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Biochemical plasticity and hormetic shifts in soybean under drought stress and salicylic acid stimuli

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ABSTRACT

This study investigates the complex genotype × treatment × environment (G×T×E) interactions driving soybean seed biochemical trait expression under salicylic acid (SA) application and water stress conditions. Using a comprehensive dataset of 55,450 observations across 13 agronomic and biochemical traits, six soybean genotypes were subjected to factorial combinations of SA (250 mg and 400 mg) and water stress (5%, 70%, and ambient field capacity). Trait responses were quantified via machine learning sensitivity analysis, three-way ANOVA, and structural equation modeling (SEM). Results reveal that genotype exerted the dominant influence across traits, followed by water stress and SA, with several traits chlorophyll concentrations, sugars, and protein contents exhibiting strong three-way interaction effects. Notably, genotypes G3 and G5 showed superior biochemical plasticity and yield stability, while G6 prioritized stress resilience at the expense of productivity. Moderate drought induced beneficial hormetic shifts in biochemical traits, and SA treatments enhanced pigment and protein expression in a genotype-dependent manner. Findings provide mechanistic insight and a scalable framework for genotype-tailored agronomy and biochemical trait optimization in soybeans.

KEYWORDS: Genotype × environment × treatment, Soybean biochemistry, Salicylic acid, Drought stress, Biochemical plasticity

INTRODUCTION

Soybean (*Glycine max* L.) plays a pivotal role in global agriculture, functioning as both a nutritional resource and a bioindicator of agroecosystem resilience (Adamič & Leskovšek, 2021; Nair *et al.*, 2023; Hadinezhad *et al.*, 2024). Its physiological and biochemical performance is highly responsive to environmental fluctuations and agronomic interventions. With rising climatic variability intensifying abiotic stresses like drought, and salicylic acid (SA) gaining traction as a mitigation agent, it becomes critical to understand how these external pressures interact with genetic variation to influence trait expression (Goulart *et al.*, 2023; Thomasz *et al.*, 2024; Hamed *et al.*, 2025). This study investigates the integrated effects of genotype (G), salicylic acid treatment (T), and environmental water stress (E) referred to collectively as G×T×E interactions on seed biochemical traits.

Key biochemical parameters such as chlorophyll content, soluble sugar levels, total protein, and protein percentage are essential to seed quality and stress response, yet their regulation arises from complex, non-additive interactions among genotypic and environmental inputs (Ebone *et al.*, 2020; Staniak *et al.*, 2021, 2023). These traits influence plant plasticity, metabolic adaptation, and reproductive viability under stress.

While past studies have documented genotype-specific drought resilience (Yan *et al.*, 2020; Hossain *et al.*, 2024; Amjid & Üstün, 2025), differential SA responsiveness (Zhao *et al.*, 2018; Beyer *et al.*, 2021; Karimi *et al.*, 2025), and biochemical robustness (Soares *et al.*, 2021; Nam *et al.*, 2025), comprehensive examinations of G×T×E dynamics across large, integrative datasets remain limited.

To bridge this gap, the study utilizes a structured dataset encompassing 55,450 observations across 13 traits, derived from factorial combinations of six genotypes, two SA treatments, and three water stress regimes. Analytical methods include machine learning sensitivity analysis, three-way ANOVA, and structural equation modeling (SEM) to uncover both independent and interactive contributions of G, T, and E to seed biochemical expression. This multi-tiered approach integrates statistical precision with physiological insight, advancing strategies for genotype selection and precision agronomic management aimed at enhancing soybean trait resilience.

METHODOLOGY

Dataset Description

The dataset consists of 55,450 observations across 13 agronomic, biochemical, and physiological traits (Table 1). It enables analysis of G×T×E interactions, trait associations, and crop productivity modeling under varying genotype, treatment, and environmental conditions (Tables 2 & 3). Each row includes a tripartite code (e.g., C1S1G5) (Table 2), specifying genotype (G1-G6), salicylic acid dose (250 mg, 450 mg, or control), and water stress level (5%, 70%, or ambient) (Table 4). Measured traits include Plant Height (PH), Number of Pods (NP), Biological Weight (BW), Sugars (Su), Relative Water Content in Leaves (RWCL), ChlorophyllA663, Chlorophyllb649, Protein Percentage (PPE), Protein Content (PCO), Seed Yield per Unit Area (SYUA), Weight of 300 Seeds (W3S), Leaf Area Index (LAI), and Number of Seeds per Pod (NSP) (Table 1). Granularity and randomization improve statistical robustness for trait classification and predictive modeling.

Analytical Software Components

Data analysis was conducted in a PyCharm 2025.1-enabled Python 3.12 IDE using Pandas 2.3.1 (wrangling), SciPy 1.16.0 (computing), Statmodels 0.14.4 (statistics), Seaborn 0.13.2, NetworkX 3.5, and Matplotlib 3.10.3 (visualization), Sklearn 1.7.1 (ML), OS 2019 (system navigation), Itertools 3.3 (task automation), and SemoPy 2.2.2 (Structural Equation Modelling).

Table 1: Codes for variables in the raw dataset

Variable	Code	Units
Parameters	parameters	-
Random	random	-
Plant Height (PH)	p-h	Centimeters (cm)
Number of Pods (NP)	n-p	Dimensionless
Biological Weight (BW)	b-w	Grams (g)
Sugars (Su)	su	Milligrams per gram (mg/g)
Relative Water Content in Leaves (RWCL)	r-w-c-l	Grams per gram (g/g)
ChlorophyllA663	chl-a	Milligrams per kilogram (mg/kg)
Chlorophyllb649	chl-b	Milligrams per kilogram (mg/kg)
Protein Percentage (PPE)	pr-p	Percentage of dry seed weight
Weight of 300 Seeds (W3S)	w-3-s	Grams (g)
Leaf Area Index (LAI)	l-a-i	Dimensionless
Seed Yield per Unit Area (SYUA)	s-y-u-a	Kilograms per hectare (kg/ha)
Number of Seeds per Pod (NSP)	n-s-p	Dimensionless
Protein Content (PCO)	pr-c	Milligrams per gram (mg/g)

Individual Contributions of Experimental Condition Levels on Biochemical Trait Expression

Machine learning via Gradient Boosting Machines (GBM) was used to assess how condition levels C, S, and G affect traits su, chl-a, chl-b, pr-p, and pr-c. After label encoding, GBM regressors evaluated impurity-based feature importance for C and S. Permutation sensitivity analysis quantified robustness by shuffling each predictor's values and tracking changes in prediction error. Both analyses (sklearn-based) revealed strong context dependence, with su and chl-b highly sensitive to S.

Influence of Combinations of Experimental Conditions on Biochemical Trait Performance/Variability

Trait variability under combined conditions (G×C×S) was assessed using multivariate means (input for trait-trait

Table 2: Number of plants sampled per repeat for each unique experimental condition

Parameters	R1 count	R2 count	R3 count
C1S1G1	514	513	513
C1S1G2	514	513	513
C1S1G3	514	513	513
C1S1G4	514	513	513
C1S1G5	514	513	513
C1S1G6	514	513	513
C1S2G1	514	513	513
C1S2G2	514	513	513
C1S2G3	514	513	513
C1S2G4	514	513	513
C1S2G5	514	513	513
C1S2G6	514	513	513
C1S3G1	514	513	513
C1S3G2	514	514	513
C1S3G3	514	514	513
C1S3G4	514	514	513
C1S3G5	514	514	513
C1S3G6	514	513	513
C2S1G1	514	514	513
C2S1G2	514	514	513
C2S1G3	1028	1027	1026
C2S1G4	514	514	513
C2S1G5	514	514	513
C2S1G6	514	514	513
C2S2G1	514	513	513
C2S2G2	514	513	513
C2S2G4	514	513	513
C2S2G5	514	513	513
C2S2G6	514	513	513
C2S3G1	514	513	513
C2S3G2	514	513	513
C2S3G3	514	513	513
C2S3G4	514	513	513
C2S3G5	514	513	513
C2S3G6	514	513	513

Table 3: Variables and codes defined after parsing the *Parameters* column

Variable	Code
Salicylic acid level	C
Water stress level	S
Soybean genotype	G

Table 4: Description of levels in parsed variables

Variable level	Description	Count
C1	250 mg salicylic acid	18
C2	400 mg salicylic acid	17
S1	Water stress at 5% of field capacity	12
S2	Water stress at 70% of field capacity	11
S3	Control	12
G1	Soybean genotype 1	06
G2	Soybean genotype 2	06
G3	Soybean genotype 3	05
G4	Soybean genotype 4	06
G5	Soybean genotype 5	06
G6	Soybean genotype 6	06

*Count refers to the number of unique experimental conditions using the variable level

correlation heatmaps), and three-way ANOVA to partition variance across main and interaction effects. GLMs with Gaussian families estimated effect sizes, standard errors, and significance for each predictor and interaction term.

Structural Equation Modeling (SEM) to Explore Causal Pathways among Biochemical Traits

Structural equation modeling (SEM) was used to define and evaluate latent causal paths among biochemical traits, including directional links (e.g., su → chl-a → chl-b → pr-p → pr-c). Parameters were optimized and assessed via residual variance, z-scores, and p-values. SEM output included both direct and mediated effects as illustrated with a path diagram. Analyses were conducted using the semopy package with graph-based and tabular reporting of coefficients, variance terms, and standardized estimates.

RESULTS

Statistical Significances of Treatment Effects Across Traits

Table 5 reports consistent, statistically significant effects of salicylic acid on soybean traits, based on ANOVA across a large sample (n=55,449). Plant Height (cm) showed strong modulation (F=1274.38, $p=5.7\times10^{-276}$; SS=11,606.4). Number of Pods (dimensionless) was highly responsive (F=4636.79, p=0; SS=1,714,356), as was Biological Weight (g) (F=9502.35, p=0; SS=19,418,448). Sugar content (mg/g) was moderately affected (F=79.48, $p=5.01\times10^{-19}$), indicating metabolic shifts. Relative Water Content in Leaves (g/g) reflected notable changes (F=1706.69, p=0; SS=11.83). Both Chlorophyll A and B (mg/kg) showed significant pigment-related responses (F=587.96 and 513.75). Protein Percentage (% dry seed weight) was sharply influenced (F=6093.59; SS=32,079.3), along with Protein Content (mg/g) (F=51.22, $p=8.35\times10^{-13}$). Seed weight (300 seeds, g) responded significantly (F=58.28, $p=2.31\times10^{-14}$), relevant for maturation dynamics under C1. Leaf Area Index (dimensionless) exhibited dramatic treatment effects (F=4079.60; SS=1.89). Seed Yield per Unit Area (kg/ha) had the strongest response (F=9849.63; $SS=1.31\times10^{10}$), especially in C2 plants under S3 watering. Number of Seeds per Pod (dimensionless) also showed pronounced responsiveness (F=1032.85, $p=1.5\times10^{-224}$).

Table 5: Statistical significance of the effect of salicylic acid treatment (C) across agronomic and biochemical traits

		sum_sq	df	F	PR(>F)
p-h	Q("C")	11606.4	1	1274.382	$5.7E^{-276}$
	Residual	504991.4	55448		
n-p	Q("C")	1714356	1	4636.79	0
	Residual	20500733	55448		
b-w	Q("C")	19418448	1	9502.354	0
	Residual	1.13E+08	55448		
su	Q("C")	4.431604	1	79.48206	$5.01E^{-19}$
	Residual	3091.56	55448		
r-w-c-l	Q("C")	11.82685	1	1706.691	0
	Residual	384.238	55448		
chl-a	Q("C")	4713.461	1	587.9584	$3.3E^{-129}$
	Residual	444507.6	55448		
chl-b	Q("C")	717.6222	1	513.7542	$3.2E^{-113}$
	Residual	77450.88	55448		
pr-p	Q("C")	32079.29	1	6093.585	0
	Residual	291902.5	55448		
w-3-s	Q("C")	1455.035	1	58.28017	$2.31E^{-14}$
	Residual	1384326	55448		
l-a-i	Q("C")	1.886093	1	4079.595	0
	Residual	25.63492	55448		
s-y-u-a	Q("C")	1.31E+10	1	9849.63	0
	Residual	7.37E+10	55448		
n-s-p	Q("C")	61.30661	1	1032.85	$1.5E^{-224}$
	Residual	3291.213	55448		
pr-c	Q("C")	4.231355	1	51.22156	$8.35E^{-13}$
	Residual	4580.496	55448		

Table 6 demonstrates that water stress significantly impacted all measured agronomic and biochemical soybean traits, with extremely high F-values and low p-values across the board. Plant Height (cm) showed an F-value of 245.27 ($p=8.9\times10^{-107}$, SS=4,530.26), while the Number of Pods had an F-value of 65.37 ($p=4.42\times10^{-29}$, SS=52,254.35). Biological Weight (g) was strongly affected (F=944.39, p=0). Sugars (mg/g) showed drastic metabolic changes (F=7,278.88, p=0, SS=643.82 mg^2/g^2). Leaf Relative Water Content (g/g) was highly responsive (F=342.32, $p=1.7\times10^{-148}$, SS=4.83 g^2/g^2), as were Chlorophyll A and B levels (A: F=2,748.20, SS=40,514.67 mg^2/kg^2; B: F=7,271.11, SS=16,241.69 mg^2/kg^2; both p=0). Protein Percentage (% dry seed) showed strong effects (F=294.16, $p=8.3\times10^{-128}$, SS=3,401.57). Weight of 300 Seeds (g) was highly impacted (F=1,210.61, p=0, SS=57,981.18), as was Leaf Area Index (F=3,708.25, p=0, SS=3.25). Seed Yield (kg/ha) dropped under stress (F=1,434.30, p=0, $SS=4.27\times10^{9}$ kg^2/ha^2). Number of Seeds per Pod showed extreme sensitivity (F=1,830.55, SS=207.65), and Protein Content (mg/g) was markedly altered (F=5,948.90, SS=809.98 mg^2/g^2), supporting the role of genotype and abiotic factors in shaping seed composition.

Table 7 shows that genotype significantly influenced all agronomic and biochemical traits, with consistently high F-values and p-values of zero. Plant height (cm) varied strongly (F=3175.519), as did pod number (dimensionless; F=895.256), especially under salicylic acid and moisture variation. Biological weight (g) had one of the highest genotype effects (F=5054.764). Sugar content (mg/g) differed markedly

(F=1004.48), tied to genotype-specific stress resilience. Leaf relative water content (g/g) was highly genotype-sensitive (F=1864.352), as were chlorophyll A and B (mg/kg; F=1717.486 and 772.682). Protein percentage (dry seed weight) also varied significantly (F=339.803). Seed weight (300 seeds, g) showed strong genetic control (F=1574.175), and leaf area index (dimensionless) had a pronounced effect (F=1439.248). Yield (kg/ha) was strongly genotype-dependent (F=1687.012), as were seeds per pod (dimensionless; F=804.980) and seed protein content (mg/g; F=1106.481).

Table 6: Statistical significance of the effect of water stress (S) across agronomic and biochemical traits

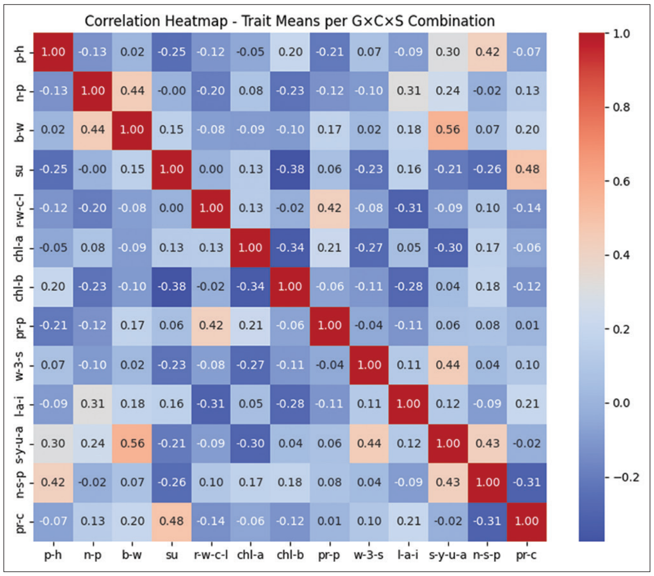
		sum_sq	df	F	PR(>F)
p-h	Q("S")	4530.259	2	245.2697	$8.9E^{-107}$
	Residual	512067.5	55447		
n-p	Q("S")	52254.35	2	65.365	$4.42E^{-29}$
	Residual	22162834	55447		
b-w	Q("S")	4372384	2	944.385	0
	Residual	1.28E + 08	55447		
su	Q("S")	643.8231	2	7278.875	0
	Residual	2452.169	55447		
r-w-c-l	Q("S")	4.830848	2	342.322	$1.7E^{-148}$
	Residual	391.234	55447		
chl-a	Q("S")	40514.67	2	2748.203	0
	Residual	408706.4	55447		
chl-b	Q("S")	16241.69	2	7271.106	0
	Residual	61926.82	55447		
pr-p	Q("S")	3401.57	2	294.1648	$8.3E^{-128}$
	Residual	320580.2	55447		
w-3-s	Q("S")	57981.18	2	1210.605	0
	Residual	1327800	55447		
l-a-i	Q("S")	3.24687	2	3708.25	0
	Residual	24.27414	55447		
s-y-u-a	Q("S")	4.27E + 09	2	1434.302	0
	Residual	8.26E + 10	55447		
n-s-p	Q("S")	207.6516	2	1830.547	0
	Residual	3144.868	55447		
pr-c	Q("S")	809.9831	2	5948.897	0
	Residual	3774.745	55447		

Table 7: Statistical significance of the effect of soybean genotype (G) across agronomic and biochemical traits

		sum_sq	df	F	PR(>F)
p-h	Q("G")	115004.9	5	3175.519	0
	Residual	401592.9	55444		
n-p	Q("G")	1659554	5	895.2557	0
	Residual	20555535	55444		
b-w	Q("G")	41559119	5	5054.764	0
	Residual	91169582	55444		
su	Q("G")	257.1562	5	1004.48	0
	Residual	2838.836	55444		
r-w-c-l	Q("G")	57.00577	5	1864.352	0
	Residual	339.0591	55444		
chl-a	Q("G")	60246.27	5	1717.486	0
	Residual	388974.8	55444		
chl-b	Q("G")	5092.064	5	772.6824	0
	Residual	73076.44	55444		
pr-p	Q("G")	9632.856	5	339.8033	0
	Residual	314348.9	55444		
w-3-s	Q("G")	172270.9	5	1574.175	0
	Residual	1213510	55444		
l-a-i	Q("G")	3.16167	5	1439.248	0
	Residual	24.35934	55444		
s-y-u-a	Q("G")	1.15E+10	5	1687.012	0
	Residual	7.54E+10	55444		
n-s-p	Q("G")	226.9011	5	804.9802	0
	Residual	3125.619	55444		
pr-c	Q("G")	415.9737	5	1106.481	0
	Residual	4168.754	55444		

Correlation Heatmap of Agronomic and Biochemical Trait Means under CxSxG Combination

The correlation heatmap (Figure 1) summarizes trait interrelationships across soybean genotypes under varying salicylic acid and water stress. Plant Height (cm) showed weak-to-moderate negative correlations with Sugars (mg/g) (-0.25), Protein Percentage, and Relative Water Content in Leaves (g/g), but modest positive links with Seed Yield (kg/ha) (0.30) and Seeds per Pod (0.42). Pod number correlated moderately with Biological Weight (g) (0.44) and Leaf Area Index (0.31), yet weakly or negatively with biochemical traits. Biological Weight (g) was a strong yield proxy (Seed Yield: 0.56) but weakly negative with Sugars (-0.08). Sugars (mg/g) co-varied with Protein Content (mg/g) (0.48) and declined with Plant Height (-0.25) and ChlorophyllB649 (-0.38). RWCL correlated positively with Protein Percentage (0.42), but not strongly with leaf or chlorophyll metrics. ChlorophyllA663 and ChlorophyllB649 had weak biochemical correlations, though ChlorophyllB649 correlated slightly with Seed Yield (0.18) and negatively with Plant Height (-0.34). Protein Percentage related positively only to RWCL and LAI. Weight of 300 Seeds (g) linked with Seed Yield (0.44), but had weak biochemical ties. LAI moderately supported Pod Number, Biological Weight, and Seed Yield. Seed Yield (kg/ha) correlated positively with Biological Weight (g), 300 Seed Weight (g), and Seeds per Pod (0.43), but negatively with Plant Height (-0.09) and Sugars (-0.21). Seeds per Pod also showed weak positive correlation with Protein Content (0.10), and stronger negative ones with Plant Height (-0.31) and Sugars (-0.24).



	p-h	n-p	b-w	su	r-w-c-l	chl-a	chl-b	pr-p	w-3-s	l-a-i	s-y-u-a	n-s-p	pr-c
p-h	1.00	-0.13	0.02	-0.25	-0.12	-0.05	0.20	-0.21	0.07	-0.09	0.30	0.42	-0.07
n-p	-0.13	1.00	0.44	-0.00	-0.20	0.08	-0.23	-0.12	-0.10	0.31	0.24	-0.02	0.13
b-w	0.02	0.44	1.00	0.15	-0.08	-0.09	-0.10	0.17	0.02	0.18	0.56	0.07	0.20
su	-0.25	-0.00	0.15	1.00	0.00	0.13	-0.38	0.06	-0.23	0.16	-0.21	-0.26	0.48
r-w-c-l	-0.12	-0.20	-0.08	0.00	1.00	0.13	-0.02	0.42	-0.08	-0.31	-0.09	0.10	-0.14
chl-a	-0.05	0.08	-0.09	0.13	0.13	1.00	-0.34	0.21	-0.27	0.05	-0.30	0.17	-0.06
chl-b	0.20	-0.23	-0.10	-0.38	-0.02	-0.34	1.00	-0.06	-0.11	-0.28	0.04	0.18	-0.12
pr-p	-0.21	-0.12	0.17	0.06	0.42	0.21	-0.06	1.00	-0.04	-0.11	0.06	0.08	0.01
w-3-s	0.07	-0.10	0.02	-0.23	-0.08	-0.27	-0.11	-0.04	1.00	0.11	0.44	0.04	0.10
l-a-i	-0.09	0.31	0.18	0.16	-0.31	0.05	-0.28	-0.11	0.11	1.00	0.12	-0.09	0.21
s-y-u-a	0.30	0.24	0.56	-0.21	-0.09	-0.30	0.04	0.06	0.44	0.12	1.00	0.43	-0.02
n-s-p	0.42	-0.02	0.07	-0.26	0.10	0.17	0.18	0.08	0.04	-0.09	0.43	1.00	-0.31
pr-c	-0.07	0.13	0.20	0.48	-0.14	-0.06	-0.12	0.01	0.10	0.21	-0.02	-0.31	1.00

Figure 1: Correlation heatmap of agronomic and biochemical traits means under CxSxG combination

Biochemical trait Sensitivity Analysis by Experimental Condition Level

Table 8 summarizes trait importance under genotype, water stress, and salicylic acid conditions for chlorophyll A663, chlorophyll B649, protein content, protein percentage, and sugars. Chlorophyll A663 was strongly genotype-dependent (mean=1.003, STD=0.0051), moderately sensitive to water stress (mean=0.988, STD=0.0055), and less responsive to salicylic acid (mean=0.729, STD=0.0045). Chlorophyll B649 was most sensitive to water stress (mean=1.476, STD=0.0144), followed by genotype effects (mean=1.237, STD=0.0115), with low importance under salicylic acid (mean=0.739, STD=0.0113). Protein content showed high genotype influence (mean=1.218, STD=0.0067), reduced under water stress (mean=1.035, STD=0.0097), and minimal under salicylic acid (mean=0.673, STD=0.0041). Protein percentage showed low overall importance, highest with salicylic acid (mean=0.507, STD=0.0039), but minimal for genotype (mean=0.384) and water stress (mean=0.331). Sugar importance peaked under water stress (mean=1.258, STD=0.0091), with lower genotype influence (mean=0.923, STD=0.0055), and negligible salicylic acid impact (mean=0.346, STD=0.0038).

Marginal means by Experimental Condition Level

Table 9 summarizes marginal means of five biochemical traits under varying salicylic acid levels, water stress, and soybean genotypes. Under 250 mg salicylic acid, Sugars were 0.49 mg/g, Chlorophyll A 4.23 mg/kg, Chlorophyll B 2.29 mg/kg, Protein Percentage 36.80%, and Protein Content 0.50 mg/g. At 400 mg, Sugars dropped to 0.47 mg/g, Chlorophyll A to 3.65 mg/kg, Chlorophyll B rose to 2.51 mg/kg, Protein Percentage declined to 35.28%, and Protein Content increased to 0.52 mg/g. Severe water stress (5% field capacity) raised Sugars to 0.56 mg/g, lowered Chlorophyll A to 2.83 mg/kg, and Chlorophyll B to 2.21 mg/kg, with Protein Percentage at 35.72% and Protein Content at 0.67 mg/g. Moderate stress (70% field capacity) yielded Chlorophyll A at 4.83 mg/kg, lower Chlorophyll B (1.82 mg/kg), and reduced Protein Content (0.40 mg/g). Control plants had 0.33 mg/g Sugars and the highest Chlorophyll B (3.13 mg/kg). Genotype 3 showed peak Sugars (0.59 mg/g) and Chlorophyll A (5.64 mg/kg), with moderate Chlorophyll B (2.06 mg/kg), Protein Content 0.56 mg/g, and Protein Percentage 36.17%. Genotype 5 had high Sugars (0.53 mg/g), peak Chlorophyll B (2.71 mg/kg), and Protein Content of 0.66 mg/g. Genotype 6 had high Chlorophyll B (2.82 mg/kg) and Protein Percentage (36.41%), with lower Sugars (0.39 mg/g). Genotypes 2 and 4 showed balanced pigments and reduced Sugars, while Genotype 1 remained intermediate across all traits.

Table 8: Sensitivity analysis of biochemical trait importance across experimental conditions (G, S, C)

Trait	Experimental condition	Mean importance	STD importance
chl-a	G	1.003294126	0.005064231
chl-a	S	0.987577944	0.005542764
chl-a	C	0.728800654	0.004459466
chl-b	S	1.475861627	0.01441629
chl-b	G	1.237122817	0.011509462
chl-b	C	0.738559221	0.011323512
pr-c	G	1.217749337	0.006699203
pr-c	S	1.035117717	0.009657578
pr-c	C	0.672819853	0.004074437
pr-p	C	0.50738156	0.003938267
pr-p	G	0.383674257	0.003335988
pr-p	S	0.331189127	0.002485706
su	S	1.257563536	0.009059644
su	G	0.923086865	0.00548681
su	C	0.345518428	0.003821381

Influence of Combinations of Experimental Conditions on Biochemical Trait Performance/Variability

The three-way ANOVA in Table 10 shows that soybean genotype, salicylic acid level, and water stress level significantly affected Chlorophyll A663 content (mg/kg), including strong interaction effects. Genotype had a major influence (SS>63,000, df=5, F≈120,070, p<0.001), indicating high genetic control over chlorophyll levels. Salicylic acid had a smaller but significant effect (SS≈1,445, F≈13,750, p<0.001), while water stress was most impactful (SS≈94,000, F>447,000, p<0.001), with a variance contribution >65 × that of salicylic acid. Notable interactions included genotype × salicylic acid (SS>52,000, F≈100,678, p<0.001), genotype × water stress (SS≈45,420, F≈43,223, p<0.001), and salicylic acid × water stress (SS≈50,082, F≈238,297, p<0.001), with the latter indicating synergism. The three-way interaction exceeded all others (SS>196,000, F>186,655, p<0.001), underscoring complex regulatory interplay. Residual variance was low (SS≈5,823), affirming model robustness.

Table 9: Marginal means of biochemical traits across experimental conditions

Experimental condition	su	chl-a	chl-b	pr-p	pr-c
C1	0.49	4.23	2.29	36.80	0.50
C2	0.47	3.65	2.51	35.28	0.52
S1	0.56	2.83	2.21	35.72	0.67
S2	0.54	4.83	1.82	36.15	0.40
S3	0.33	4.32	3.13	36.29	0.43
G1	0.49	3.53	2.42	35.45	0.47
G2	0.43	3.91	2.40	36.45	0.44
G3	0.59	5.64	2.06	36.17	0.56
G4	0.43	4.55	1.99	35.48	0.52
G5	0.53	2.19	2.71	36.29	0.66
G6	0.39	3.79	2.82	36.41	0.39

Table 10: Three-Way ANOVA for chl-a trait variation

	sum_sq	df	F	PR(>F)
Q("G")	63086.86867	5	120070.0139	0
Q("C")	1444.906979	1	13750.08814	0
Q("S")	93995.57865	2	447242.4559	0
Q("G"):Q("C")	52898.16993	5	100678.3841	0
Q("G"):Q("S")	45420.52195	10	43223.27938	0
Q("C"):Q("S")	50082.06203	2	238296.5746	0
Q("G"):Q("C"):Q("S")	196143.7539	10	186655.1926	0
Residual	5823.200506	55415		

Table 11 shows that genotype, salicylic acid level, water stress level, and all interactions significantly affected chlorophyll b concentration (mg/kg), with p-values<0.001. Genotype explained 5958.183099 (SS), df=5, F=14,793.44, p=0; salicylic acid: 575.08 (SS), df=1, F=7,139.30, p=0; and water stress: 1794.03 (SS), df=2, F=11,135.87, p=0 confirming genotype-specific responses and stress-driven pigment variation. The genotype × salicylic acid interaction was also significant (SS=5214.05, F=12,945.84), as was genotype × water stress (SS=16,616.83, F=20,628.77), reinforcing genotype-dependent chlorophyll b dynamics under drought. Salicylic acid × water stress (SS=1447.41, F=8,984.33) showed compounded priming effects. The strongest effect came from the three-way interaction (genotype × salicylic acid × water stress): SS=31,705.70, F=39,360.67, underscoring complex trait modulation across treatments. Residuals totaled 4463.77 over 55,415 df, indicating precise variance partitioning.

Table 12 presents a three-way ANOVA on soybean seed protein content (mg/g) across genotypes, salicylic acid levels, and water stress conditions. Genotype had a dominant effect (SS=205.55; F>6900; p≈0), highlighting strong genetic control over protein levels. Salicylic acid showed a modest but significant impact (SS=0.12; F=20.11), consistent with prior subtle hormonal modulations in chlorophyll and relative water content. Water stress was the most influential factor (SS=731.99; F>61,000), profoundly affecting biochemical composition. All two-way interactions were highly significant: genotype × salicylic acid (SS=731.08; F=24,547), genotype × water stress (SS=1120.67; F=18,814), and salicylic acid × water stress (SS=261.82; F>21,977), indicating strong condition-dependent biochemical responses. The three-way interaction (SS=551.71; F=9262.50) confirmed that protein accumulation depends on integrated effects of genotype, stress response, and hormonal signaling. Residual error was low (SS=330.07; df=55,415), confirming excellent model fit.

Table 11: Three-Way ANOVA for chl-b trait variation

	sum_sq	df	F	PR(>F)
Q(“G”)	5958.183099	5	14793.43525	0
Q(“C”)	575.0828357	1	7139.299474	0
Q(“S”)	1794.026899	2	11135.86991	0
Q(“G”):Q(“C”)	5214.048585	5	12945.84085	0
Q(“G”):Q(“S”)	16616.83159	10	20628.77374	0
Q(“C”):Q(“S”)	1447.406938	2	8984.333168	0
Q(“G”):Q(“C”):Q(“S”)	31705.69978	10	39360.67497	0
Residual	4463.773436	55415		

Table 12: Three-Way ANOVA for pr-c trait variation

	sum_sq	df	F	PR(>F)
Q(“G”)	205.5525687	5	6901.934922	0
Q(“C”)	0.119810474	1	20.11466213	7.30816E^{-06}
Q(“S”)	731.9889386	2	61445.83902	0
Q(“G”):Q(“C”)	731.0800767	5	24547.81833	0
Q(“G”):Q(“S”)	1120.667373	10	18814.58677	0
Q(“C”):Q(“S”)	261.8182121	2	21977.982	0
Q(“G”):Q(“C”): Q(“S”)	551.7097791	10	9262.508894	0
Residual	330.0725296	55415		

Table 13 presents a three-way ANOVA showing protein percentage in dry soybean seed weight was significantly affected by genotype (SS=12,287.41; F=736.63; p<0.0001), salicylic acid level (SS=29,737.23; F=8,913.67; p≈0), and water stress (SS=2,309.10; F=346.07; p=4.294E^{-150}). Genotypic variability across G1-G6 was a major source of protein synthesis differences. Salicylic acid (250 mg vs. 400 mg) induced strong biochemical effects more pronounced than in seed yield metrics. Water stress (5% vs. 70% field capacity) also altered protein pathways. Interactions were all statistically significant: genotype × salicylic acid (SS=14,039.69; F=841.67), genotype × water stress (SS=36,347.84; F=1,089.52), salicylic acid × water stress (SS=20,076.68; F=3,008.97), and the triple interaction (SS=16,506.33; F=494.77). Compared to traits like leaf index or sugars, protein percentage displayed stronger regulatory complexity, including notable ABA/ethylene crosstalk. Residual variance was 184,872.01 across 55,415 degrees of freedom, yet explained variance was dominant, confirming protein percentage as a sensitive marker for genotype-by-treatment-by-environment effects.

Table 14 presents a three-way ANOVA showing that sugar content (mg/g) in soybean was significantly affected by genotype (F=6238.81, SS=208.68, p=0), salicylic acid level (F=1199.59, SS=8.02, $p \approx 4.57\times10^{-260}$), and water stress level (F=47486.14, SS=635.33, p=0). Genotypic effect mirrored patterns seen in protein and chlorophyll traits, while salicylic acid (250 mg or 400 mg) and water stress (5%, 70%, control) induced sharp biochemical changes. The genotype × salicylic acid interaction (F=3234.36, SS=108.18) revealed genotype-dependent hormonal responses. The genotype × water stress interaction had the largest SS (1055.28) and F-value (15774.91), indicating strong genotype-specific sugar regulation under stress. The salicylic acid × water stress interaction (F=13009.14, SS=174.05) reflected hormonal modulation of osmotic adjustment. The three-way interaction (F=8675.68, SS=580.37) showed complex sugar trait regulation across

Table 13: Three-Way ANOVA for pr-p trait variation

	sum_sq	Df	F	PR(>F)
Q(“G”)	12287.40819	5	736.6250117	0
Q(“C”)	29737.22913	1	8913.672603	0
Q(“S”)	2309.100135	2	346.0739822	4.294E^{-150}
Q(“G”):Q(“C”)	14039.69145	5	841.6736644	0
Q(“G”):Q(“S”)	36347.84492	10	1089.519094	0
Q(“C”):Q(“S”)	20076.67901	2	3008.971395	0
Q(“G”):Q(“C”):Q(“S”)	16506.33247	10	494.773884	0
Residual	184872.0079	55415		

Table 14: Three-Way ANOVA for su trait variation

	sum_sq	df	F	PR(>F)
Q(“G”)	208.6767001	5	6238.806031	0
Q(“C”)	8.02478711	1	1199.585057	4.5657E^{-260}
Q(“S”)	635.3299581	2	47486.13972	0
Q(“G”):Q(“C”)	108.1833172	5	3234.355975	0
Q(“G”):Q(“S”)	1055.284243	10	15774.91329	0
Q(“C”):Q(“S”)	174.052765	2	13009.13614	0
Q(“G”):Q(“C”):Q(“S”)	580.3715678	10	8675.682615	0
Residual	370.7061664	55415		

genetic, chemical, and stress contexts. Residual variance was low (SS=370.71, df=55,415), confirming excellent model fit, consistent with previous ANOVA results for protein and leaf water traits.

Structural Equation Modeling (SEM) to Explore Causal Pathways among Biochemical Traits

Table 15 presents a structural path model detailing trait interaction in soybean under salicylic acid, water stress, and genotype effects. Sugars (mg/g) were significantly reduced by salicylic acid (−0.01788, z=-8.92, $p<0.001$), while Chlorophyll A (mg/kg) increased with sugars (1.1088, z=22.82), but declined with salicylic acid (-0.4507) and rose under water stress (1.0126). Chlorophyll B (mg/kg) was suppressed by Chlorophyll A (-0.1312), sugars (-1.3571), and water stress (-0.0565), reflecting pigment trade-offs. Protein Percentage (of dry seed weight) increased with both Chlorophyll A (0.1328) and B (0.0335), suggesting pigment-linked biosynthesis. Protein Content (mg/g) showed a weak inverse link with Protein Percentage (-0.00088, $p=0.052$) but was strongly inhibited by Chlorophyll B (-0.0427), water stress (-0.1483), and genotype (-0.0296; all $p<0.001$), confirming stress-driven suppression. Residual variances (“~~”) were highest for Chlorophyll A (7.30) and Protein Percentage (5.71), and lower for Sugars, Protein Content, and Chlorophyll B, indicating differential model fit and trait stability.

Figure 2 illustrates direct and indirect effects of salicylic acid, water stress, and genotype on biochemical and agronomic traits via standardized regression. Salicylic acid significantly reduced sugars (-0.018, $p<0.001$) and chlorophyll A (-0.451, $p<0.001$), suggesting stress-induced pigment inhibition. Water stress increased chlorophyll A (1.013, $p<0.001$) but decreased chlorophyll B (-0.057, $p<0.001$), indicating compensatory biosynthesis and isoform-specific regulation. Genotype negatively impacted protein content (-0.030, $p<0.001$), reflecting potential proteome constraints. Internally, chlorophyll A unexpectedly showed a negative effect on chlorophyll B (-0.131, $p<0.001$), likely due to modeling artifacts, while positively influencing protein percentage (0.133, $p<0.001$). Chlorophyll B reduced protein content (-0.148, $p<0.001$), consistent with drought-related protein suppression. Protein percentage had mixed effects on protein content positive (0.034, $p<0.001$) and negative (-0.043, $p<0.001$) suggesting multicollinearity or indirect mediation pathways.

Table 15: Parameter estimates for biochemical trait interactions and effects in structural path model

lval	op	rval	Estimate	Std. Err	z-value	p-value
su	~	C	-0.01788	0.002006	-8.91576	0
chl_a	~	su	1.108777	0.04859	22.81923	0
chl_a	~	C	-0.4507	0.023019	-19.58	0
chl_a	~	S	1.012553	0.014119	71.71652	0
chl_b	~	chl_a	-0.13121	0.001671	-78.5007	0
chl_b	~	su	-1.35707	0.019271	-70.4184	0
chl_b	~	S	-0.05653	0.005824	-9.7071	0
pr_p	~	chl_b	0.033509	0.009107	3.679577	0.000234
pr_p	~	chl_a	0.132789	0.003801	34.93182	0
pr_c	~	pr_p	-0.00088	0.000452	-1.94374	0.051927
pr_c	~	chl_b	-0.04274	0.000925	-46.1872	0
pr_c	~	S	-0.14834	0.001359	-109.147	0
pr_c	~	G	-0.02963	0.000642	-46.1383	0
chl_a	~~	chl_a	7.300373	0.043844	166.5083	0
chl_b	~~	chl_b	1.138694	0.006839	166.5083	0
pr_p	~~	pr_p	5.710342	0.034295	166.5083	0
su	~~	su	0.055764	0.000335	166.5083	0
pr_c	~~	pr_c	0.066025	0.000397	166.5083	0

DISCUSSION

Genotype significantly influenced all measured traits across ~55,450 observations (ANOVA/MANOVA, $F>800$, $p<0.001$), explaining most variance in plant height ($F\approx3,175$), pods ($F\approx895$), seeds per pod ($F\approx805$), biological weight ($F\approx5,054$), seed yield ($F\approx1,687$), chlorophyll a/b ($F\approx1,717$ and 773), protein %/content ($F\approx340$ and 1,106), sugars ($F\approx1,004$), leaf RWC ($F\approx1,864$), leaf area index, and seed weight. MANOVA identified genotype as the dominant source of trait covariance (Wilks’ Lambda≈0, $F>1,900$, $p<0.001$), with strong genotype × treatment effects under salicylic acid (SA) and water stress.

Abiotic stresses (e.g. drought, salinity, heat) triggered excess ROS (O_2^-, H_2O_2, $\cdot OH$, $^1O_2$), inducing lipid peroxidation,

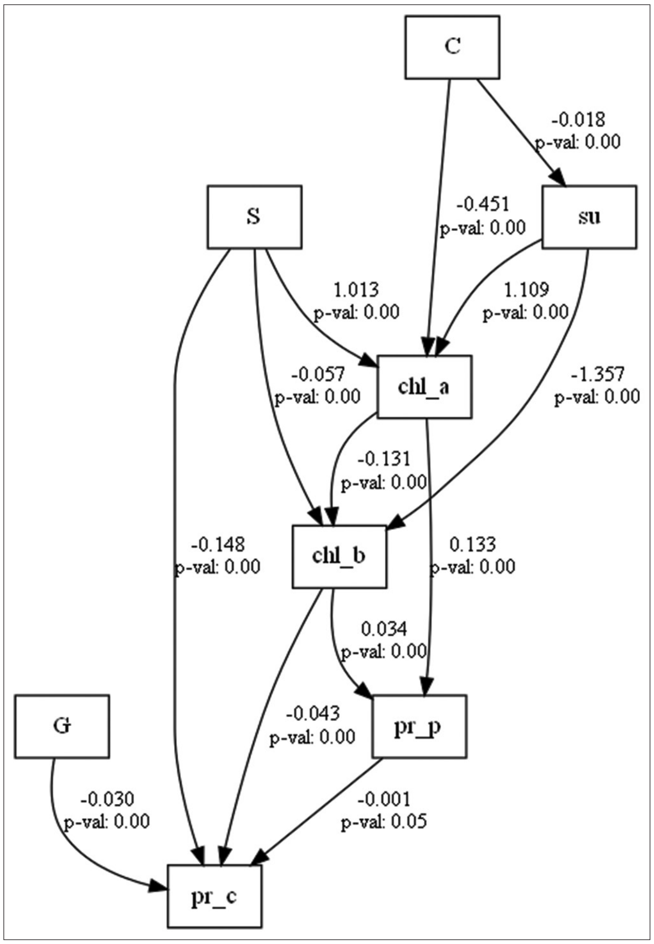


Figure 2: Path diagram of direct and indirect effects on biochemical (pr-c) trait expression

membrane damage, ion leakage, and cell death. SA activated enzymatic defenses SOD ($O_2^- \rightarrow H_2O_2 + O_2$), CAT ($H_2O_2$ breakdown), PODs like APX (peroxide detoxification) reducing lipid degradation and improving chlorophyll stability, protein content, and seed biochemical quality under stress (citations retained). SA also enhanced non-enzymatic antioxidants (ascorbate, glutathione, carotenoids, flavonoids, phenolics), which stabilized redox states via radical scavenging and energy dissipation. Biosynthesis regulation occurred via SA-induced signaling cascades. SA efficacy was dose-dependent and genotype-specific; 400 mg doses yielded optimal stress resilience.

Genotypes G3 and G5 showed elevated enzyme-based antioxidant activity, while G6's tolerance reflected stable chlorophyll b and leaf water content. Water stress treatments severe (5% FC, S1), moderate (70% FC, S2), control (S3) showed expected declines in growth and yield under S1, but S2 unexpectedly boosted biochemical traits like seed protein and sugar, indicating hormetic resource reallocation.

Moderate drought enhances osmoprotectants (sucrose, raffinose, fructans) to maintain osmotic balance and membrane integrity, with soybean seeds showing elevated sugar levels tied to both osmotic adjustment and carbon storage, plus gene regulation (Fraire-Velázquez *et al.*, 2013; Sharma *et al.*, 2019; Ghosh *et al.*, 2021; Mehta & Vyas, 2023). Protein rises via nitrate reductase and glutamine synthetase activity, reallocating nitrogen to seeds despite yield loss. ABA and SA trigger protective responses; SA boosts antioxidants and stabilizes chlorophyll and proteins (Ren *et al.*, 2023; Decsi *et al.*, 2025). Stress prioritizes seed quality, a trend seen across crops under moderate salinity and drought (Razmi *et al.*, 2017; Kuchlan & Kuchlan, 2021; Zamani *et al.*, 2024).

Under SA treatments and drought, six soybean genotypes (G1-G6) showed varied responses: G3 had top sugar levels under 400 mg SA at 70% field capacity, while G5 led in protein percentage, absolute protein, and yield (Goel & Singh, 2015; Hajibarat & Saidi, 2022; Zayed *et al.*, 2023; Ruiz-Romero *et al.*, 2024; Liu *et al.*, 2025). G3 retained chlorophyll b; G5 showed pigment stability, photosynthetic efficiency, and seed fill. Both had high biomass, height, pod number, and seeds per pod, aligning with known SA-induced, genotype-specific drought tolerance (Razmi *et al.*, 2017; Kuchlan & Kuchlan, 2021; Abebe *et al.*, 2024). G6 prioritized biochemical stability with highest chlorophyll b and leaf water content but had lower biomass, seed yield, and pod/seed count, with large trait shifts hinting at survival-oriented regulation (Chaves & Oliveira, 2004; Balestrini *et al.*, 2018; Khan *et al.*, 2018; Wahab *et al.*, 2022; Alafari *et al.*, 2024).

Genotypic trade-offs are evident: G6 favors antioxidant defense and chlorophyll b retention under stress, aiding drought escape but lowering yield (Sharma *et al.*, 2021; Zhao *et al.*, 2021; Wang *et al.*, 2022; Ren *et al.*, 2023), while G3 and G5 maintain yield and trait stability, especially under salicylic acid (SA). SA at 250 mg and 400 mg enhances traits like protein and chlorophyll in G3/G5, but G6 may need alternate strategies (Razmi *et al.*, 2017; Kuchlan & Kuchlan, 2023; Decsi *et al.*, 2025). Genotype strongly influences traits (e.g., F>3000 for height, F>1000 for protein), as confirmed by MANOVA (Sharma *et al.*, 2021; Abebe *et al.*, 2024; Vymyslický *et al.*, 2025), with proteomics supporting nutrient stress tolerance (Zhao *et al.*, 2021).

Water stress at 5% FC reduces yield, while 70% FC boosts protein and sugars. Genotype × stress × treatment (G×S×C) effects reveal context-specific trait expression, requiring multi-factorial trials (MacMillan *et al.*, 2006; Vaidya & Stinchcombe, 2020; Lee *et al.*, 2022). Key traits include chlorophyll a/b, protein, and sugar (SEM, ML), guiding breeding for resilience and performance (Sharma *et al.*, 2021). The study used six genotypes (G1-G6), two SA doses, and three water regimes, but lacked field variability and broad germplasm, limiting generalizability (La *et al.*, 2019; Azam *et al.*, 2020). Omics layers were not included, and trials were single-location.

Further research can explore expanding stress gradients, incorporate diverse genetics and multi-omics, and conduct field trials in sub-Saharan Africa for G×E×M modeling (Majidian *et al.*, 2024; Abebe *et al.*, 2024; Vymyslický *et al.*, 2025), while refining SA use with biochar, silicon, microbes, and hormone profiling (Alizadeh *et al.*, 2024; Nyzhnyk *et al.*, 2025).

CONCLUSION

This study presents a rigorous, multidimensional examination of soybean biochemical trait expression across factorial combinations of genotype, salicylic acid (SA) application, and water stress. Through integrated machine learning sensitivity analyses and statistical modeling, it reveals that genotype not only governs phenotypic variability but also shapes response plasticity to abiotic stressors and biochemical treatments. Notable three-way interactions highlight the necessity of genotype-specific agronomic strategies, especially under changing environmental conditions. Genotypes G3 and G5 emerged as optimal for both productivity and resilience, while G6 demonstrated a trade-off prioritizing stress tolerance. Moderate drought exerted hormetic effects, enhancing key traits, and SA applications amplified pigment and protein expression depending on contextual factors. These findings deepen mechanistic insights into G×T×E dynamics and offer a scalable framework for precision phenotyping, stress physiology, and trait optimization in legume agronomy.

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