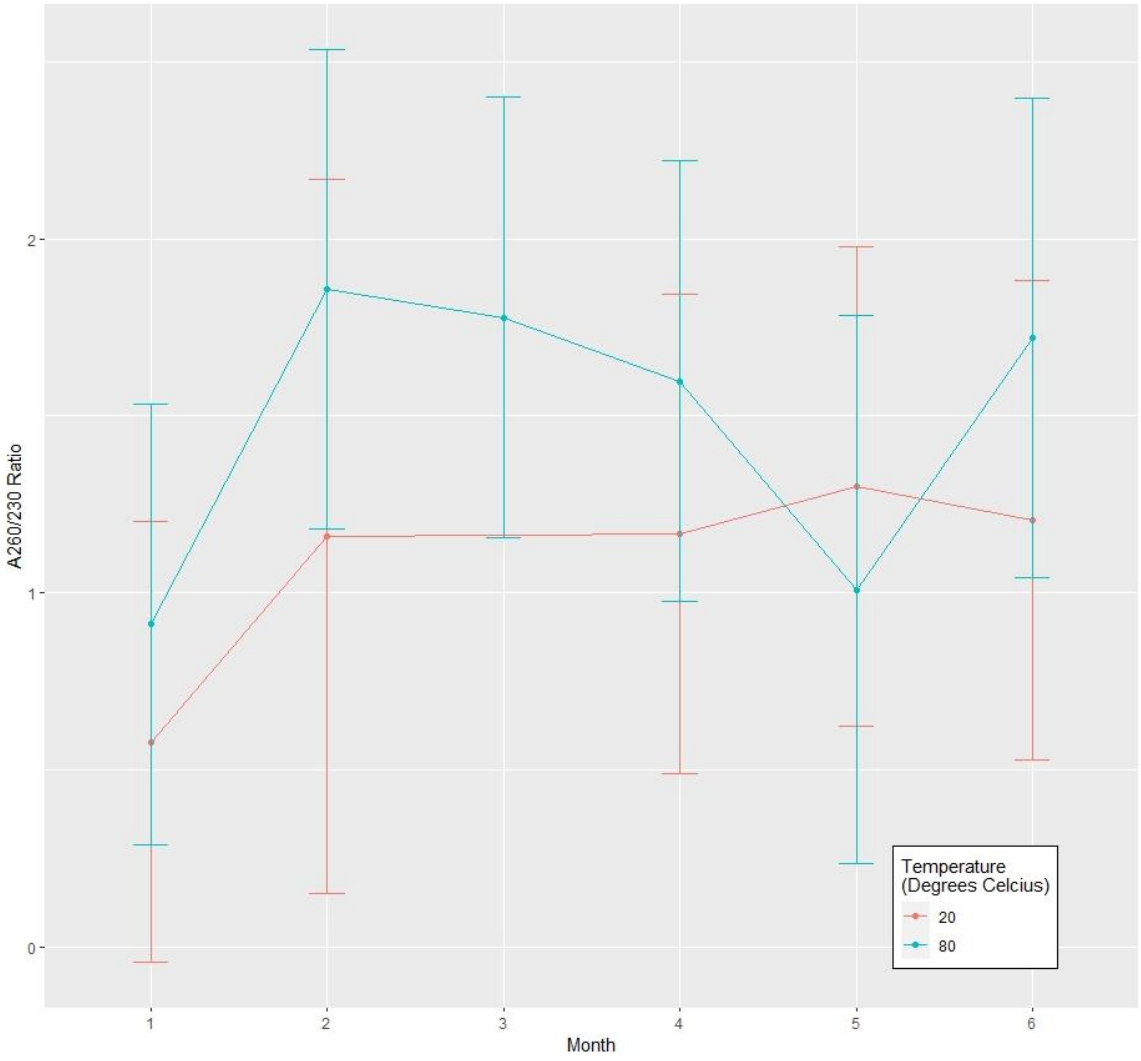

Figure 2.13: LS-Means for $A_{260/230}$ Ratio Response to Treatments-Seed

Comparison of the seed tissues' $A_{260/230}$ response to storage length (1-6 months) and storage temperature (-20℃ and -80℃) using 95% confidence intervals. No significant differences were seen for storage length and temperature, but the ratio decreased from month two through six. Samples stored in -80℃ produced higher ratios, > 1.0, across the six months.