Impacts of charcoal rot (*Macrophomina phaseolina)* epidemiology on drought resistant soybean cellular metabolism and accompanying tissue microbiome for identifying alternative breeding targets under increasing environmental stress, 31-2022

Annual Report

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ANNUAL REPORT OF PROGRESS/ACTIVITY

Charcoal rot disease of soybean, caused by a common soilborne fungus known as *Macrophomina phaseolina* (*Mp*), is a major pathogen in soybean production areas in Mississippi and worldwide. This disease resulted in the estimated loss 220 billion dollars from 2010 to 2014. The fungus causes stalk rot or charcoal rot disease in more than 500 plant species worldwide, including agricultural, horticultural, woody shrub, and tree species. Charcoal rot disease is an endemic problem in Mississippi where mid to late summer is relatively dry and often under drought conditions. The disease onset coincides with plant stress from heat and drought, especially when pods are being set and nutrients are being used for pod fill. Traditional methods for controlling this disease such as fungicide have only shown limited success. Progress in breeding for resistance is slow, largely due to multi-gene disease associations. Researchers attempting to identify genetic targets influencing severity of *Mp* infection have not been able to locate redundant data pinpointing specific genes, thus suggesting that the molecular mechanisms for resistance to this fungus are complex. Alternatively, diagnostic biomarkers associated with plant cellular metabolites may provide evidence for selection of varieties exhibiting. When plants are under drought stress, chemical signaling initiates physical and physiological changes to reduce water loss. This signaling cascade improves tolerance to stress, but prolonged exposure to stressors results in increased levels of reactive oxidative species (ROS), which results in a feedback loop that causes further cellular damage. Since ROS species are transient, to determine the extent of ROS occurrence activity, we must indirectly qualify and quantify antioxidant scavenging metabolites and enzymes. These can include glutathione, ascorbate, catalase and superoxide dismutase, and malondialdehyde, among others. In addition, the presence of glycolytic and citric acid cycle associated amino acids, ketone bodies and intermediate metabolites for energy formation may provide key metabolic differences between drought-resistant soybean varieties and those that are susceptible. The impacts of infection and drought processes are intertwined, and investigating both is a high research priority.

Another indirect approach potentially implicated in *Mp* resistance is found in soybeans' endophytic communities. Vascular plants host a hyper-diverse microbial community of endophytic bacteria and fungi, which colonize internal tissues of their host plants but do not cause any visible signs of tissue damage or adverse effects on the host. The effects of endophytes on host plants can be direct or indirect; studies with fungal endophytes have shown reduced disease and/or improved plant growth. These endophytes conduct important ecosystem functions such as absorption of non-mobile nutrients from the soil and their translocation to host plants, facilitation of interplant transfer of nutrients, and beneficial modification of plant-water relationships. Analysis of soybean endophytes can direct efforts to understanding if (and if so, which) key microbes are associated with temperature/drought resistant soybean varieties and can potentially serve as indicators for selection of disease resistant varieties.

Metabolomics research using the ^{1}H proton nuclear magnetic resonance (NMR) platform can provide metabolic profiles for plant disease or other stress factors, and these metabolic fingerprints could provide biomarkers for healthy/drought resistant *Mp* infected/uninfected soybeans during the pathogen's disease

cycle. Critical stages of *Mp* infection in soybean initiate host responses that impact the metabolome. NMR is a rapid, inexpensive, and high throughput detection technique for determining metabolites that may indicate potential defense mechanisms within host plants. NMR spectra can be used to analyze the presence of a variety of compounds, from carbohydrates to amino acids. Many of these metabolites are associated with host cell responses, redox signaling, and energy production critical to plant growth. If hybrid resistance to *Mp* does occur through drought resistance, these metabolic biomarkers could provide targets for downstream genetic marker identification, thereby guiding new breeding strategies. Metabolomic analysis platform using ^{1}H NMR can be used to assess metabolic shifts in the plants during pathogen invasion and progression that may guide or support research into genetic resistance or other control measures for *Mp.* Results from the profiles can provide breeders with information for new approaches or genetic markers for new control strategies. The objectives for this research initiative included:

Objective (1) Microbial whole community analysis of fungal/bacterial endophytes in drought tolerant and susceptible varieties infected with *Mp* in two greenhouse trials (normal watering in Trial 1 and drought stressed in Trial 2). Lab isolation confirmations of *Mp* from root tissue samples will be done to confirm MP infection during both greenhouse trials.

Research Completed: Two greenhouse tests were established in 2022. The first greenhouse failed due to issues with greenhouse temperature controls. Consequently, an additional study design was evaluated. **Reddy Soybean Environmental Study:** This study included drought resistance, temperature tolerance, and CO_2 levels as main treatments. Dr. Reddy, MSU, suggested we use samples from his soybean study with plants grown at high temperatures, drought and CO_2 differences in humidity-controlled chambers (SPAR unites) using a high temperature tolerant variety (DS25-1 (HT)) compared with a standard control variety (DS31-243 (DT)). We used the samples from the study to evaluate levels of essential ROS-associated compounds, metabolic variation of important cellular metabolites, and to determine the microbial communities under these conditions.

Microbial community portion of study delayed (6-7 Months): This portion was delayed for two reasons: 1) Funds from MSU were not delivered to University of Memphis until September 2022. 2) Failure of the first greenhouse study required delay until the termination of the additional study. Following completion of the Reddy study, plant tissue samples were sent to University of Memphis in October. As a result of this delay, the microbial community research could not begin after January 2023.

Despite delays in receiving samples at University of Memphis, analyses have proceeded in a timely fashion. All DNAs (n=240) from the Reddy study have been extracted and libraries have been prepared. However, we are still waiting on the second greenhouse study. Unfortunately, at the time of this report we do not have any results to show as these sequencing libraries are currently in queue in the sequencing center, Kansas State University. These molecular-based community analyses are time consuming and are difficult to finish within a 12-month window, but we are nearing the finality of this process. We will generate one full sequencing reaction (~25 million sequences) for both the fungal and bacterial endophytic communities associated with differential climate change scenarios. These results will provide insight into how soybean-associated microbial communities will be impacted by potential climate alterations. We are hopeful that these results will inform management and best practices to minimize harm and yield losses associated with differential carbon dioxide levels, temperature, and water availability that Mississippi will experience soon. We anticipate sequences to be returned in the next few weeks, with the analyses beginning immediately. It is our expectation that we will have and understanding of how climate dynamic impacts endophyte communities within the next three months and these results will be presented in a peer reviewed journal article to be submitted soon thereafter. Below is outlined the metagenomic library preparation conducted and a summary of analytical methods to be conducted along with expected outcomes.

Objective (2) Plant tissue subsamples collected from Reddy treatments in (1), an untargeted and targeted metabolomic analysis using NMR was conducted emphasizing oxidative stress and energy producing metabolites. **Thirty metabolites were selected for preliminary analysis,** given their essential roles in potential drought and stress tolerance and metabolism. A broader metabolite search will be done as year 2 data is completed. The selected metabolites are detailed in Table 2. These metabolites do not constitute an exhaustive list of the metabolites detected, given the convergence of signals, especially around 3 ppm. Many of the metabolites detected with NMR are associated with cellular health and plant energy. For example, proline, generally synthesized in leaves, is transported to roots from stress dependent accumulation due to water deficit, thereby supporting osmotic pressure while maintaining protein and cellular membrane stability and reducing damaging ROS free radicals. Depending upon further studies of soybean plants pre- and during *Mp* infections, metabolites such as proline could be critical biomarker in future breeding for research. Several other metabolites associated with ROS reduction such as ascorbate and myo-inositol were detected, as well as other essential pathway metabolites associated with energy (ATP) production such as pyruvate, glucose, and formate. Further repetitive studies confirming their roles in cellular health and environmental tolerances will be conducted.

Reactive Oxygen Species Biomarker Study (Reddy Study Samples)

Reactive oxygen species (ROS) are essential components of metabolism. Although they contain free radicals, these molecules are produced because of metabolic processes and serve a variety of functions ranging from signaling to plant defense from pathogens. Despite their essential roles, production of ROS can be stimulated by both abiotic and biotic stressors, resulting in an overaccumulation that can initiate fatty acid peroxidation, DNA degradation, and further adverse effects.

Mp is believed to benefit from plant cellular damage following biotic or abiotic stress, as the production of ROS and fatigue on hosts' antioxidant capabilities may provide a foothold for *Mp* infection. Furthermore, given *Mp's* predilection for arid and hot conditions, we hypothesized that soybeans that are already under abiotic stress are likely more susceptible to *Mp* infection, and that these ROS levels are correlated with the severity of infection. To investigate this matter, four compounds were selected for analysis: **glutathione peroxidase, glutathione reductase, hydrogen peroxide, and malondialdehyde**. The majority of ROS are extremely volatile and therefore difficult to quantify, necessitating the analysis of the compounds that directly engage with ROS instead. Below, Figures 1-4 show results of the ROS assays, and demonstrate meaningful concentration not only between the soybean varieties but also between treatments.

Results and Discussion

ROS Based Data: The results of the ROS kits were analyzed using IBM SPSS software. From the results of the ANOVA performed on the samples for each assay, it is the overall main effect of treatments has a significant impact on the ROS activity of the soybeans. This is to be expected, as free radical scavenging under ideal environmental conditions is likely to be significantly different than the activity necessary to maintain homeostasis while under varying stressors. By contrast, the relative insignificance of the cultivar on the ROS activity is a surprising result but may be a result of the pooling of the samples across dates.

However, the only assay that did demonstrate a significant difference in levels between the two cultivars, MDA, may be key to maintaining soybean cellular integrity. The capacity to mediate ROS attack on cell integrity is critical to maintaining integrity of the cell wall, which has a high lipid content. A weak endogenous defense leads to damage to cell structures and molecules such as lipids, proteins, and DNA, ultimately contributing to the pathogenesis by *Mp* and other soilborne pathogens. Although there was a

significant difference in MDA content between the two cultivars, this difference was highly variable across treatment groups.

Of all the assays, only glutathione peroxidase demonstrated a statistically significant interaction effect between the cultivar and the experimental group, although glutathione reductase would have a significant interaction effect at a 90% confidence interval. This is interesting given the fact that glutathione peroxidase directly interacts with the hydrogen peroxide as a scavenger molecule, and a significant interaction effect between the cultivar and group for this assay points towards an underlying metabolic difference between DS31-243 (DT) and DS25-1 (HT) that may involve the preference towards utilizing one antioxidant pathway over another. However, these findings are preliminary needing additional study.

For the control treatment (SPAR1), variety HT exhibited lower glutathione reductase activity and hydrogen peroxide concentration, but higher glutathione peroxidase activity than variety DT. This data indicates that under ideal conditions, HT is potentially capable of converting hydrogen peroxide into water more efficiently. This trend is also observed in SPAR5, the drought treatment.

However, for the temperature-related challenges SPAR2 (high temperature), SPAR3 (low temperature), and SPAR4 (high night temperature), the DT variety exhibited higher glutathione peroxidase activity. Despite this, for SPAR2 and SPAR4, DT exhibited statistically significant higher concentrations of hydrogen peroxide and similar concentrations of hydrogen peroxide to cultivar HT for SPAR3. The glutathione reductase activities in SPAR2 and SPAR4 were similar for both cultivars, but for SPAR3 cultivar DT actually exhibited significantly higher glutathione reductase activity. The persistently high levels of hydrogen peroxide content in cultivar DT, despite upregulation of glutathione peroxidase, indicates that DT is likely not as efficient as HT in managing ROS through this pathway.

Results and Discussion

NMR-Based Metabolite Data: Following optimization of the extraction protocol, NMR spectra were successfully obtained. Analysis of the metabolites detected was performed using Chenomx which revealed significant variations in metabolite concentrations. For preliminary analysis of metabolic variations, a two-way mixed design ANOVA using IBM SPSS was utilized with metabolite concentration as the dependent variable, variety as the independent variable, and date of harvest as a covariate.

In the control treatment (SPAR1), 6 metabolites exhibited a statistically significant difference in concentration between the two varieties across all sampling dates: arginine, aspartate, cysteine, phenylalanine, pyruvate, and tryptophan. For all of these metabolites (except pyruvate), the HT variety exhibited a higher concentration than DT. Many of these metabolites are tied to pyruvate metabolism, which is critical to energy production. Additionally, the high concentration of cysteine in the HT variety is of interest, as cysteine serves as a substrate for the synthesis of glutathione. Even though high levels of serine were not significantly different between the two varieties, the amino acid serine has a fundamental role in plant metabolism, plant development, and cell signaling. In addition to being a building block for proteins, L-Ser participates in the biosynthesis of several biomolecules required for cell proliferation, including amino acids, nitrogenous bases, phospholipids, and sphingolipids. Further research will center on this amino acid role in cell health during plant stress and *Mp* pathogenesis (Tables 2-10).

In the high temperature treatment (SPAR2), 7 metabolites exhibited a statistically significant difference in concentration between the two varieties across all sampling dates: arginine, asparagine, aspartate, citrulline, myo-inositol, proline, and trehalose. In plants, arginine biosynthesis occurs in the chloroplast, and is a key component of the pathway for the synthesis of proline, which can serve as an osmoprotectant. In response to stress, asparagine accumulation can occur. However, whether this accumulation serves as a stress mitigator or if it is a consequence of stress endurance is not yet understood. Given that the HT variety exhibited consistently lower asparagine concentrations in comparison to DT, HT may be capable of mitigating the consequences of the high temperature conditions, thus minimizing the accumulation of

asparagine. Very important organic N for protein formation/transport is building block, or precursor to formation of many key metabolites associated with cellular health and in seed development.

In the low temperature treatment (SPAR3), 4 metabolites exhibited a statistically significant difference in concentration between the two varieties across all sampling dates: citrate, formate, malate, maltose. Formate is a terminal product of several essential metabolic pathways, such as photorespiration and the Krebs cycle, and feeds into additional pathways such as the Calvin-Benson cycle and the production of tetrahydrofolic acid (THF) which is an essential vitamin. Malate and citrate are intermediary metabolites in the TCA cycle, and the decreased levels of these metabolites in the HT variety compared to the DT variety may indicate a decrease in efficient ATP production in low temperatures.

In the high night temperature treatment (SPAR4), only 1 metabolite exhibited a statistically significant difference in concentration between the two varieties across all sampling dates: (glycine) betaine. Glycine betaine is implicated as an osmoprotectant, and in SPAR4 the DT variety exhibited consistently higher concentrations in comparison to the HT variety. Although several other compounds appear to have significant variations between the two varieties, when pooled across all three sampling dates, the wide variations in these concentrations resulted in higher standard deviations and thus a lack of statistical significance between the concentrations.

In the drought stress treatment (SPAR5), 4 metabolites exhibited a statistically significant difference in concentration between the two varieties across all sampling dates: arginine, glutamine, phenylalanine, and tyrosine. The shikimate pathway has two key branch points that determine the resultant products: chorismate can either be converted to prephenate or to anthranilate in a mechanism that converts glutamine to glutamate and pyruvate. If the former path is followed, arogenate is converted to either phenylalanine or tyrosine; if the latter path is followed, the product is tryptophan. The HT variety exhibited consistently higher phenylalanine content in comparison to the DT variety, but also exhibited consistently lower tyrosine content. This may indicate that HT uses tyrosine over phenylalanine as the primary entrance into the phenylpropanoid pathway, which is essential to plant development and is central to the production of most plant metabolites. The low levels of glutamine but high levels of arginine exhibited by the HT variety in comparison to the DT variety may indicate an upregulation in the production of arginine, which is an essential precursor to polyamine production and may mitigate drought stress.

From these data, arginine may be an essential metabolite in efficient response to abiotic stress in soybean. At present, the role of arginine in plant metabolism is not fully understood. Current research has indicated that it may serve as a precursor to proline biosynthesis and as a precursor to nitric oxide formation, the latter of which can be an ROS in high concentrations but is also an essential signaling molecule and is essential to regulating a plant's response to ROS because of abiotic stress.

Objective (3) Through strong statistical inference and experimentation, the holistic data will be analyzed to elucidate and three-party interactions among endophytic microbes, the fungal pathogen, and the host plant.

University of Memphis Metagenomic Bioinformatics Portion of Study: Sequencing analyses and Bioinformatics

To test if fungal and bacterial communities differ across treatments and time, a permutational multivariate analysis of variance of average Bray-Curtis dissimilarity values (iteratively subsampled as above) will be conducted in the program R with the package vegan (function adonis2 with 999 iterations, strata=individual plant to facilitate repeated measures) and post-hoc multiple comparisons will be examined using the package RVAIDeMemoire (function pairwise.perm.manova with FDR corrections, 999 iterations). To visualize communities, nonmetric multidimensional scaling (NMDS) will be conducted. Further, we will examine patterns of common core taxa (OTUs) across our experimental

framework. To identify core taxa, we will compile a list of OTUs that are present in at least 90% of the samples in our experiment. Core OTUs will be tested using repeated measures ANOVA (relative abundance, logit transformed, sampling effort [time] as a categorical variable) to examine if these core taxa change in abundance across treatments and time, with Kenward-Roger first order approximations with Kacker-Harville corrections. When treatment have a significant effect, Tukey HSD post-hoc tests will be conducted to identify how treatments differ and effect sizes were deter-mined using partial eta-squared (partial $\eta 2$). Additionally, to visualize changes in the relative abundances of core taxa over time (continuous), we will fit Kernel Smoothing lines using linear local fits, tri-cube weighing, and four iterations to derive best fit lines. Since we expect to observe significant community and OTU-based time and treatment effects, we will aim to identify biomarker OTUs for treatment conditions for each timepoint. To do so, we used the mothur implementation of LEfSe which will identify biomarker OTUs separately for each sampling timepoint after Kruskal Wallis and Wilcoxon tests to determine a signed LDA log-score and associated p-values.

Connecting Metabarcoding data and Metabolite Data – These metabarcoding results will be integrated with metabolome data (ROS and NMR) generated by Dr. Baird's lab at Mississippi State University. Using these results, we will use a neural networks approach to connect experimentally generated microbiomes with metabolites using a biclustered interaction network (MiMeNet). These results will be invaluable for identifying groupings of associated microbes and metabolites that are predicated to be associated with different climate change scenarios. Depending on the identity of the associated microbes and metabolites, we can begin to fundamentally understand of how predicted changes in climate regimes will impact soybean-microbial interactions, which will have major implications for plant productivity and health.

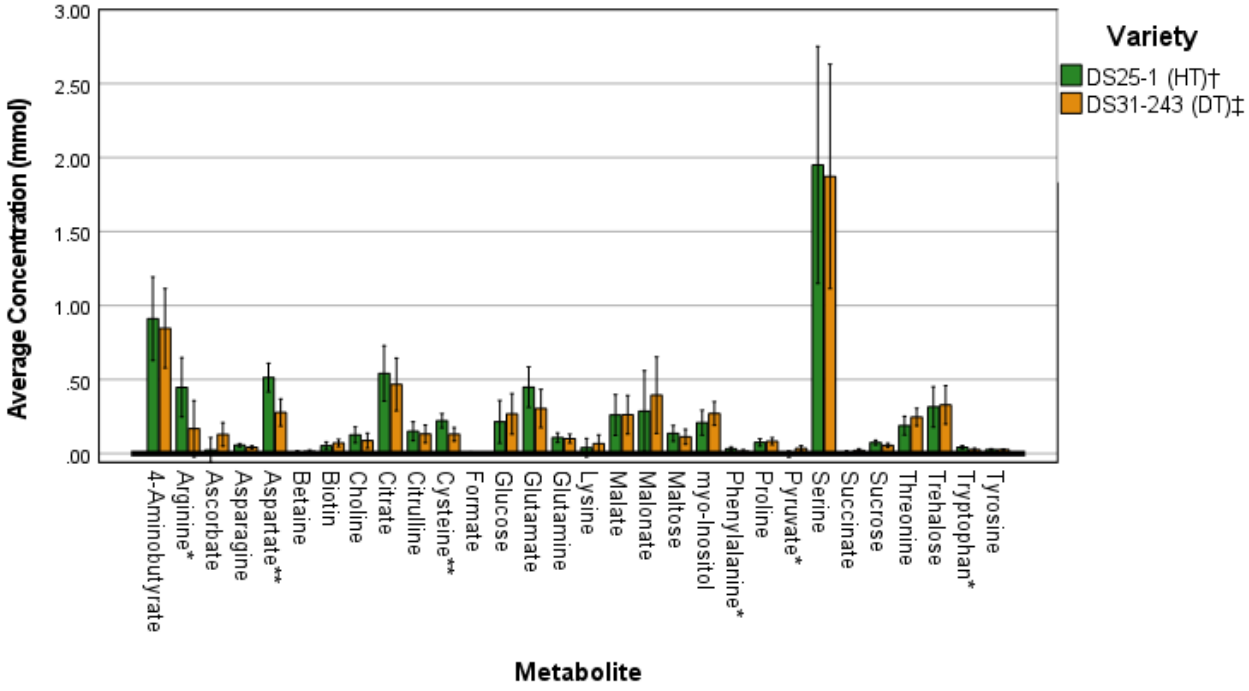
Tables and Figures

Table 1. Timeline for Metagenomic (Microbial) Results in Year 1.

		2023					
Experiment	**Task**	**July**	**Aug.**	**Sept.**	**Oct.**	**Nov.**	**Dec.**
Reddy Trial	Bioinformatics						
	Statistical Analyses						
	Integration of Metabarcoding and Metabolomics						
	Manuscript Publication and Dissemination						

Figures and Tables (Year 1)

Figure 1. Average Metabolite Concentrations Between Soybean Varieties Across All Harvesting Dates in the SPAR1[a] Treatment



[a] SPAR1 (Control) – Day: 30°C; Night: 22°C
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

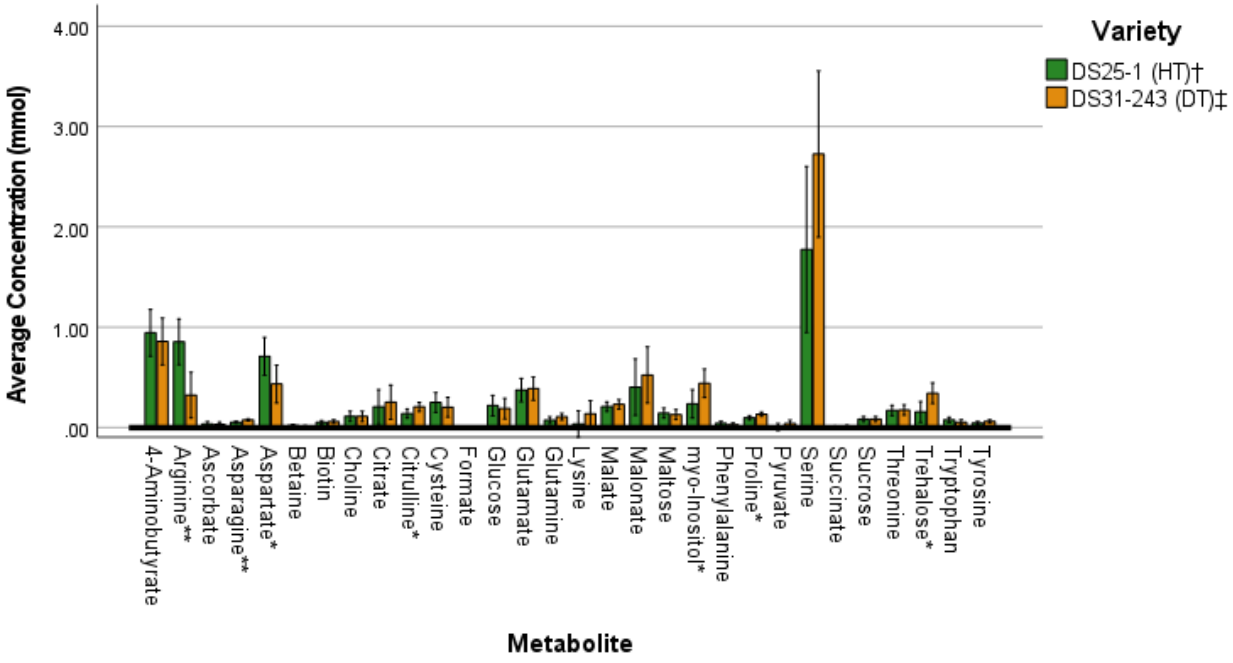
Table 2. Average Metabolite Concentrations Between Soybean Varieties for Each Harvesting Date in the SPAR1[a] Treatment

	Date of Harvest					
	7/25/22		8/15/22		9/2/22	
	Variety					
	HT[b]	DT[c]	HT	DT	HT	DT
Metabolite	**Average Concentration (mmol)**					
4-Aminobutyrate	0.497	0.554	0.652	0.671	1.461	1.226
Arginine*	0.263	0.124	0.948	0.115	0.301	0.329
Ascorbate	0.027	0.100	0.014	0.105	0.035	0.181
Asparagine	0.032	0.039	0.051	0.027	0.083	0.058
Aspartate**	0.341	0.180	0.362	0.184	0.774	0.427

Betaine	0.014	0.012	0.014	0.009	0.000	0.021
Biotin	0.062	0.084	0.023	0.056	0.065	0.071
Choline	0.134	0.075	0.057	0.094	0.163	0.080
Citrate	0.030	0.290	0.066	0.124	1.337	0.869
Citrulline	0.110	0.131	0.088	0.090	0.231	0.164
Cysteine**	0.112	0.093	0.336	0.105	0.249	0.200
Formate	0.009	0.008	0.001	0.001	0.001	0.001
Glucose	0.154	0.174	0.216	0.228	0.277	0.392
Glutamate	0.252	0.221	0.350	0.319	0.669	0.337
Glutamine	0.057	0.060	0.062	0.076	0.185	0.152
Lysine	0.041	0.151	0.051	0.027	0.037	0.034
Malate	0.177	0.226	0.071	0.196	0.464	0.327
Malonate	0.126	0.262	0.177	0.422	0.483	0.461
Maltose	0.117	0.076	0.146	0.075	0.157	0.187
myo-Inositol	0.202	0.215	0.283	0.276	0.170	0.328
Phenylalanine*	0.018	0.013	0.074	0.015	0.019	0.022
Proline	0.036	0.085	0.079	0.054	0.111	0.109
Pyruvate*	0.000	0.009	0.000	0.003	0.000	0.090
Serine	0.975	0.873	0.557	1.819	3.663	2.565
Succinate	0.005	0.029	0.002	0.004	0.014	0.029
Sucrose	0.071	0.043	0.051	0.042	0.097	0.076
Threonine	0.132	0.169	0.064	0.212	0.324	0.330
Trehalose	0.205	0.224	0.040	0.318	0.582	0.385
Tryptophan*	0.024	0.027	0.069	0.024	0.042	0.030
Tyrosine	0.012	0.016	0.034	0.017	0.037	0.044

[a] SPAR1 (Control) – Day: 30°C; Night: 22°C
[b] DS25-1 (HT): Heat-tolerant soybean variety
[c] DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

Figure 2. Average Metabolite Concentrations of Both Soybean Cultivars Across All Harvesting Dates in the SPAR2[a] Treatment



[a] SPAR2 (High Temperature) – Day: 38°C; Night: 30°C
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

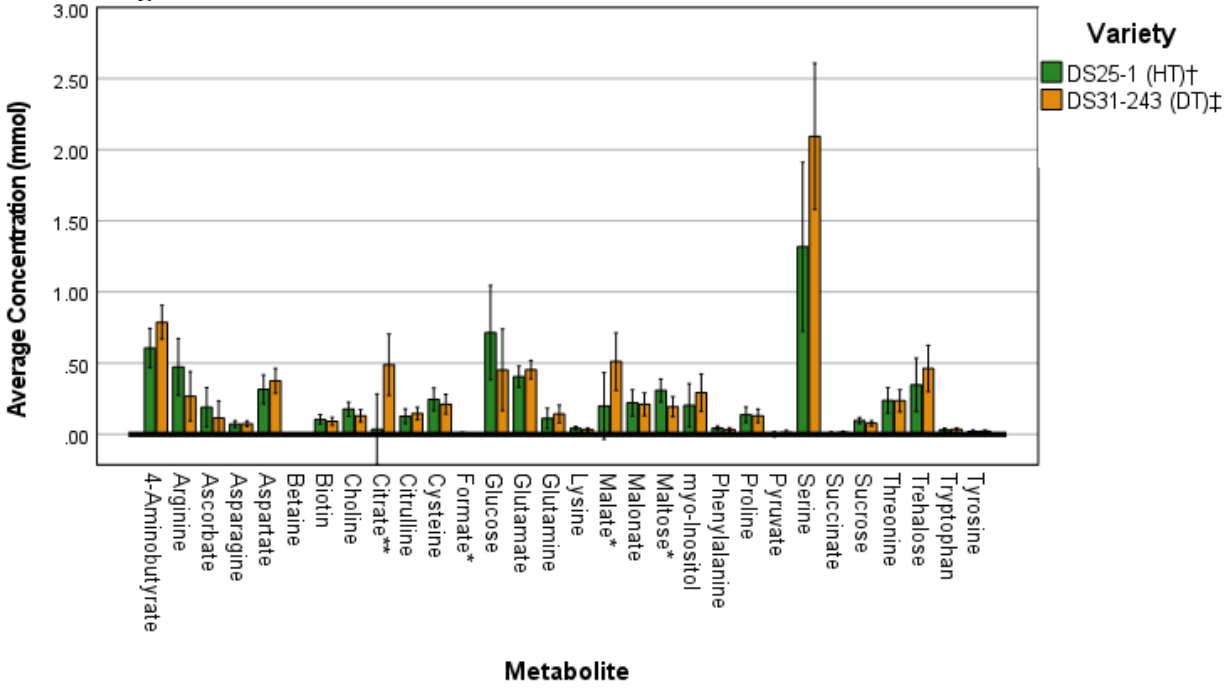
Table 3. Average Metabolite Concentrations Between Soybean Varieties for Each Harvesting Date in the SPAR2[a] Treatment

	Date of Harvest					
	7/25/22		8/15/22		9/2/22	
	Variety					
	HT[b]	DT[c]	HT	DT	HT	DT
Metabolite	**Average Concentration (mmol)**					
4-Aminobutyrate	1.161	0.632	0.928	1.082	0.815	0.901
Arginine**	0.749	0.395	1.059	0.150	0.738	0.375
Ascorbate	0.024	0.032	0.064	0.025	0.021	0.033
Asparagine**	0.036	0.040	0.060	0.079	0.065	0.109

Aspartate*	0.931	0.492	0.576	0.271	0.645	0.534
Betaine	0.007	0.000	0.038	0.018	0.017	0.007
Biotin	0.045	0.065	0.078	0.063	0.035	0.050
Choline	0.177	0.055	0.121	0.166	0.058	0.123
Citrate	0.229	0.045	0.184	0.474	0.237	0.272
Citrulline*	0.137	0.156	0.148	0.307	0.145	0.173
Cysteine	0.399	0.182	0.197	0.212	0.184	0.229
Formate	0.006	0.002	0.004	0.000	0.001	0.000
Glucose	0.096	0.190	0.350	0.222	0.198	0.148
Glutamate	0.453	0.232	0.408	0.559	0.301	0.399
Glutamine	0.069	0.051	0.081	0.192	0.082	0.094
Lysine	0.025	0.050	0.046	0.060	0.037	0.283
Malate	0.162	0.219	0.252	0.288	0.211	0.193
Malonate	0.228	0.326	0.700	0.719	0.294	0.522
Maltose	0.167	0.116	0.159	0.077	0.112	0.186
myo-Inositol*	0.194	0.189	0.337	0.776	0.213	0.398
Phenylalanine	0.069	0.033	0.026	0.021	0.036	0.028
Proline*	0.080	0.083	0.128	0.157	0.091	0.159
Pyruvate	0.000	0.012	0.000	0.092	0.022	0.024
Serine	2.108	0.985	1.704	4.086	1.770	3.272
Succinate	0.011	0.000	0.002	0.027	0.011	0.018
Sucrose	0.100	0.072	0.094	0.064	0.065	0.115
Threonine	0.215	0.225	0.163	0.164	0.137	0.142
Trehalose*	0.138	0.103	0.180	0.601	0.180	0.355
Tryptophan	0.085	0.044	0.059	0.031	0.087	0.081
Tyrosine	0.042	0.064	0.032	0.060	0.059	0.060

[a] SPAR2 (High Temperature) – Day: 38°C; Night: 30°C
[b] DS25-1 (HT): Heat-tolerant soybean variety
[c] DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

Figure 3. Average Metabolite Concentrations of Both Soybean Cultivars Across All Harvesting Dates in the SPAR3[a] Treatment



[a] SPAR3 (Low Temperature) – Day: 22°C; Night: 14°C
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

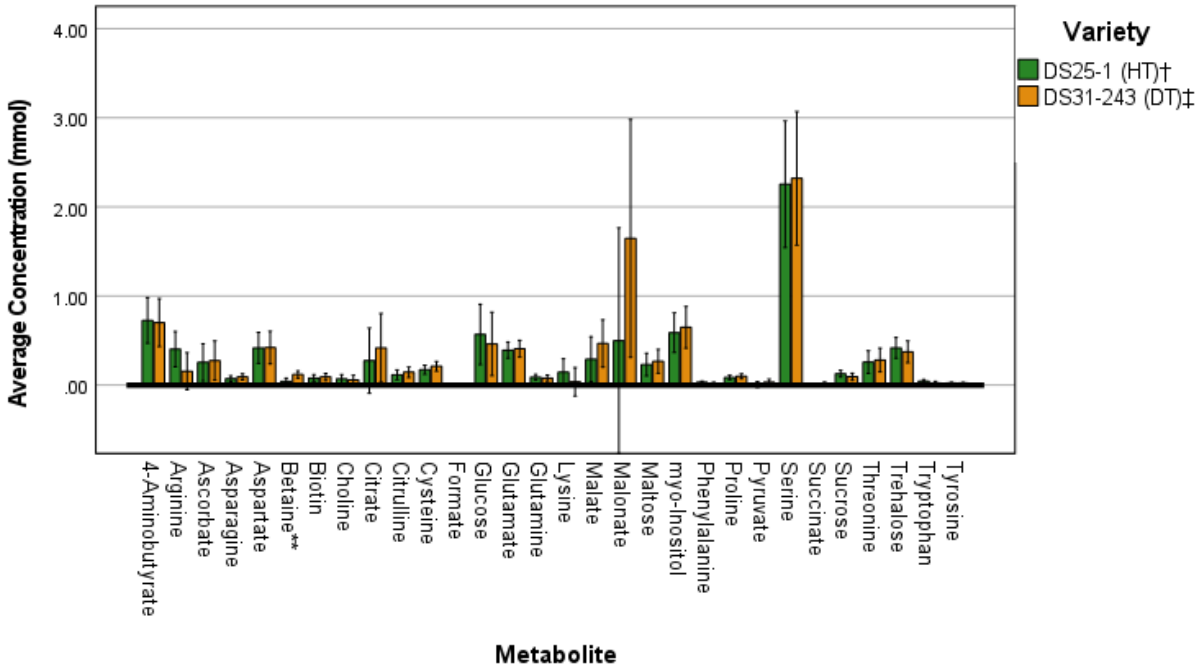
Table 4. Average Metabolite Concentrations Between Soybean Varieties for Each Harvesting Date in the SPAR3[a] Treatment

	Date of Harvest					
	7/25/2022		8/15/2022		9/2/2022	
	Variety					
	HT[b]	DT[c]	HT	DT	HT	DT
Metabolite	**Average Concentration (mmol)**					
4-Aminobutyrate	0.903	0.724	0.551	0.820	0.487	0.833
Arginine	0.316	0.204	0.552	0.191	0.520	0.383
Ascorbate	0.201	0.171	0.221	0.118	0.151	0.071
Asparagine	0.057	0.042	0.084	0.058	0.079	0.116
Aspartate	0.414	0.482	0.216	0.366	0.322	0.300
Betaine	0.010	0.000	0.000	0.000	0.006	0.000
Biotin	0.098	0.079	0.111	0.080	0.108	0.112
Choline	0.255	0.148	0.123	0.119	0.173	0.128
Citrate**	0.129	0.336	0.028	0.320	0.024	0.782
Citrulline	0.127	0.134	0.137	0.165	0.121	0.141

Cysteine	0.149	0.148	0.300	0.187	0.272	0.284
Formate*	0.019	0.007	0.007	0.002	0.005	0.001
Glucose	0.395	0.261	0.991	0.363	0.719	0.685
Glutamate	0.455	0.475	0.362	0.486	0.406	0.410
Glutamine	0.241	0.137	0.052	0.144	0.086	0.155
Lysine	0.030	0.033	0.035	0.020	0.058	0.048
Malate*	0.279	0.357	0.140	0.515	0.226	0.639
Malonate	0.250	0.116	0.235	0.121	0.217	0.378
Maltose*	0.270	0.200	0.383	0.178	0.272	0.206
myo-Inositol	0.180	0.134	0.212	0.381	0.231	0.341
Phenylalanine	0.049	0.032	0.041	0.026	0.046	0.043
Proline	0.123	0.100	0.064	0.103	0.213	0.175
Pyruvate	0.007	0.000	0.000	0.003	0.000	0.035
Serine	1.036	1.776	0.891	2.187	1.873	2.228
Succinate	0.015	0.012	0.006	0.028	0.001	0.002
Sucrose	0.114	0.097	0.079	0.075	0.099	0.066
Threonine	0.232	0.260	0.255	0.192	0.228	0.257
Trehalose	0.217	0.357	0.316	0.397	0.471	0.599
Tryptophan	0.057	0.027	0.024	0.030	0.025	0.049
Tyrosine	0.02	0.02	0.01	0.02	0.02	0.03

[a] SPAR3 (Low Temperature) – Day: 22°C; Night: 14°C
[b] DS25-1 (HT): Heat-tolerant soybean variety
[c] DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

Figure 4. Average Metabolite Concentrations of Both Soybean Cultivars Across All Harvesting Dates in the SPAR4[a] Treatment



[a] SPAR4 (High Night Temperature) – Day: 30°C; Night: 27.8°C
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

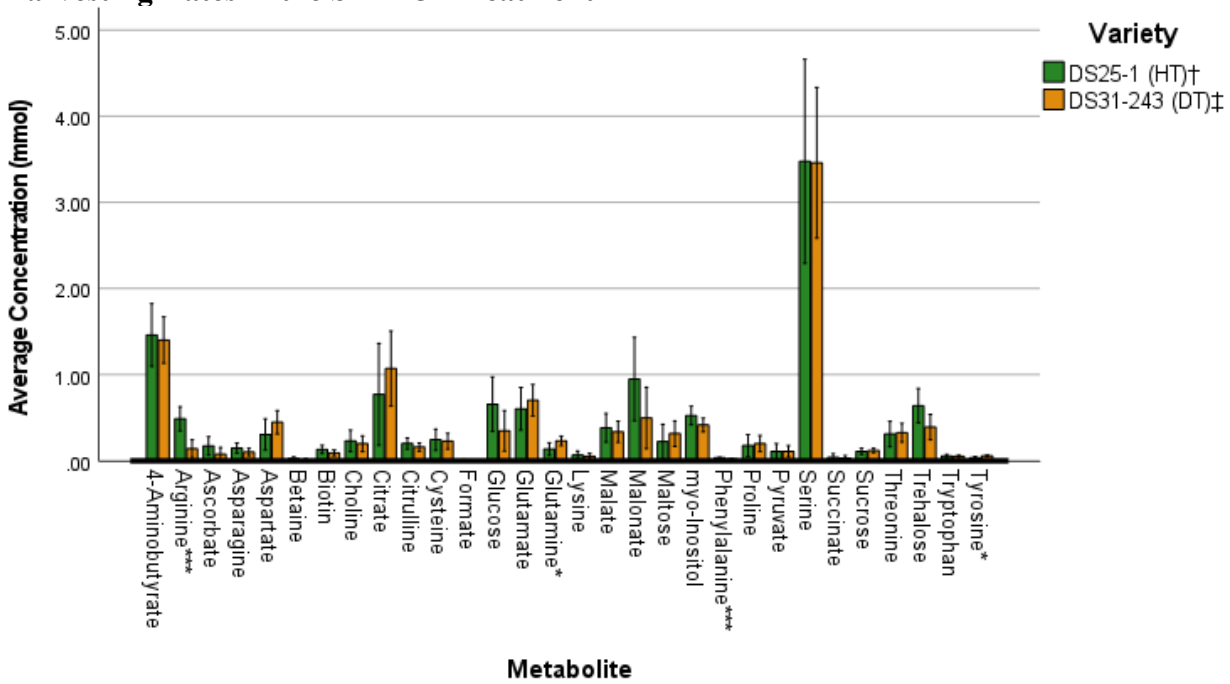
Table 5. Average Metabolite Concentrations Between Soybean Varieties for Each Harvesting Date in the SPAR4[a] Treatment

	Date of Harvest					
	7/25/2022		8/15/2022		9/2/2022	
	Variety					
	HT[b]	DT[c]	HT	DT	HT	DT
Metabolite	**Average Concentration (mmol)**					
4-Aminobutyrate	0.641	0.642	0.656	0.591	0.831	0.812
Arginine	0.465	0.180	0.350	0.177	0.396	0.130
Ascorbate	0.200	0.425	0.128	0.144	0.377	0.246
Asparagine	0.053	0.057	0.047	0.115	0.106	0.109
Aspartate	0.356	0.347	0.287	0.364	0.551	0.519
Betaine**	0.038	0.062	0.012	0.081	0.063	0.186
Biotin	0.095	0.112	0.073	0.088	0.073	0.081
Choline	0.007	0.038	0.174	0.048	0.046	0.074

Citrate	0.103	0.116	0.101	0.035	0.502	0.869
Citrulline	0.134	0.073	0.098	0.125	0.114	0.212
Cysteine	0.132	0.132	0.145	0.171	0.221	0.297
Formate	0.003	0.005	0.004	0.000	0.002	0.008
Glucose	0.547	0.496	0.492	0.644	0.649	0.343
Glutamate	0.293	0.379	0.433	0.476	0.445	0.390
Glutamine	0.056	0.029	0.126	0.069	0.086	0.119
Lysine	0.043	0.025	0.059	0.052	0.281	0.045
Malate	0.279	0.424	0.195	0.430	0.365	0.527
Malonate	0.203	0.544	0.365	0.350	0.748	3.198
Maltose	0.366	0.247	0.165	0.194	0.172	0.327
myo-Inositol	0.270	0.558	0.569	0.616	0.840	0.738
Phenylalanine	0.026	0.015	0.032	0.011	0.036	0.027
Proline	0.087	0.084	0.069	0.081	0.096	0.122
Pyruvate	0.004	0.000	0.000	0.000	0.012	0.078
Serine	0.827	1.827	3.196	1.895	2.668	2.855
Succinate	0.000	0.005	0.008	0.004	0.009	0.039
Sucrose	0.167	0.104	0.103	0.089	0.115	0.093
Threonine	0.209	0.290	0.240	0.225	0.307	0.310
Trehalose	0.326	0.366	0.493	0.367	0.437	0.377
Tryptophan	0.034	0.022	0.031	0.011	0.064	0.036
Tyrosine	0.016	0.015	0.020	0.013	0.035	0.035

[a] SPAR4 (High Night Temperature) – Day: 30°C; Night: 27.8°C
[b] DS25-1 (HT): Heat-tolerant soybean variety
[c] DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

Figure 5. Average Metabolite Concentrations of Both Soybean Cultivars Across All Harvesting Dates in the SPAR5[a] Treatment



[a] SPAR5 (Drought) – Day: 30°C; Night: 22°C; Water: 50% of control
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

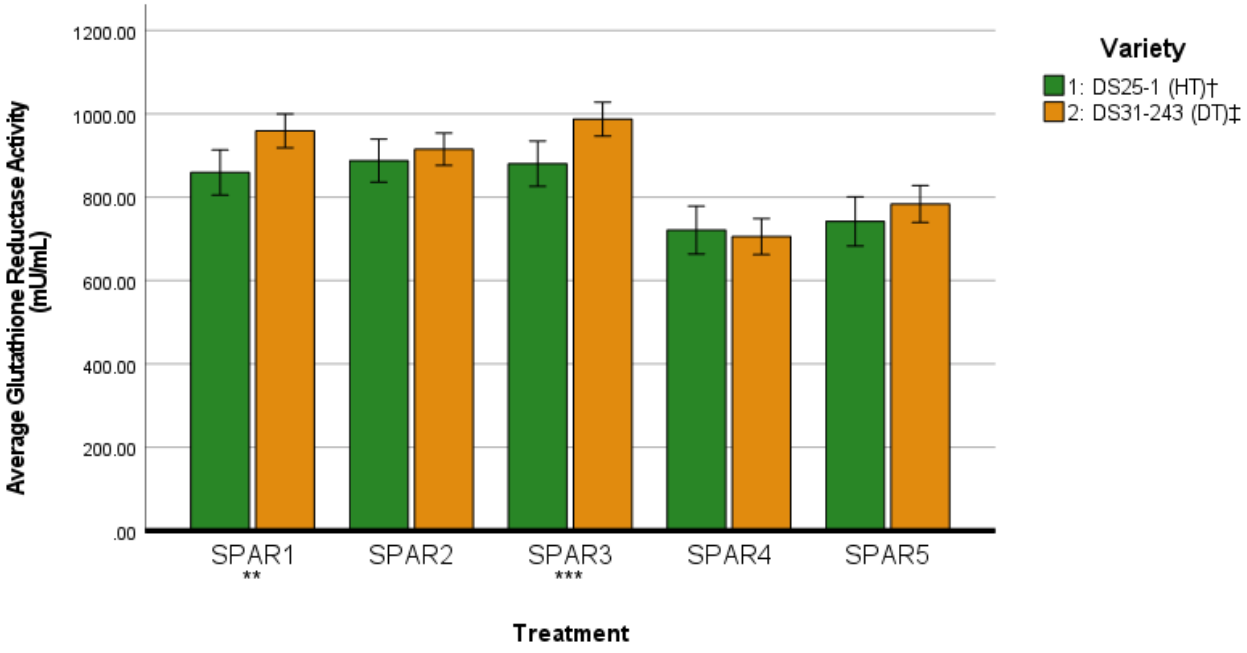
Table 6. Average Metabolite Concentrations Between Soybean Varieties for Each Harvesting Date in the SPAR5[a] Treatment

	Date of Harvest					
	7/25/2022		8/15/2022		9/2/2022	
	Variety					
	HT	DT	HT	DT	HT	DT
Metabolite	**Average Concentration (mmol)**					
4-Aminobutyrate	1.437	0.941	1.308	1.817	1.655	1.453
Arginine***	0.667	0.165	0.351	0.172	0.435	0.072
Ascorbate	0.288	0.075	0.197	0.076	0.023	0.083
Asparagine	0.163	0.047	0.112	0.125	0.174	0.137
Aspartate	0.391	0.247	0.216	0.684	0.303	0.402
Betaine	0.000	0.017	0.035	0.012	0.039	0.000
Biotin	0.158	0.098	0.153	0.097	0.079	0.079
Choline	0.335	0.152	0.181	0.289	0.170	0.146

Citrate	0.990	0.161	0.362	1.450	1.044	1.724
Citrulline	0.157	0.153	0.189	0.183	0.259	0.135
Cysteine	0.313	0.140	0.153	0.298	0.271	0.249
Formate	0.022	0.004	0.000	0.004	0.000	0.002
Glucose	1.148	0.285	0.665	0.421	0.124	0.359
Glutamate	0.591	0.338	0.669	1.141	0.535	0.613
Glutamine*	0.074	0.083	0.130	0.271	0.223	0.366
Lysine	0.053	0.054	0.076	0.075	0.068	0.019
Malate	0.371	0.239	0.300	0.361	0.493	0.423
Malonate	1.956	0.404	0.460	0.535	0.404	0.590
Maltose	0.341	0.159	0.115	0.347	0.231	0.473
myo-Inositol	0.349	0.261	0.839	0.619	0.371	0.362
Phenylalanine***	0.038	0.018	0.036	0.025	0.030	0.019
Proline	0.232	0.087	0.150	0.319	0.144	0.192
Pyruvate	0.118	0.103	0.097	0.052	0.118	0.189
Serine	2.935	1.551	3.606	4.721	4.002	4.248
Succinate	0.082	0.002	0.000	0.048	0.040	0.040
Sucrose	0.140	0.077	0.082	0.155	0.108	0.128
Threonine	0.414	0.254	0.215	0.381	0.305	0.346
Trehalose	0.815	0.295	0.474	0.519	0.623	0.354
Tryptophan	0.049	0.032	0.059	0.068	0.058	0.056
Tyrosine*	0.018	0.018	0.056	0.077	0.038	0.081

[a] SPAR5 (Drought) – Day: 30°C; Night: 22°C; Water: 50% of control
[b] DS25-1 (HT): Heat-tolerant soybean variety
[c] DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

Figure 6. Average Glutathione Reductase Activity of Both Soybean Varieties Across All Harvesting Dates for All Treatments[a]



[a] SPAR1 (Control) Day: 30°C, Night: 22°C; SPAR2 (High Temperature) Day: 38°C; Night: 30°C; SPAR3 (Low Temperature) Day: 22°C; Night: 14°C; SPAR4 (High Night Temperature) Day: 30°C; Night: 27.8°C; SPAR5 (Drought) – Day: 30°C; Night: 22°C; Water: 50% of control
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

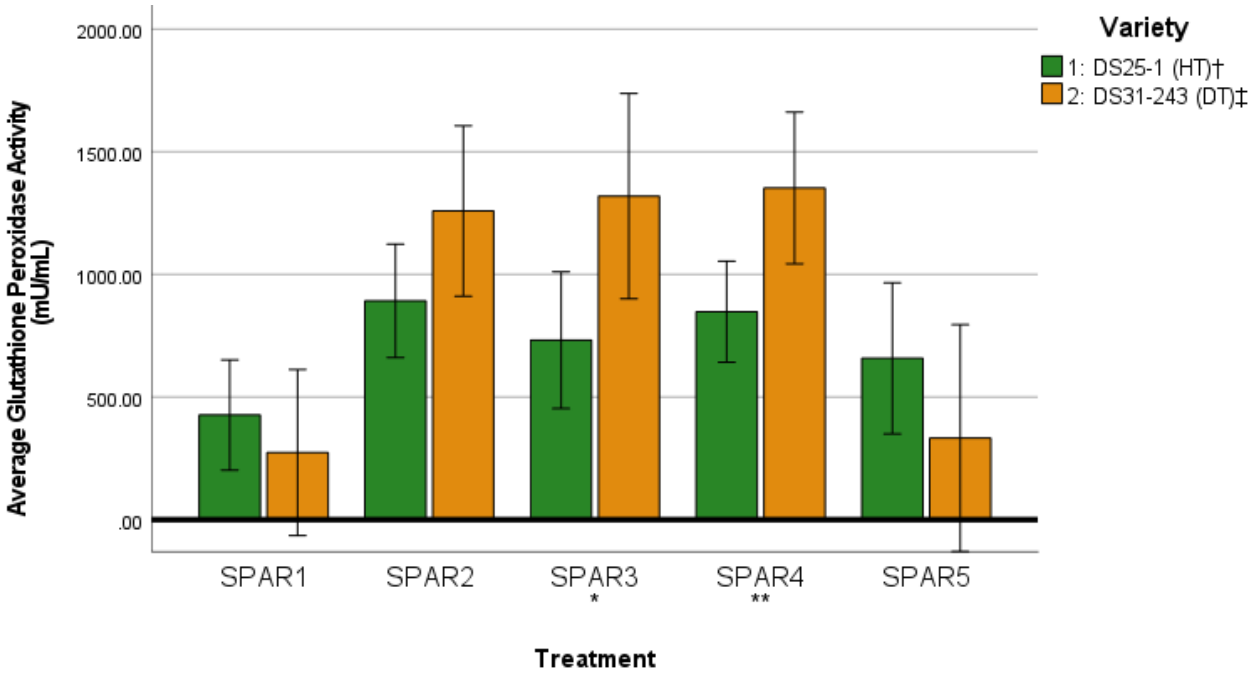
Table 7. Two-way Mixed-Design ANOVA Results for Glutathione Reductase Activity in Two Soybean Cultivars Across Five Treatments

		Type III Sum of Squares	df	F	Sig.	Partial Eta Squared
Within-Subjects Effects	*variety*	995.636	1	0.104	0.748	0.001
	variety x group	94283.9	4	2.457	0.052	0.104
	Error(variety)	94283.9	4	2.457	0.052	0.104
Between-Subjects Effects	*Intercept*	48710547	1	3925.475	<.001	0.979
	*date1**	55455.68	1	4.469	0.037	0.05
	date2	6016.252	1	0.485	0.488	0.006
	*group****	1375484	4	27.712	<.001	0.566

	Error	1054750	85

*: $p < .05$, **: $p < .01$, *** $p < .001$.

Figure 7. Average Glutathione Peroxidase Activity of Both Soybean Varieties Across All Harvesting Dates for All Treatments[a]



[a] SPAR1 (Control) Day: 30°C, Night: 22°C; SPAR2 (High Temperature) Day: 38°C; Night: 30°C; SPAR3 (Low Temperature) Day: 22°C; Night: 14°C; SPAR4 (High Night Temperature) Day: 30°C; Night: 27.8°C; SPAR5 (Drought) – Day: 30°C; Night: 22°C; Water: 50% of control
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

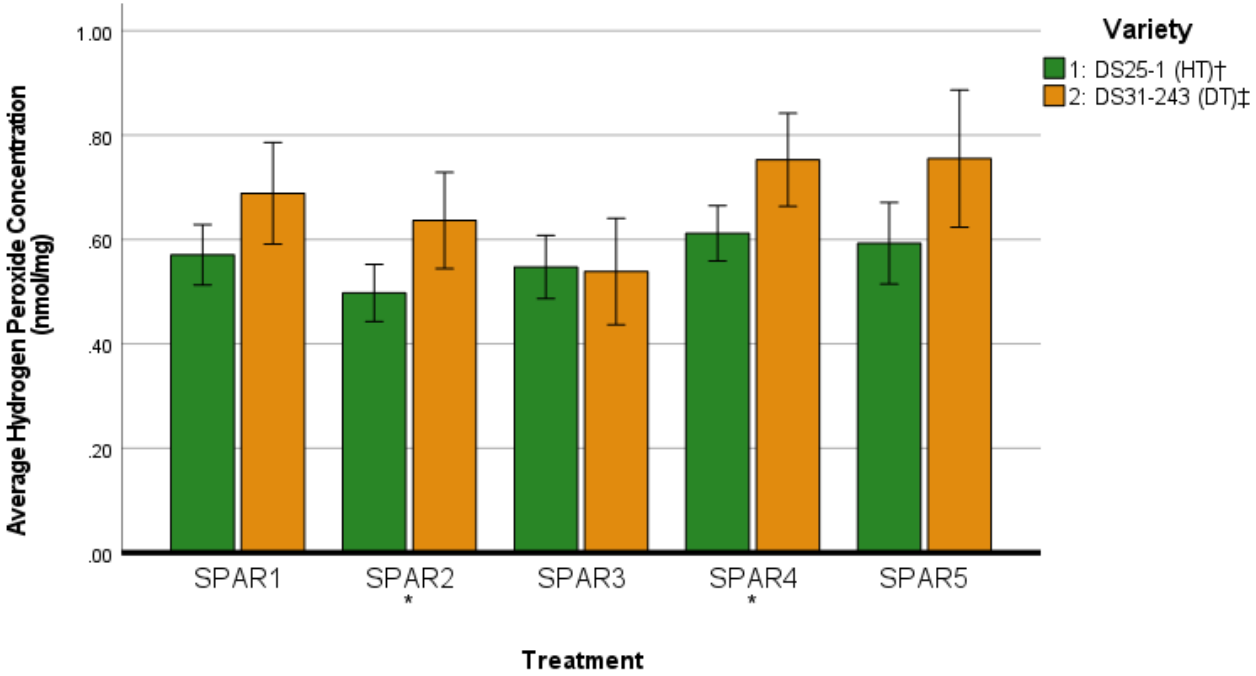
Table 8. Two-way Mixed-Design ANOVA Results for Glutathione Peroxidase Activity in Two Soybean Cultivars Across Five Treatments

		Type III Sum of Squares	df	F	Sig.	Partial Eta Squared
Within-Subjects Effects	*variety*	373756.2	1	1.055	0.308	0.016
	*variety x group**	4224578	4	2.982	0.025	0.153
	Error(variety)	23372690	66			

	Intercept	18773019	1	55.841	<.001	0.458
	*date1**	1509070	1	4.489	0.038	0.064
	*date2**	1657203	1	4.929	0.03	0.069
	*group****	15420266	4	11.467	<.001	0.41
Between-Subjects Effects	*Error*	22188179	66			

Note: *: $p < .05$, **: $p < .01$, *** $p < .001$.

Figure 8. Average Hydrogen Peroxide Concentrations of Both Soybean Varieties Across All Harvesting Dates for All Treatments[a]

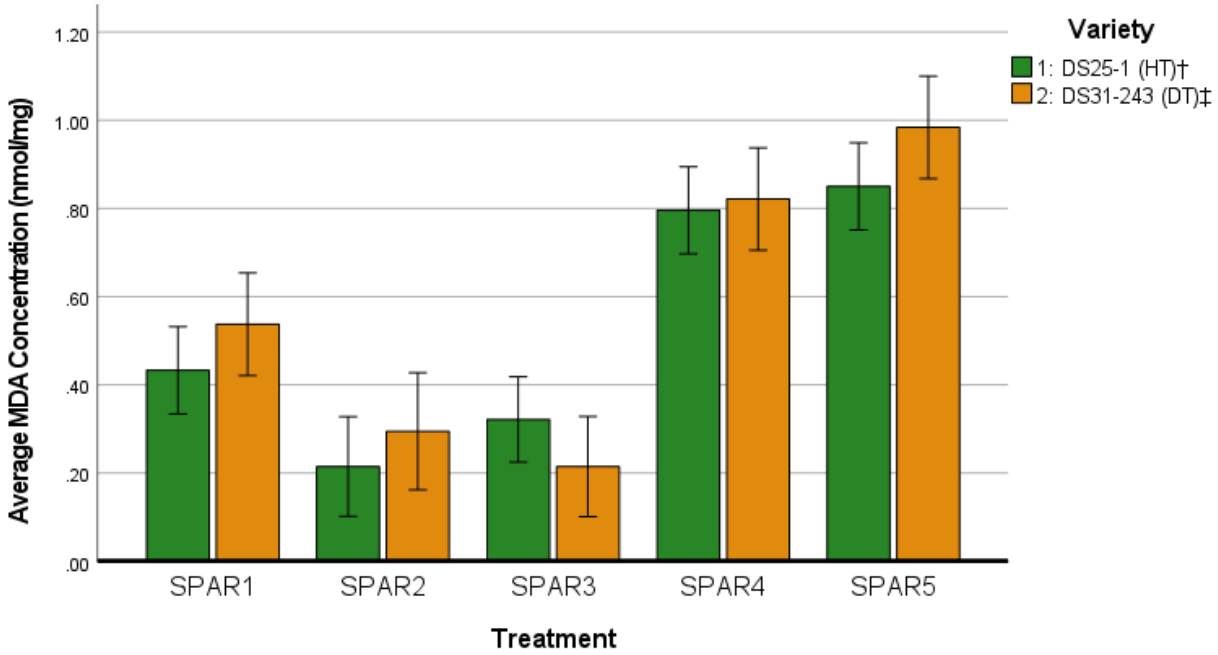


[a] SPAR1 (Control) Day: 30°C, Night: 22°C; SPAR2 (High Temperature) Day: 38°C; Night: 30°C; SPAR3 (Low Temperature) Day: 22°C; Night: 14°C; SPAR4 (High Night Temperature) Day: 30°C; Night: 27.8°C; SPAR5 (Drought) – Day: 30°C; Night: 22°C; Water: 50% of control
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

Table 9. Two-way Mixed-Design ANOVA Results for Glutathione Peroxidase Activity in Two Soybean Cultivars Across Five Treatments

		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Within-Subjects Effects	*variety*	0.113	1	0.113	3.835	0.054	0.052
	variety x group	0.128	4	0.032	10.87	0.370	0.058
	Error(variety)	2.059	70	0.029			
Between-Subjects Effects	*Intercept*	20.607	1	20.607	946.706	<.001	0.931
	*date1**	0.104	1	0.104	4.757	0.033	0.064
	*date2**	0.087	1	0.087	4.009	0.049	0.054
	*group***	0.474	4	0.118	5.441	<.001	0.237
	Error	1.524	70	0.022			

Figure 8. Average MDA Concentrations of Both Soybean Varieties Across All Harvesting Dates for All Treatments[a]



[a] SPAR1 (Control) Day: 30°C, Night: 22°C; SPAR2 (High Temperature) Day: 38°C; Night: 30°C; SPAR3 (Low Temperature) Day: 22°C; Night: 14°C; SPAR4 (High Night Temperature) Day: 30°C; Night: 27.8°C; SPAR5 (Drought) – Day: 30°C; Night: 22°C; Water: 50% of control
† DS25-1 (HT): Heat-tolerant soybean variety
‡ DS31-243 (DT): Standard soybean variety
*: $p < .05$; **: $p < .01$; ***: $p < .001$; results generated from a two-way mixed-design ANOVA at a 95% confidence interval with date of harvest added as a covariate

Table 10. Two-way Mixed-Design ANOVA Results for Glutathione Peroxidase Activity in Two Soybean Cultivars Across Five Treatments

		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Within-Subjects Effects	*variety***	0.632	1	0.632	9.612	0.003	0.088
	variety x group	0.405	4	0.101	1.539	0.197	0.059
	Error(variety)	6.506	99	0.066			
Between-Subjects Effects	*Intercept*	18.194	1	18.194	284.443	<.001	0.742
	date1	0.224	1	0.224	3.501	0.064	0.034
	date2	0.192	1	0.192	3.006	0.086	0.029
	*group****	15.64	4	3.91	61.128	<.001	0.712
	Error	6.332	99	0.064			