

Article

Biochemical Defenses of Tomato in Response to Multiple Herbivory Damage Under Contrasting Irrigation Environments

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Abstract

Water limitation and herbivore attack frequently co-occur in agricultural systems, yet defense-related biochemical responses under these combined conditions remain difficult to predict, particularly during simultaneous attack by multiple herbivore species. We investigated how defense responses in field-grown tomato (*Solanum lycopersicum* L.) differed between irrigation environments and according to herbivore species identity and combination. Well-watered and water-deficit plants were exposed to the spider mites *Tetranychus evansi* and *Tetranychus urticae* and the tomato leafminer *Tuta absoluta*, individually or in interspecific combinations involving two or three species. Plants received either 100% or 50% of the estimated crop water requirements, with leaves collected before and after seven days of herbivore feeding. Gravimetric soil moisture was 21.1% in the water-deficit sector and 29.0% in the well-watered sector. Hormonal responses differed between irrigation environments and among herbivore treatments, with significant interactions for abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), indole-3-acetic acid (IAA), and zeatin. ABA and SA concentrations were generally higher in the water-deficit environment, whereas JA concentrations were generally higher in the well-watered environment. IAA and zeatin exhibited distinct responses associated with *T. urticae* infestation under water-deficit conditions. Proline concentrations were generally higher in the water-deficit environment. Simultaneous infestations involving two or three herbivore species were associated with distinct biochemical response patterns, particularly in lipoxygenase (LOX), polyphenol oxidase (PPO), and trypsin inhibitory activity (TIA). Fruit production also differed between irrigation environments, whereas the proportion of fruits damaged by *Neoleucinodes elegantalis* was similar (29.3% and 30.8% in well-watered and water-deficit environments, respectively). Overall, these findings indicate that tomato hormonal and biochemical responses to herbivory vary with irrigation environment and herbivore community composition, highlighting the context-dependent nature of plant responses to combined abiotic and biotic challenges.



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1. Introduction

Climate change is increasing the frequency and severity of water limitation in agricultural systems, exposing crops to increasingly complex combinations of abiotic and biotic stresses [1]. These effects are highly context-dependent, varying with stress intensity and duration, plant genotype, herbivore identity, feeding guild, and host specialization [1,2]. Consequently, plant responses to simultaneous abiotic and biotic stresses may differ substantially from responses to either stress alone and can result in additive, synergistic, or antagonistic effects [2,3]. In tomato (*Solanum lycopersicum* L.), these interactions involve a coordinated defense network in which jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA) interact with metabolic, oxidative, and protein-based defenses [4,5]. Because water deficit strongly activates ABA-dependent signaling and alters cellular redox and metabolic status, it can modify several components of herbivore-induced defense.

Herbivore identity further shapes these responses because arthropods differ in their ability to induce, tolerate, or manipulate plant defenses. In tomato, the two-spotted spider mite *Tetranychus urticae* Koch generally induces JA- and SA-associated defenses, whereas *Tetranychus evansi* Baker & Pritchard can suppress components of these pathways and create a more favorable feeding environment [6]. These contrasting strategies become particularly relevant when both species share the same host: *T. evansi* can suppress defenses previously induced by *T. urticae*, whereas *T. urticae* can induce defenses in tissues previously attacked by *T. evansi* [7–9]. Simultaneous infestation studies likewise show that *T. evansi* can attenuate *T. urticae*-induced tomato responses [9]. The tomato leafminer *Tuta absoluta* (Meyrick), another major tomato pest, also induces hormonal and biochemical defenses, particularly those associated with JA signaling and antinutritive responses [10]. Together, these contrasting herbivore strategies indicate that plant responses to multiple herbivores cannot necessarily be predicted from the effects of individual species.

Water availability adds another layer of complexity to these interactions. Moderate water deficit can alter free sugar and amino acid concentrations in tomato and improve host nutritional quality for *T. evansi*, increasing feeding damage and mite performance [1]. Water limitation also modifies ABA-, JA-, and SA-associated responses during *T. evansi* infestation, while the mite can retain its ability to suppress important components of tomato defense under water-limited conditions [11]. Similarly, water availability can modify bottom-up effects on *T. absoluta*, with outcomes depending on host physiological status and stress severity [12], and combined water deficit and *T. absoluta* herbivory can produce non-additive physiological and metabolic responses [3]. Thus, water limitation can simultaneously modify plant physiology, defense-associated responses, and herbivore performance.

Despite this progress, most mechanistic studies have examined relatively simplified plant–herbivore systems, frequently involving a single herbivore species under controlled conditions [11,13]. Much less is known about how water availability shapes plant responses when herbivores with contrasting capacities to induce or suppress host defenses occur together. Here, we investigated how water deficit and herbivore community composition interact to shape tomato defense-associated responses and crop performance under field conditions. Plants under well-watered or water-deficit conditions were exposed to *T. evansi*, *T. urticae*, and *T. absoluta* individually and in interspecific combinations. We quantified phytohormones and proline, measured lipoxygenase (LOX), polyphenol oxidase (PPO),

and trypsin inhibitory activity (TIA), and evaluated fruit production and damage caused by the fruit borer *Neoleucinodes elegantalis*.

We hypothesized that water deficit would alter herbivory-associated hormonal and biochemical responses rather than uniformly strengthening or weakening tomato defense. We further predicted that the magnitude and direction of these responses would depend on herbivore identity and community composition because *T. urticae*, *T. evansi*, and *T. absoluta* differ in feeding strategy, host specialization, and their capacity to induce or suppress tomato defenses. Finally, we predicted that water limitation would modify fruit production traits and the incidence of fruit-borer damage.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Tomato plants (*Solanum lycopersicum* L., cv. Thaíse; Feltrin Seeds, Farroupilha, RS, Brazil) were used throughout the study. Seeds were sown in 8 × 16-cell plastic trays containing commercial substrate (Carolina Soil®, Santa Cruz do Sul, Brazil), covered with plastic film, and maintained in a germination chamber at 30 °C for 3 days. After germination, trays were transferred to a greenhouse equipped with an automatic irrigation system, and seedlings received two fertigation applications before transplantation.

Thirty days after sowing, seedlings were transplanted into previously prepared and fertilized soil in the experimental field of the Department of Plant Science, Federal University of Viçosa (UFV), Viçosa, Minas Gerais, Brazil (20°45'14" S, 42°52'53" W; 648 m a.s.l.), during the May–October growing season. A drip-irrigation system and vertical trellising system were installed (Figure S1). The experimental area comprised two irrigation sectors, each consisting of six rows with 14 plants per row. Plants were spaced 0.6 m within rows and 1 m between rows.

Plants were maintained under standard irrigation for 2 weeks after transplantation. Thereafter, contrasting irrigation regimes were established. The well-watered regime corresponded to 100% of the estimated crop water requirement, equivalent to approximately 3 L plant^{−1} day^{−1} under the conditions used in this study [14]. Irrigation was progressively restricted in the water-deficit sector during establishment of the treatment, and the two regimes were stabilized at 100% and 50% of crop water requirements before herbivore infestations were initiated.

Standard crop-management practices, including fertigation, weeding, pruning, and plant training, were performed according to local recommendations for tomato production [15]. Preventive pesticide applications were performed twice weekly for approximately 6 weeks after transplantation and were discontinued 20 days before the experimental herbivore infestations were initiated. The products used during this pre-experimental crop-management period are detailed in Table S1. No pesticide applications were performed during the 20-day interval preceding the experimental infestations or during the experimental period.

2.2. Irrigation Management and Soil Water Monitoring

Irrigation requirements were determined every 2–3 days using meteorological data obtained from an IrriPlus E5000 field weather station. Crop evapotranspiration (ET_c) was estimated from reference evapotranspiration (ET_o) using crop coefficients and meteorological, soil, water, plant, and irrigation-system parameters according to the FAO-56 Penman–Monteith approach [16,17]. These estimates were used to determine the irrigation requirement for each irrigation regime.

Soil water status was independently assessed by gravimetric determination of soil moisture. Soil samples were collected at approximately 40 cm depth using a manual auger.

Fresh soil mass was recorded immediately after sampling, after which samples were oven-dried at 100 °C for 24 h and weighed again. Gravimetric soil moisture was calculated as $[(\text{fresh soil mass} - \text{dry soil mass}) / \text{dry soil mass}] \times 100$. Gravimetric soil moisture was lower in the water-deficit sector at the time of sampling (Figure S2). Additional irrigation calculations are provided in the Supplementary Material.

2.3. Herbivore Colonies

Colonies of the spider mites *Tetranychus evansi* Baker & Pritchard and *Tetranychus urticae* Koch were obtained from the Acarology Laboratory, whereas *Tuta absoluta* (Meyrick) adults were obtained from the Integrated Pest Management Laboratory, both at the Department of Entomology, UFV.

New colonies were established and maintained on tomato plants cv. Santa Clara I-5300 under controlled conditions at 25 ± 5 °C, $70 \pm 5\%$ relative humidity, and a 12 h photoperiod until arthropods were used for field infestations.

2.4. Experimental Design and Herbivore Infestation

The experiment consisted of two independent irrigation sectors corresponding to well-watered (100% crop water requirement) and water-deficit (50% crop water requirement) conditions. Within each irrigation sector, four rows were used as experimental blocks. Ten herbivory treatments were randomly assigned to individual tomato plants within each block, resulting in four biological replicates for each herbivory treatment within each irrigation regime. Border rows and plants at the ends of rows were maintained to minimize edge effects.

The irrigation regime was imposed at the sector level, with one field sector assigned to each water regime. Four plots within each sector provided biological replication for the herbivory treatments. However, the irrigation treatment itself was not independently replicated across multiple field sectors. Therefore, comparisons between well-watered and water-deficit plants should be interpreted as contrasts between the two irrigation environments established in this experiment, rather than independently replicated causal effects of irrigation regime.

The ten treatments were (i) non-infested plants sampled before infestation (CBI); (ii) non-infested plants sampled after the infestation period (CAI); (iii) 100 adult females of *T. evansi*; (iv) 100 adult females of *T. urticae*; (v) nine third-instar *T. absoluta* larvae; (vi) mechanical damage; (vii) 50 *T. evansi* females + 50 *T. urticae* females; (viii) 100 *T. evansi* females + nine *T. absoluta* larvae; (ix) 100 *T. urticae* females + nine *T. absoluta* larvae; and (x) 50 *T. evansi* females + 50 *T. urticae* females + nine *T. absoluta* larvae.

CBI and CAI were included as temporal non-infested controls. CBI represented the baseline condition and was sampled immediately before the infestation period. CAI consisted of non-infested plants sampled seven days later, at the same time as the herbivore treatments. Thus, CAI served as the time-matched non-infested control for the herbivore treatments. Because CBI and CAI were sampled at different time points, differences between them may reflect temporal changes and should not be interpreted as effects of herbivore infestation.

Immediately after herbivore introduction, each treated leaf was individually enclosed in an organza bag to confine the arthropods to the designated experimental leaf and minimize movement among plants and treatments. The organza bags remained in place throughout the seven-day infestation period until leaf sampling.

Mechanical damage was imposed using fine forceps to puncture the selected fourth fully expanded leaf. Unlike the herbivore treatments, in which arthropods remained on the selected leaves for seven days until sample collection, the mechanical-damage treat-

ment represented a physical-injury stimulus. The original experimental records did not provide quantitative information on the number of punctures or the total damaged leaf area; therefore, this treatment should be interpreted as a qualitative mechanical-damage control rather than as a quantitatively matched simulation of herbivore feeding.

2.5. Leaf Sampling

After the 7-day infestation period, leaflets from the treated leaves were excised with scissors, immediately wrapped in aluminum foil, flash-frozen in liquid nitrogen, and transferred to a -80°C freezer until biochemical and metabolite analyses.

2.6. Phytohormone and Proline Quantification

Frozen tomato leaf tissue was ground to a fine powder in liquid nitrogen and weighed. For each 100 mg of tissue, 200 μL of extraction solution consisting of methanol:isopropanol:acetic acid (20:79:1, $v/v/v$) was added. Samples were sonicated for 30 min and centrifuged at $14,000\times g$ for 30 min at 4°C . The supernatant was collected, and the extraction procedure was repeated. Extracts were stored at -80°C until chromatographic analysis.

Approximately 300 μL of each extract was filtered and transferred to chromatographic vials, and 5 μL was injected into the UHPLC-MS/MS system at the Nucleus of Biomolecule Analysis (NuBioMol), Federal University of Viçosa. Chromatographic separation was performed using an Agilent Eclipse Plus RRHD column (1.8 μm , 2.1×50 mm), at a flow rate of 0.3 mL min^{-1} , coupled online to an Agilent triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

The compounds analyzed were abscisic acid (ABA), jasmonic acid (JA), methyl jasmonate (MeJA), salicylic acid (SA), 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA), zeatin, and proline. ACC was quantified as the immediate biosynthetic precursor of ethylene rather than as ethylene itself. The mass spectrometer was operated in alternating positive and negative ionization modes according to the retention characteristics of each compound, and analytes were monitored in multiple reaction monitoring (MRM) mode.

Four biological replicates were analyzed per treatment. Mass spectra were processed using MassHunter software (version B.08.00, Agilent Technologies) to obtain extracted-ion chromatograms for each transition. Peak-area data were further processed using Skyline software (version 4.1, MacCoss Laboratory, University of Washington, Seattle, WA, USA). Calibration curves ranging from 0.1 to 300 ng mL^{-1} were used to convert peak areas into metabolite concentrations, expressed as ng g^{-1} fresh leaf tissue.

2.7. Preparation of Leaf Extracts for Biochemical Assays

Frozen plant material was weighed, ground in liquid nitrogen, and homogenized at a tissue-to-buffer ratio of 1:3 (w/v). For lipoxygenase assays, tissue was extracted in 50 mM sodium phosphate buffer (pH 6.5). For trypsin inhibitory activity assays, tissue was extracted in 100 mM Tris-HCl buffer (pH 8.2) containing 20 mM CaCl_2 . Extracts were centrifuged at $17,200\times g$ for 60 min at 4°C , and the supernatants were collected for biochemical analyses [18].

Soluble protein concentration was determined according to Bradford [19], using bovine serum albumin (BSA; $0\text{--}0.2\text{ mg mL}^{-1}$) as the standard. Spectrophotometric assays were performed using a Hitachi U-5100 ratio-beam spectrophotometer at the Enzymes, Proteins and Peptides Biochemistry Laboratory, Institute of Biotechnology Applied to Agriculture (BIOAGRO), UFV, Viçosa, MG, Brazil.

2.8. Lipoxygenase Activity

Lipoxygenase (LOX) activity was determined using linoleic acid as substrate according to Axelrod et al. [20]. The reaction mixture contained 10 μL of leaf extract, 20 μL of sodium linoleate stock solution, and sodium phosphate buffer to a final volume of 1.0 mL. Formation of conjugated dienes was monitored at 234 nm every 30 s for 150 s. Enzyme activity was calculated from the change in absorbance between 30 and 150 s using a molar extinction coefficient of $25,000 \text{ M}^{-1} \text{ cm}^{-1}$ and normalized to soluble protein concentration. The equations used for activity calculations are provided in the Supplementary Material.

2.9. Trypsin Inhibitory Activity

Trypsin inhibitory activity (TIA) in tomato leaf extracts was determined by measuring the inhibition of commercial trypsin using *N* α -benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) as substrate, based on Erlanger et al. [21]. BAPNA was used at a final concentration of 1.2 mM. The trypsin control contained 100 μL of $4.7 \times 10^{-5} \text{ M}$ trypsin solution and 1100 μL of Tris-HCl buffer before addition of substrate. For samples, 100 μL of leaf extract was mixed with 100 μL of trypsin solution and 500 μL of buffer. After 5 min of pre-incubation at 25 $^{\circ}\text{C}$, the mixture was transferred to a reaction containing 500 μL of buffer and 500 μL of BAPNA substrate solution. BAPNA hydrolysis was monitored spectrophotometrically at 410 nm every 30 s for 150 s. Trypsin inhibition was calculated from the difference in activity between the trypsin control and samples containing leaf extract and expressed as milligrams of trypsin inhibited per gram of protein.

2.10. Polyphenol Oxidase Activity

Polyphenol oxidase (PPO) activity was determined using catechol as substrate according to Soliva et al. [22]. Each reaction contained 960 μL of 0.2 M catechol and 40 μL of plant extract. Product formation was monitored at 410 nm every 30 s for 150 s. Enzyme activity was calculated from the change in absorbance between 30 and 150 s using a molar extinction coefficient of $1470 \text{ M}^{-1} \text{ cm}^{-1}$ and normalized to soluble protein concentration. The equations used for activity calculations are provided in the Supplementary Material.

2.11. Tomato Fruit Production and Fruit-Borer Damage

Ripe tomato fruits were evaluated for fruit number, size, and weight. Fruits were classified into four diameter classes (10, 8, 6.5, and 5 cm), and fruit groups were weighed using an analytical balance. Damage caused by the tomato fruit borer *Neoleucinodes elegantalis* was also recorded.

2.12. Statistical Analyses

Four biological replicates ($n = 4$) were used for phytohormone and proline analyses, whereas five biological replicates ($n = 5$) were used for the enzymatic assays (LOX, PPO, and TIA) for each herbivory treatment within each irrigation environment. Herbivory treatments were randomized among four experimental plots within each irrigation sector. Because each irrigation regime was imposed at the sector level, with one sector assigned to each water regime, the four plots represented biological replication of the herbivory treatments within each irrigation environment but not independent replication of the irrigation treatment itself. Consequently, statistical comparisons between irrigation environments were interpreted cautiously as contrasts between the two field environments established in this experiment rather than as independently replicated causal effects of water regime.

Gravimetric soil moisture was compared between the well-watered and water-deficit irrigation environments using the Mann–Whitney U test. Five independent soil samples were analyzed per irrigation environment ($n = 5$), and differences were considered signifi-

cant at $p < 0.05$. Phytohormone and proline data were analyzed by two-way analysis of variance (ANOVA), considering water regime, infestation treatment, and their interaction, followed by pairwise comparisons using the Student–Newman–Keuls method at a significance level of $p < 0.05$. Statistical analyses were performed using SigmaPlot 12 (Systat Software, Inc., San Jose, CA, USA). Enzymatic data were originally analyzed using linear mixed-effects models in R version 3.4.1 (R Core Team, 2017), with treatment contrasts evaluated by general linear hypothesis testing. The statistical outputs obtained from these analyses were retained, but their interpretation was restricted to the observed differences among experimental groups.

Spearman rank correlations were used as exploratory analyses to examine patterns of association among biochemical variables. No correction for multiple comparisons was applied; therefore, these correlations were considered hypothesis-generating rather than confirmatory. Significant associations were not interpreted as evidence of direct regulatory or causal relationships among variables. Data are presented as mean $\pm$ standard error of the mean (SEM). Graphs were generated using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Soil Moisture Differed Between Irrigation Environments

Gravimetric soil moisture differed significantly between the two irrigation environments. Soil moisture averaged 21.1% in the water-deficit sector and 29.0% in the well-watered sector, representing an approximately 27% lower gravimetric soil moisture under water deficit. The difference between irrigation environments was significant (Mann–Whitney $U = 0$, exact $p = 0.008$; Figure S2).

3.2. Water Deficit and Herbivore Infestation Differentially Affect Tomato Phytohormonal and Stress-Related Responses

Water regime, herbivore infestation, and their interaction differentially affected the phytohormonal and stress-related responses of tomato plants (Table 1).

Table 1. Two-way ANOVA results for the effects of water regime, herbivore infestation, and their interaction on phytohormone and proline levels in tomato plants.

Two-Way ANOVA Parameters					
Variable	Source of Variation	D.F.	M.S.	F	p
Absciscic acid	Water regime	1	2269.587	233.511	<0.001
	Infestation	9	154.315	15.877	<0.001
	Interaction	9	46.264	4.760	<0.001
	Residual	60	9.719		
	Total	79	58.961		
1-Aminocyclopropane-1-carboxylic acid (ACC)	Water regime	1	585,190.4	37.188	<0.001
	Infestation	9	71,339.758	4.534	<0.001
	Interaction	9	25,732.466	1.635	0.126
	Residual	60	15,735.895		
	Total	79	30,417.647		
Indole-3-acetic acid	Water regime	1	0.526	1.783	0.187
	Infestation	9	0.589	1.997	0.055
	Interaction	9	0.832	2.820	0.008
	Residual	60	0.295		
	Total	79	0.392		

Table 1. Cont.

Two-Way ANOVA Parameters					
Variable	Source of Variation	D.F.	M.S.	F	p
Jasmonic acid	Water regime	1	1532.058	20.404	<0.001
	Infestation	9	354.425	4.720	<0.001
	Interaction	9	236.918	3.155	0.004
	Residual	60	75.087		
	Total	79	143.790		
Methyl jasmonate	Water regime	1	58.858	3.598	0.063
	Infestation	9	20.840	1.274	0.270
	Interaction	9	22.178	1.356	0.229
	Residual	60	16.358		
	Total	79	18.070		
Proline	Water regime	1	2,984,902,272	27.993	<0.001
	Infestation	9	473,841,584	4.444	<0.001
	Interaction	9	197,697,446	1.854	0.077
	Residual	60	106,629,941		
	Total	79	535,324,943		
Salicylic acid	Water regime	1	617,681.826	87.819	<0.001
	Infestation	9	34,065.069	4.843	<0.001
	Interaction	9	34,128.900	4.852	<0.001
	Residual	60	7033.570		
	Total	79	20,929.642		
Zeatin	Water regime	1	9933.325	1.434	0.236
	Infestation	9	25,271.707	3.649	0.001
	Interaction	9	24,713.651	3.569	0.001
	Residual	60	6924.752		
	Total	79	11,079.578		

D.F. = degrees of freedom; M.S. = mean square; F = F statistic; p = p-value.

Significant water regime $\times$ infestation interactions were detected for abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA), salicylic acid (SA), and zeatin, indicating that the effects of herbivore infestation on these compounds differed between irrigation environments. In contrast, 1-aminocyclopropane-1-carboxylic acid (ACC) and proline were significantly affected by water regime and infestation, but not by their interaction. Methyl jasmonate (MeJA) was not significantly affected by water regime, infestation, or their interaction.

ABA concentrations were significantly affected by water regime ($F = 233.511$, $p < 0.001$), infestation ($F = 15.877$, $p < 0.001$), and their interaction ($F = 4.760$, $p < 0.001$; Table 1). Overall, ABA concentrations were higher in water-deficit than in well-watered plants, although the magnitude of this increase varied among infestation treatments (Figure 1A).

Before the experimental infestation period, control plants under water deficit exhibited the highest ABA concentrations. After the experimental period, ABA concentrations differed among herbivore treatments under water deficit, with comparatively high concentrations in plants infested with *T. urticae*. In contrast, ABA concentrations varied less markedly among herbivore treatments under well-watered conditions.

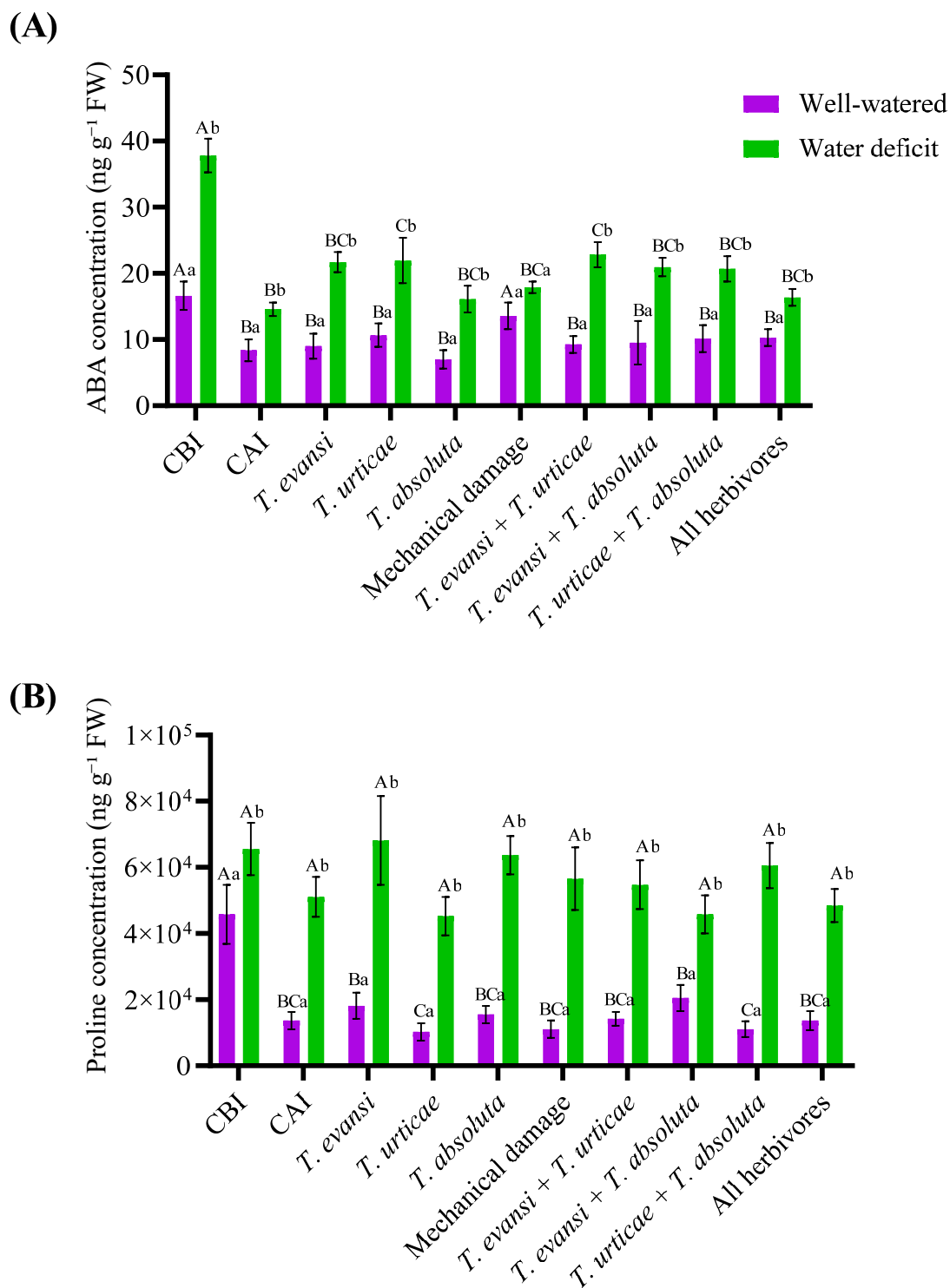


Figure 1. Abscissic acid (ABA) (A) and proline (B) concentrations in tomato plants under well-watered and water-deficit conditions. Treatments included non-infested controls evaluated before (CBI) and after (CAI) seven days of the experiment, and plants subjected for seven days to *Tetranychus evansi*, *Tetranychus urticae*, *Tuta absoluta*, mechanical damage, *T. evansi* + *T. urticae*, *T. evansi* + *T. absoluta*, *T. urticae* + *T. absoluta*, or all three herbivores. Bars represent the mean $\pm$ standard error of the mean (SEM) ($n = 4$). Different lowercase letters indicate significant differences between irrigation environments within each treatment, whereas different uppercase letters indicate significant differences among treatments within the same irrigation environment, according to the Student–Newman–Keuls test ($p < 0.05$).

Proline concentrations were significantly affected by water regime ($F = 27.993$, $p < 0.001$) and infestation ($F = 4.444$, $p < 0.001$), whereas the water regime $\times$ infestation interaction was not significant ($F = 1.854$, $p = 0.077$; Table 1). Proline concentrations were generally higher in the water-deficit environment across infestation treatments (Figure 1B). Under well-watered conditions, proline varied among treatments, with pre-infestation control plants showing comparatively high concentrations and *T. urticae*-infested plants among the lowest. In contrast, proline concentrations showed less differentiation among herbivore treatments under water deficit.

ACC concentrations were significantly affected by water regime ($F = 37.188$, $p < 0.001$) and infestation ($F = 4.534$, $p < 0.001$), whereas the water regime $\times$ infestation interaction was not significant ($F = 1.635$, $p = 0.126$; Table 1). Overall, ACC concentrations were higher in the well-watered environment than in the water-deficit environment (Figure 2A).

Pre-infestation control plants showed comparatively high ACC concentrations, followed by an overall decrease after the experimental period. Under well-watered conditions, relatively high ACC concentrations occurred in plants exposed to *T. evansi*, *T. absoluta*, mechanical damage, and the combination of *T. evansi* + *T. absoluta*. Under water deficit, ACC concentrations remained comparatively low across most herbivore treatments.

IAA was not significantly affected by water regime as a main effect ($F = 1.783$, $p = 0.187$) or by infestation ($F = 1.997$, $p = 0.055$), whereas the water regime $\times$ infestation interaction was significant ($F = 2.820$, $p = 0.008$; Table 1). IAA concentrations varied little among infestation treatments under well-watered conditions (Figure 2B). Under water deficit, however, plants infested with *T. urticae* showed a pronounced increase in IAA relative to the other treatments. Thus, differences in IAA between irrigation regimes depended on herbivore treatment rather than reflecting a general effect of water deficit.

Zeatin was not significantly affected by water regime as a main effect ($F = 1.434$, $p = 0.236$), whereas infestation ($F = 3.649$, $p = 0.001$) and the water regime $\times$ infestation interaction ($F = 3.569$, $p = 0.001$) were significant (Table 1). Water-deficit plants infested with *T. urticae* exhibited comparatively high zeatin concentrations among the treatments (Figure 2C). Differences between irrigation environments were also observed for specific mixed-herbivore treatments, indicating that zeatin responses varied according to herbivore species identity and combination.

3.3. Jasmonic and Salicylic Acid Responses Depend on Water Regime and Herbivore Community Composition

JA concentrations were significantly affected by water regime ($F = 20.404$, $p < 0.001$), herbivore infestation ($F = 4.720$, $p < 0.001$), and their interaction ($F = 3.155$, $p = 0.004$; Table 1). Under well-watered conditions, herbivore infestation generally increased JA concentrations relative to non-infested controls, although the magnitude of induction varied among treatments (Figure 3A).

Particularly high JA concentrations were observed in plants simultaneously infested with *T. urticae* + *T. absoluta* and in plants exposed to all three herbivore species. Mechanical damage was also associated with relatively high JA concentrations.

Under water-deficit conditions, a different pattern was observed. Plants infested with *T. urticae* alone showed comparatively high JA concentrations, whereas JA concentrations were lower than those observed under well-watered conditions for several other infestation treatments, including some mixed-herbivore combinations (Figure 3A). Overall, the significant water regime $\times$ infestation interaction indicates that differences in JA concentrations between irrigation environments varied according to herbivore species identity and combination.

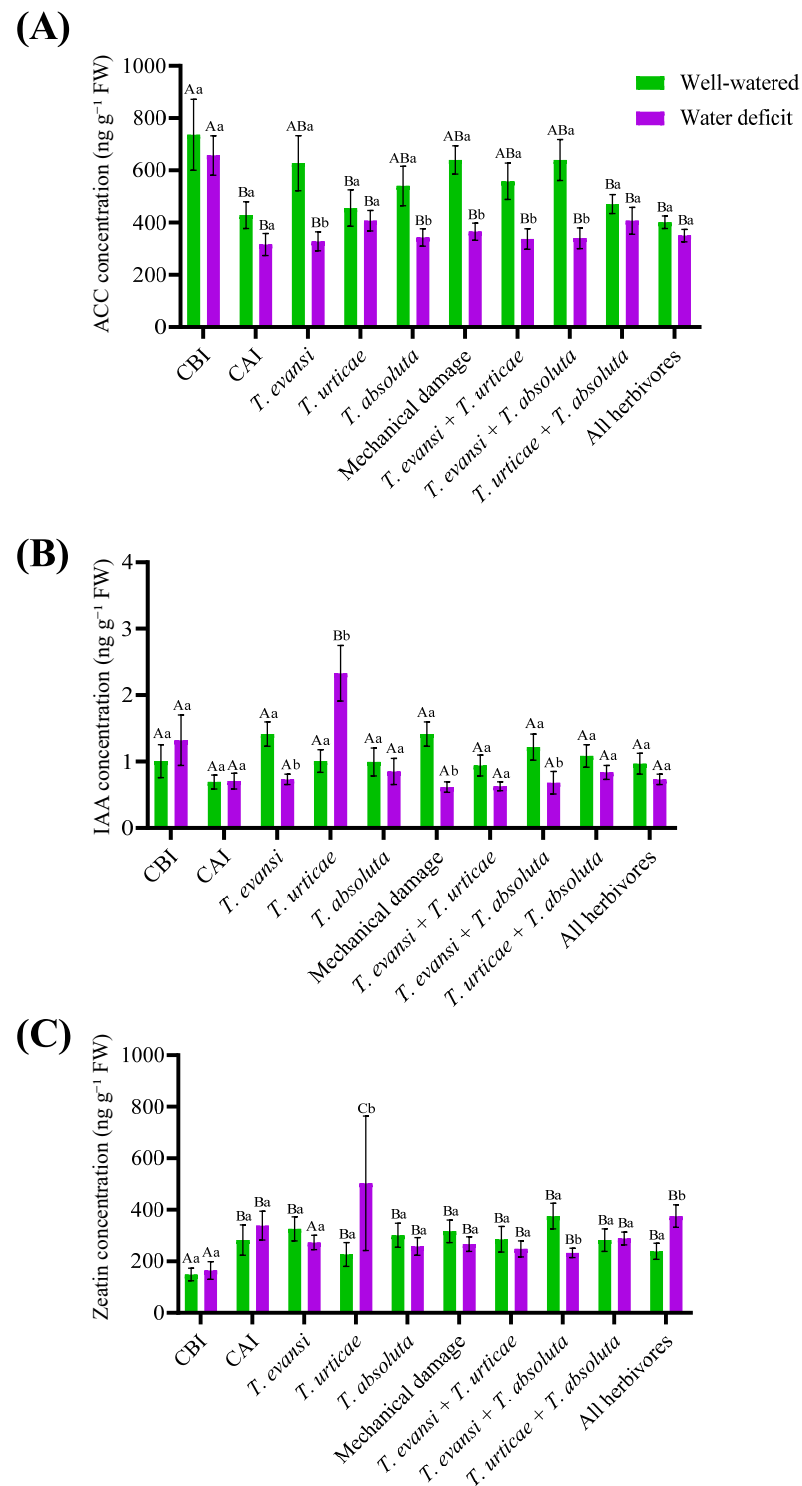


Figure 2. Concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC) (A), indole-3-acetic acid (IAA) (B), and zeatin (C) in tomato plants under well-watered and water-deficit conditions. Treatments included non-infested controls evaluated before (CBI) and after (CAI) seven days of the experiment, and plants subjected for seven days to *Tetranychus evansi*, *Tetranychus urticae*, *Tuta absoluta*, mechanical damage, *T. evansi* + *T. urticae*, *T. evansi* + *T. absoluta*, *T. urticae* + *T. absoluta*, or all three herbivores. Bars represent the mean $\pm$ standard error of the mean (SEM) ($n = 4$). Different lowercase letters indicate significant differences between irrigation environments within each treatment, whereas different uppercase letters indicate significant differences among treatments within the same irrigation environment, according to the Student–Newman–Keuls test ($p < 0.05$).

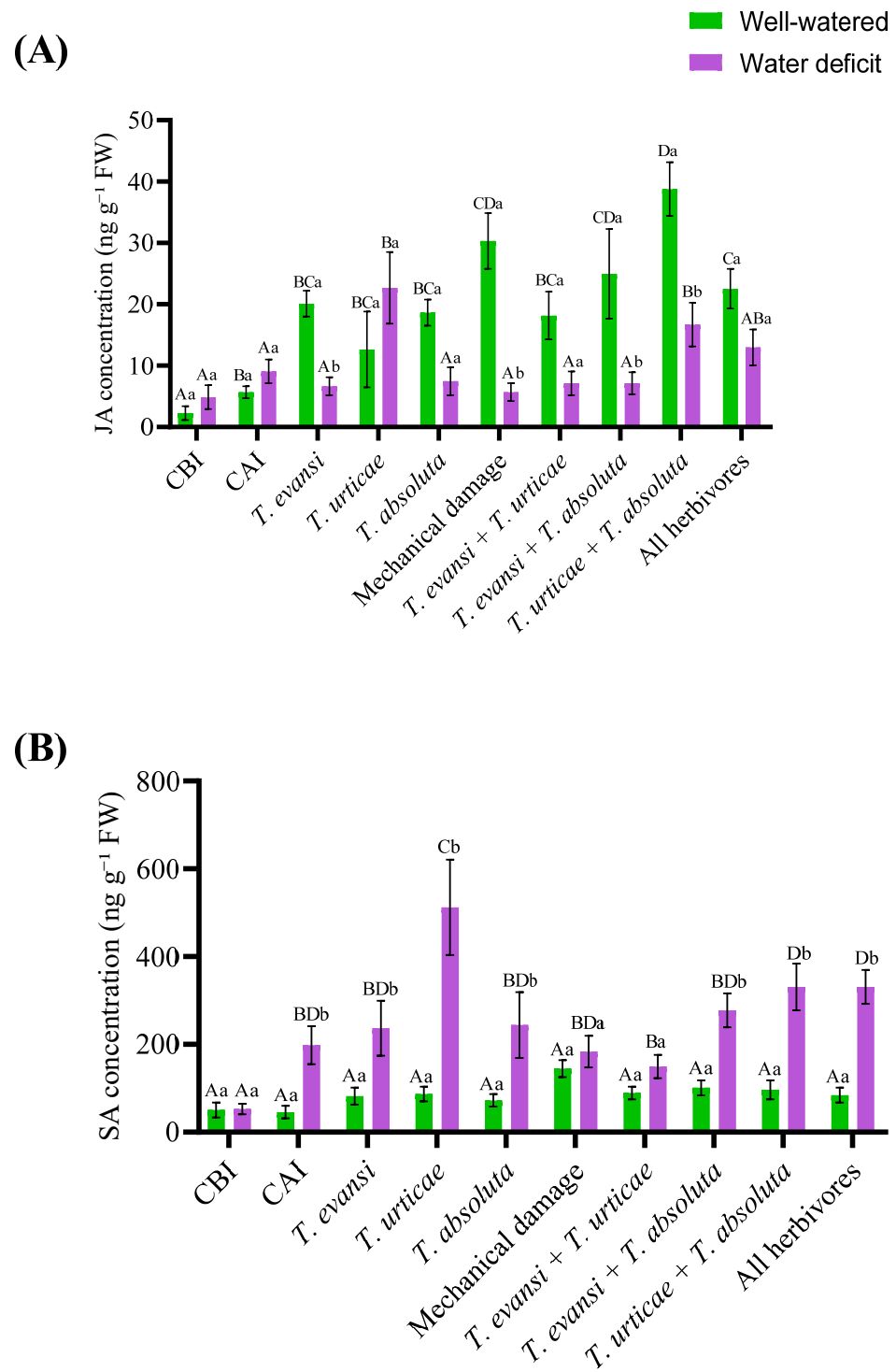


Figure 3. Jasmonic acid (JA) (A) and salicylic acid (SA) (B) concentrations in tomato plants under well-watered and water-deficit conditions. Treatments comprised non-infested controls sampled before (CBI) and after (CAI) the seven-day experimental period; *Tetranychus evansi*; *Tetranychus urticae*; *Tuta absoluta*; mechanical damage; *T. evansi* + *T. urticae*; *T. evansi* + *T. absoluta*; *T. urticae* + *T. absoluta*; and all three herbivores combined. Bars represent the mean $\pm$ standard error of the mean (SEM) ($n = 4$). Different lowercase letters indicate significant differences between irrigation environments within each treatment, whereas different uppercase letters indicate significant differences among treatments within each irrigation environment (Student–Newman–Keuls test, $p < 0.05$).

SA concentrations were significantly affected by water regime ($F = 87.819$, $p < 0.001$), infestation ($F = 4.843$, $p < 0.001$), and their interaction ($F = 4.852$, $p < 0.001$; Table 1).

Overall, SA concentrations were higher in the water-deficit environment than in the well-watered environment (Figure 3B). Well-watered plants exhibited comparatively low SA concentrations, with limited variation among infestation treatments. Under water deficit, SA concentrations varied more markedly among treatments. Comparatively high SA concentrations were observed in plants infested with *T. urticae* and in some treatments involving *T. urticae* in combination with other herbivores.

In contrast to JA and SA, MeJA concentrations were not significantly affected by water regime ($F = 3.598$, $p = 0.063$), infestation ($F = 1.274$, $p = 0.270$), or their interaction ($F = 1.356$, $p = 0.229$; Table 1).

3.4. Herbivore-Induced Biochemical Defenses Differ Between Irrigation Environments

LOX, TIA, and PPO varied among irrigation environments and herbivore treatments (Table 2). The observed patterns differed according to the biochemical response and treatment combination, indicating context-dependent variation in these defense-related traits.

Table 2. Deviance analysis for linear mixed-effects models with Poisson distribution for enzymatic activities.

Enzymatic Activity	Source of Variation	DF	Deviance	Residual DF	Residual Deviance	<i>p</i> Value
Lipoxygenase activity	Water regime	1	39.802	98	329.90	2.811×10^{-10}
	Infestation	9	92.952	89	236.95	4.154×10^{-16}
	Interaction	9	75.560	80	161.39	1.225×10^{-12}
	Total			99	369.70	
Trypsin inhibitory activity	Water regime	1	31.685	98	342.54	8.879×10^{-5}
	Infestation	9	93.662	89	248.88	5.651×10^{-6}
	Interaction	9	101.581	80	147.30	1.833×10^{-6}
	Total			99	374.23	
Polyphenol oxidase activity	Water regime	1	8526.6	98	28,984	$<2.2 \times 10^{-16}$
	Infestation	9	10,744.8	89	18,239	$<2.2 \times 10^{-16}$
	Interaction	9	6060.2	80	12,179	$<2.2 \times 10^{-16}$
	Total			99	37,511	

LOX activity was significantly affected by water regime ($p = 2.81 \times 10^{-10}$), infestation ($p = 4.15 \times 10^{-16}$), and their interaction ($p = 1.23 \times 10^{-12}$; Table 2). Under well-watered conditions, LOX activity varied markedly among treatments, with particularly high activities following mechanical damage and simultaneous infestation by all three herbivore species (Figure 4A).

LOX activity differed substantially between irrigation environments, with lower activity observed in the water-deficit environment for several treatments that showed pronounced responses under well-watered conditions. The magnitude of these differences, however, varied substantially among herbivore treatments.

TIA differed significantly between irrigation environments ($p = 8.88 \times 10^{-5}$), among infestation treatments ($p = 5.65 \times 10^{-6}$), with a significant interaction between irrigation environment and infestation treatment ($p = 1.83 \times 10^{-6}$; Table 2). Under water-deficit conditions, relatively high TIA was observed in non-infested plants and following several single-species infestations (Figure 4B). In contrast, simultaneous infestation by multiple herbivore species was generally associated with lower TIA, with a particularly pronounced reduction in plants attacked by all three herbivores. Under well-watered conditions, TIA also varied among infestation treatments, but the pattern differed from that observed under water-deficit conditions.

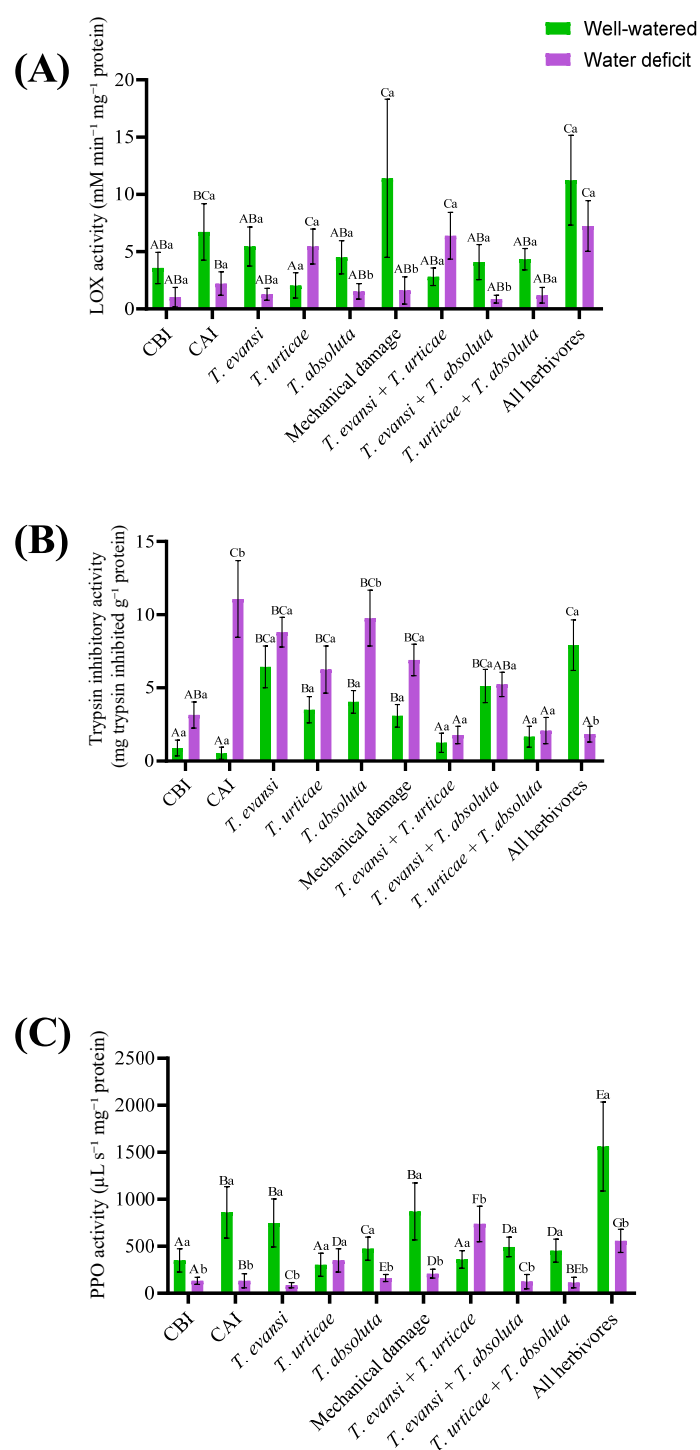


Figure 4. Biochemical defense responses of tomato plants under well-watered and water-deficit conditions and different herbivore treatments. **(A)** Lipoxygenase (LOX) activity, **(B)** trypsin inhibitory activity (TIA), and **(C)** polyphenol oxidase (PPO) activity. CBI and CAI represent non-infested controls evaluated before and after the seven-day experimental period, respectively. Infestation treatments consisted of *Tetranychus evansi*, *Tetranychus urticae*, *Tuta absoluta*, mechanical damage, *T. evansi* + *T. urticae*, *T. evansi* + *T. absoluta*, *T. urticae* + *T. absoluta*, and all three herbivore species combined. Bars represent the mean $\pm$ standard error of the mean (SEM) ($n = 5$). Within each panel, different lowercase letters indicate significant differences between irrigation environments within the same treatment, whereas different uppercase letters indicate significant differences among treatments within the same irrigation environment, according to general linear hypothesis testing ($p < 0.05$).

PPO activity differed significantly according to irrigation environment, infestation treatment, and their interaction (all $p < 2.2 \times 10^{-16}$; Table 2). Under well-watered conditions, PPO activity was relatively high in non-infested control plants and varied considerably among herbivore treatments (Figure 4C). The highest PPO activity was observed in plants simultaneously attacked by all three herbivore species. Under water deficit, PPO activity was substantially lower in several treatments, including the three-herbivore combination.

3.5. Patterns of Association Among Hormonal and Biochemical Responses Differ Between Irrigation Environments

Spearman correlation analysis revealed multiple significant positive and negative associations among hormonal, stress-related, and biochemical defense variables (Table 3).

Table 3. Spearman rank correlations among the biochemical and phytohormonal variables measured in tomato plants across both irrigation environments. Values indicate Spearman's correlation coefficient (ρ) and the corresponding p -value ($n = 20$).

	PPO	TIA	ABA	JA	SA	IAA	ACC	Zeatin	Proline
LOX	0.892 ** <0.001	−0.414 0.068	−0.685 ** <0.001	0.527 * 0.016	−0.559 * 0.010	0.530 * 0.016	0.672 ** 0.001	−0.475 * 0.033	−0.781 ** <0.001
PPO		−0.349 0.129	−0.604 ** 0.005	0.493 * 0.026	−0.436 0.053	0.329 0.153	0.416 0.066	−0.281 0.225	−0.723 ** <0.001
TIA			0.202 0.388	0.0256 0.911	0.463 * 0.039	−0.070 0.762	−0.513 * 0.021	0.778 ** <0.001	0.408 0.073
ABA				−0.454 * 0.044	0.511 * 0.021	−0.192 0.410	−0.407 0.073	0.239 0.305	0.691 ** <0.001
JA					0.138 0.555	0.598 ** 0.005	0.213 0.360	0.0895 0.700	−0.621 ** 0.003
SA						−0.239 0.305	−0.672 ** 0.001	0.562 ** 0.009	0.485 * 0.029
IAA							0.637 ** 0.003	−0.231 0.320	−0.407 0.073
ACC								−0.631 ** 0.003	−0.434 0.055
Zeatin									0.196 0.399

LOX = lipoxygenase; PPO = polyphenol oxidase; TIA = trypsin inhibitory activity; ABA = abscisic acid; JA = jasmonic acid; SA = salicylic acid; IAA = indole-3-acetic acid; ACC = 1-aminocyclopropane-1-carboxylic acid. * $p < 0.05$; ** $p < 0.01$.

Across both irrigation environments, the strongest positive association was observed between LOX and PPO activities ($\rho = 0.892$, $p < 0.001$).

LOX activity was positively associated with JA ($\rho = 0.527$, $p = 0.016$), IAA ($\rho = 0.530$, $p = 0.016$), and ACC ($\rho = 0.672$, $p = 0.001$), whereas negative associations were observed with ABA ($\rho = -0.685$, $p < 0.001$), SA ($\rho = -0.559$, $p = 0.010$), zeatin ($\rho = -0.475$, $p = 0.033$), and proline ($\rho = -0.781$, $p < 0.001$). PPO activity was positively associated with JA ($\rho = 0.493$, $p = 0.026$) and negatively associated with ABA ($\rho = -0.604$, $p = 0.005$) and proline ($\rho = -0.723$, $p < 0.001$). The negative association between PPO and SA did not reach the conventional significance threshold ($\rho = -0.436$, $p = 0.053$).

TIA was positively associated with zeatin ($\rho = 0.778$, $p < 0.001$) and SA ($\rho = 0.463$, $p = 0.039$), whereas it was negatively associated with ACC ($\rho = -0.513$, $p = 0.021$). No significant association was detected between TIA and JA.

Among the signaling compounds, ABA was positively associated with SA ($\rho = 0.511$, $p = 0.021$) and proline ($\rho = 0.691$, $p = 0.001$) and negatively associated with JA ($\rho = -0.454$, $p = 0.044$). JA was positively associated with IAA ($\rho = 0.598$, $p = 0.005$) and negatively associated with proline ($\rho = -0.621$, $p = 0.003$). SA was negatively associated with ACC ($\rho = -0.672$, $p = 0.001$) and positively associated with zeatin ($\rho = 0.562$, $p = 0.009$) and proline ($\rho = 0.485$, $p = 0.029$). IAA was positively associated with ACC ($\rho = 0.637$, $p = 0.003$), whereas ACC and zeatin were negatively associated ($\rho = -0.631$, $p = 0.003$).

When the two irrigation environments were examined separately, the pattern of significant associations differed (Figures 5 and 6).

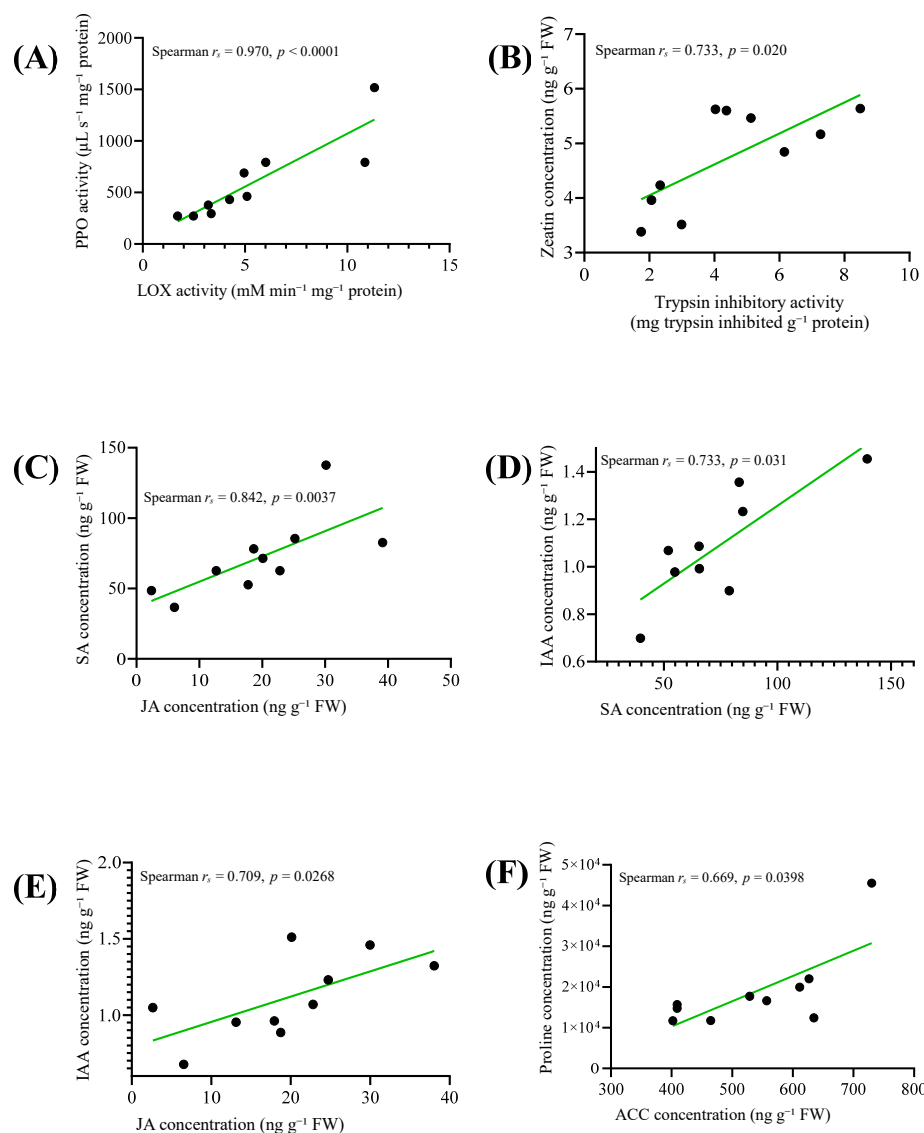


Figure 5. Spearman rank correlations among biochemical and phytohormonal variables in tomato plants under well-watered conditions. (A) Lipoxxygenase (LOX) activity versus polyphenol oxidase (PPO) activity; (B) trypsin inhibitory activity (TIA) versus zeatin concentration; (C) jasmonic acid (JA) versus salicylic acid (SA) concentrations; (D) salicylic acid (SA) versus indole-3-acetic acid (IAA) concentrations; (E) jasmonic acid (JA) versus indole-3-acetic acid (IAA) concentrations; and (F) 1-aminocyclopropane-1-carboxylic acid (ACC) versus proline concentration. Spearman's rank correlation coefficients (r_s) and corresponding p -values are shown in each panel. Solid lines represent linear trends for visualization only. Correlations were considered exploratory and were not interpreted as evidence of causal relationships.

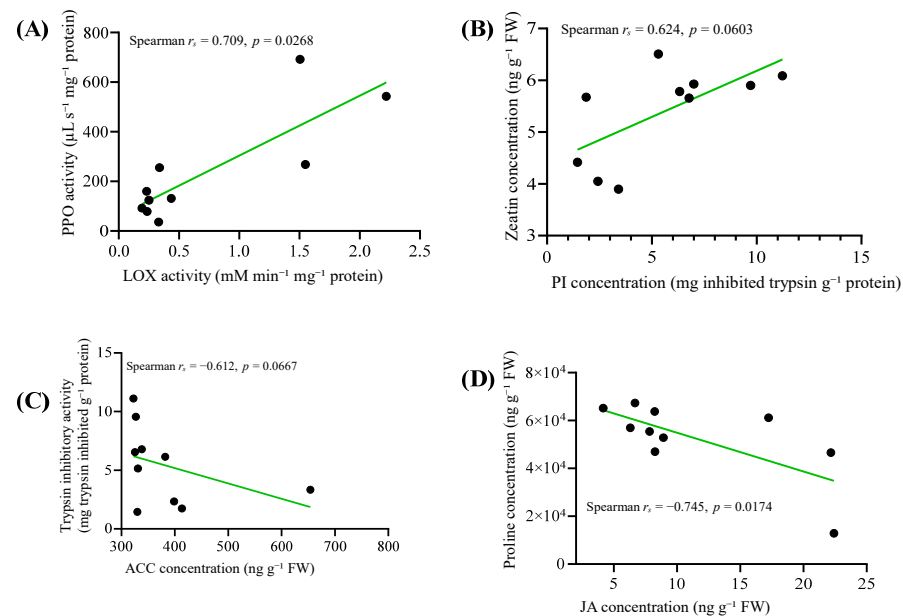


Figure 6. Spearman rank correlations among biochemical and phytohormonal variables in tomato plants under water-deficit conditions. (A) Lipoyxygenase (LOX) activity versus polyphenol oxidase (PPO) activity; (B) trypsin inhibitory activity (TIA) versus zeatin concentration; (C) trypsin inhibitory activity (TIA) versus 1-aminocyclopropane-1-carboxylic acid (ACC) concentration; and (D) jasmonic acid (JA) versus proline concentration. Spearman's rank correlation coefficients (r_s) and corresponding p -values are shown in each panel. Solid lines represent linear trends for visualization only. Correlations were considered exploratory and were not interpreted as evidence of causal relationships.

Under well-watered conditions, six significant positive associations were detected (Figure 5). LOX and PPO activities were positively associated ($\rho = 0.970$, $p < 0.0001$), and TIA was positively associated with zeatin ($\rho = 0.733$, $p = 0.0202$). Positive associations were also observed between JA and SA ($\rho = 0.842$, $p = 0.0037$), SA and IAA ($\rho = 0.733$, $p = 0.0311$), JA and IAA ($\rho = 0.709$, $p = 0.0268$), and ACC and proline ($\rho = 0.669$, $p = 0.0398$).

Under water-deficit conditions, fewer significant associations were detected (Figure 6). The positive association between LOX and PPO activities was maintained ($\rho = 0.709$, $p = 0.0268$), whereas JA was negatively associated with proline ($\rho = -0.745$, $p = 0.0174$). In contrast, the positive association between TIA and zeatin ($\rho = 0.624$, $p = 0.0603$) and the negative association between TIA and ACC ($\rho = -0.612$, $p = 0.0667$) did not reach statistical significance.

3.6. Water Deficit Alters Tomato Fruit Production Traits and Fruit-Borer Damage

Water regime significantly affected fruit production traits, whereas the original two-way analysis detected no significant effects of herbivore infestation or water regime $\times$ infestation interactions on fruit production. Therefore, we subsequently compared fruit traits between irrigation regimes (Table 4).

Fruit production traits differed between irrigation environments. Plants under water-deficit conditions produced 21.74 ± 0.83 fruits plant⁻¹, compared with 15.14 ± 0.73 fruits plant⁻¹ under well-watered conditions ($F = 35.769$, $p < 0.001$; Table 4). This greater fruit number was accompanied by lower individual fruit size and weight. Mean fruit size was 7.416 ± 0.080 under water-deficit conditions compared with 8.155 ± 0.075 under well-watered conditions ($F = 45.633$, $p < 0.001$). Similarly, mean fruit weight was 152.522 ± 4.355 under water-deficit conditions compared with 206.085 ± 7.374 under well-watered conditions ($F = 39.120$, $p < 0.001$).

Table 4. One-way ANOVA of tomato fruit traits comparing well-watered and water-deficit irrigation environments.

Fruit Variable	Irrigation Environment	Mean	SEM	Residual DF	Residual MS	F Statistic	p-Value
Number of fruits per plant	Well-watered	15.140	0.725	98	30.445	35.769	<0.001
	Water deficit	21.740	0.832				
Fruit diameter (cm)	Well-watered	8.155	0.0752	98	0.300	45.633	<0.001
	Water deficit	7.416	0.0796				
Fruit weight (g)	Well-watered	206.085	7.374	98	1833.432	39.120	<0.001
	Water deficit	152.522	4.355				
Damaged fruits (no. plant ^{−1})	Well-watered	4.440	0.371	98	12.580	10.150	0.002
	Water deficit	6.700	0.605				

SEM = standard error of the mean; DF = degrees of freedom.

4. Discussion

Our findings indicate that hormonal and biochemical responses to herbivory differed between well-watered and water-deficit environments, and that the magnitude and direction of these differences varied by herbivore species identity and combination. Significant water regime $\times$ herbivory interactions were observed for several measured traits, indicating context-dependent patterns across the two irrigation environments. Because irrigation treatments were imposed at the sector level and were not independently replicated, these differences should be interpreted as contrasts between the irrigation environments established in this experiment rather than as fully replicated causal effects of water regime [23].

ABA and proline concentrations were generally higher in plants from the water-deficit irrigation environment. These patterns are consistent with previous studies showing increased ABA and proline accumulation in tomato under reduced water availability [24,25]. ABA plays a central role in tomato responses to water deficit by coordinating stomatal regulation and multiple metabolic and transcriptional responses, whereas proline accumulation has been associated with osmotic adjustment and cellular protection under drought conditions [26]. However, plant water status was not directly measured in the present experiment, and soil moisture was assessed at a single sampling time. Therefore, the observed ABA and proline responses should not be interpreted as independent confirmation of the magnitude or persistence of plant water stress. In addition, these metabolites were quantified on a fresh-weight basis, and differences in leaf water content between irrigation environments could have contributed to the observed concentration differences [27]. Their responses to herbivory nevertheless differed: ABA showed a significant water regime $\times$ herbivory interaction, whereas proline concentrations were predominantly associated with irrigation environment. This pattern suggests that ABA responses were more dependent than proline responses on herbivore identity and community composition across the two irrigation environments.

These responses are relevant in the tomato–spider mite system because physiological adjustments to reduced water availability may also modify host quality for herbivores. Previous studies reported changes in sugars, amino acids, and proline in tomato under water limitation, together with changes in *T. evansi* performance [11]. Our observations indicate that ABA and proline responses occurred alongside changes in other hormonal and biochemical variables, although the present experiment does not establish causal relationships among these responses.

In contrast to ABA and proline, ACC concentrations were markedly lower under water deficit. Because ACC is the immediate biosynthetic precursor of ethylene rather than a direct measurement of ethylene production, this result should be interpreted as evidence of altered ethylene-associated metabolism rather than reduced ethylene signaling

per se. This distinction is particularly important because ABA, ethylene, jasmonates, and SA participate in interconnected stress-response networks rather than functioning as independent signaling pathways. Contemporary models of plant stress physiology increasingly emphasize this network organization and the importance of interactions among growth- and defense-associated hormones in determining the final phenotype [28]. Consistent with this interpretation, ACC was negatively associated with SA and zeatin across treatments, suggesting that the reduction in ACC under water deficit occurred as part of a broader shift in the hormonal state of the plant.

One of the clearest herbivore-specific responses occurred in water-deficit plants attacked by *T. urticae*. In this treatment, both IAA and zeatin reached particularly high concentrations, accompanied by pronounced JA and SA accumulation. In contrast, neither IAA nor zeatin showed an overall main effect of water regime, demonstrating that their responses were specifically associated with the interaction between water availability and herbivore identity. Thus, water deficit alone was insufficient to generate this hormonal phenotype.

Auxins and cytokinins are primarily recognized for regulating growth, cell division, source–sink relationships, and developmental processes, but their functions extend to plant responses to biotic and abiotic stress. Current concepts of phytohormonal regulation emphasize extensive integration between these growth-related hormones and classical stress-associated signals such as ABA, JA, SA, and ethylene [28]. The simultaneous alteration of IAA, zeatin, JA, and SA in *T. urticae*-infested plants therefore suggests that herbivory under limited water availability involves coordination between growth-related and defense-associated processes rather than activation of a single canonical defense pathway.

This result is particularly interesting when contrasted with *T. evansi*. The two spider mites share a cell-content feeding mode but can elicit markedly different tomato defense phenotypes. *T. evansi* is well known for its capacity to manipulate tomato defenses, including suppression of JA- and SA-associated responses, although the magnitude of suppression varies considerably among mite populations [6]. Previous work under water limitation has likewise demonstrated that tomato genotype and drought status can modify hormonal and defense responses to *T. evansi*, emphasizing that the outcome of this interaction is strongly context-dependent [11]. Our findings extend this comparison by indicating that the hormonal phenotype elicited by spider mites also depends on the presence of additional herbivore species sharing the same host.

The responses of JA and SA further demonstrate that water limitation does not uniformly enhance or suppress herbivore-induced defense. Under well-watered conditions, herbivore attack generally promoted JA accumulation, although the magnitude of this response varied considerably among herbivore treatments. Particularly high JA concentrations occurred during simultaneous infestation by *T. urticae* and *T. absoluta* and when all three herbivore species shared the plant. Under water deficit, however, this pattern changed substantially. *T. urticae* alone maintained a pronounced JA response, whereas JA accumulation was reduced in several other herbivore treatments relative to their well-watered counterparts.

SA displayed a contrasting response, with greater accumulation under water-deficit conditions, particularly following infestation by *T. urticae*. High SA concentrations were also observed in water-deficit plants exposed to *T. urticae* together with *T. absoluta* and in the three-herbivore treatment. Overall, the patterns of JA and SA accumulation varied according to irrigation environment and herbivore species identity and combination.

Importantly, these patterns should not be interpreted as evidence of simple JA–SA antagonism. Although antagonistic interactions between JA and SA are well established in plant immunity, hormonal interactions are highly context-dependent, and positive or

coordinated responses can also occur. In our study, JA and SA were not significantly correlated when all treatments were considered together, whereas they were strongly positively associated under well-watered conditions. Therefore, our data indicate a water-dependent change in the balance and coordination of JA- and SA-associated responses rather than a universal switch between two mutually exclusive signaling pathways. This interpretation is consistent with recent perspectives that describe phytohormonal stress responses as dynamic networks whose outputs depend on the combination and temporal context of environmental signals [28].

The behavior of the different herbivore species further supports this context-dependent interpretation. *T. urticae* is generally associated with induction of tomato defenses, whereas *T. evansi* can actively suppress components of JA- and SA-associated signaling and downstream defenses. Furthermore, substantial intraspecific variation in defense suppression occurs among *T. evansi* populations [6]. *T. absoluta* represents a different feeding strategy as an endophytic leafminer, potentially generating a distinct spatial and biochemical pattern of plant damage. Previous work combining drought and *T. absoluta* in tomato found that simultaneous abiotic and biotic stress generated additive, synergistic, and antagonistic effects depending on the physiological or metabolic trait considered [3]. These findings are consistent with our observation that mixed-herbivore treatments frequently produced responses that differed from those elicited by the corresponding single-herbivore treatments.

The hormonal differences observed under combined water limitation and herbivory were accompanied by substantial variation in downstream biochemical defenses. LOX, PPO, and TIA showed significant irrigation environment $\times$ herbivory interactions, indicating that biochemical defense responses differed according to irrigation environment and herbivore treatment. Similar context-dependent responses have been reported in tomato, where water deficit and *T. evansi* infestation were associated with changes in oxidative enzymes and protease inhibitor-related responses, supporting the view that biochemical defense patterns can vary under combined abiotic and biotic stress conditions [11].

LOX and PPO showed broadly parallel responses across treatments and were positively associated in the exploratory correlation analysis. Both enzymes are components of wound- and herbivore-responsive plant defenses, although they act through distinct biochemical mechanisms. LOX participates in oxylipin metabolism and reactions associated with jasmonate-mediated defense, whereas PPO oxidizes phenolic substrates following tissue disruption, generating reactive quinones that can decrease the nutritional quality of plant tissues for herbivores [29]. PPO is particularly relevant in tomato, where its activity is induced by herbivore-associated defense signals, including jasmonates, and has long been associated with resistance to herbivorous insects. The positive LOX–PPO association observed here is therefore consistent with parallel variation in distinct components of the tomato biochemical defense response. However, because the correlation analysis was exploratory, this association should not be interpreted as evidence of direct regulatory coupling or causality.

However, neither LOX nor PPO responded uniformly to water limitation. Under well-watered conditions, particularly high activities occurred in specific treatments, including mechanical damage and/or simultaneous attack by all three herbivore species, whereas water deficit substantially altered these patterns. These observations indicate that LOX and PPO activities differed among herbivory treatments and between the two irrigation environments rather than showing a uniform pattern under reduced water availability. This context dependence is consistent with the complex nature of plant responses to phytophagous mites, in which herbivore perception and feeding-induced damage trigger interconnected Ca^{2+} - and ROS-associated signaling processes that contribute to downstream hormonal and biochemical defense responses [30]. A similar complexity has been

reported in tomato exposed to water deficit and *T. evansi*, where drought effects on hormonal signaling, defense-related genes, and proteins depended on tomato genotype and the specific defense component considered [11].

TIA revealed an especially interesting pattern associated with herbivore community composition. Under water-deficit conditions, relatively high TIA was observed in non-infested plants and following several single-species infestations, whereas simultaneous attack by multiple herbivores was generally associated with lower TIA, with a particularly pronounced reduction when all three herbivore species shared the host. This pattern is especially relevant considering the ability of *T. evansi* to manipulate tomato defenses. Previous studies have demonstrated that *T. evansi* can suppress downstream JA- and SA-associated defense responses in tomato [31], including reductions in proteinase inhibitor-related defenses previously induced by *T. urticae* [7]. However, defense suppression is not a fixed trait, as its magnitude can vary substantially among *T. evansi* populations [6]. Our results extend these findings by suggesting that, under water-limited conditions, trypsin inhibitory responses may be associated not only with the presence of a defense-suppressing herbivore but also with the composition of the herbivore community sharing the host.

Our results suggest that, under water-limited conditions, trypsin inhibitory responses may depend on herbivore community composition rather than solely on the presence of a known defense-suppressing herbivore [32,33]. However, because expected additive responses were not formally compared with the observed responses, the combined-herbivore effects cannot be classified as additive, antagonistic, or synergistic [34]. Non-additive responses to combined drought and *T. absoluta* have previously been reported in tomato, although that study focused mainly on plant physiology and volatile profiles rather than the broader hormonal and biochemical defense responses evaluated here [35].

This distinction represents an important extension of previous drought–herbivory research. Much of the existing literature has examined drought in combination with a single herbivore species [13]. Recent reviews identify herbivore identity, feeding guild, drought intensity, and stress duration as major determinants of combined-stress outcomes and highlight the need to understand these interactions in more ecologically complex systems [23,36,37]. By comparing single-, two-, and three-species herbivore assemblages, our results indicate that herbivore community composition is associated with distinct tomato defense phenotypes under limited water availability.

The contrasting correlation patterns between irrigation environments provide an additional descriptive perspective on the biochemical responses observed in this study. Under well-watered conditions, six significant positive associations were detected among the analyzed variables. Under water-deficit conditions, only two of the relationships shown in Figure 6 reached statistical significance: the positive association between LOX and PPO activities and the negative association between JA and proline. The associations between TIA and zeatin and between TIA and ACC did not reach the conventional significance threshold.

Differences between irrigation environments also extended to tomato reproductive traits. Plants under water-deficit conditions produced more fruits, but individual fruits were smaller and lighter than those under well-watered conditions, suggesting a shift in reproductive allocation between fruit number and fruit size. Such responses are consistent with evidence that tomato reproductive performance under water limitation varies with stress intensity and timing, irrigation strategy, cultivar, and environmental conditions [38,39]. Because fruit number and individual fruit traits were evaluated separately, however, these differences should not be interpreted as direct measures of total crop yield. Determination of total fruit mass per plant would be required to establish whether the observed pattern resulted in an overall yield reduction.

The greater absolute number of fruits damaged by *Neoleucinodes elegantalis* under water deficit should be interpreted in the context of this increased fruit production. When damage was expressed relative to total fruit number, damaged fruits represented approximately 29.3% of fruits in well-watered plants and 30.8% in water-deficit plants. Thus, despite the greater absolute number of damaged fruits under water deficit, the proportional incidence of damage was descriptively similar between irrigation environments. Consequently, our data do not support the conclusion that water deficit increased tomato susceptibility to *N. elegantalis*; rather, the larger absolute number of damaged fruits appears to largely reflect the greater number of fruits available under water-deficit conditions.

More broadly, the absence of a pronounced difference in proportional fruit damage illustrates the context dependence of water–plant–herbivore interactions. Water limitation can modify plant nutritional quality, defense chemistry, growth, phenology, and tolerance, but these changes do not consistently translate into increased or decreased herbivore damage. Their consequences depend on stress severity and duration, plant and herbivore identity, feeding guild, and the ecological context in which the interaction occurs [13,36]. Therefore, the reproductive responses observed here complement the hormone and enzyme-level responses. They show that substantial differences in plant physiology and reproductive allocation between irrigation environments did not translate into greater proportional damage by the subsequent fruit-feeding herbivore.

These findings should nevertheless be interpreted within the limitations of the experimental design. Each irrigation regime was established in a single field sector. The four experimental plots within each sector provided biological replication for the herbivory treatments, but not independent replication of the irrigation treatment. Consequently, sector-specific environmental variation cannot be fully separated from variation associated with water availability. Comparisons between well-watered and water-deficit plants should therefore be interpreted as contrasts between the two irrigation environments established in this experiment. They should not be interpreted as independently replicated causal effects of irrigation regime. Future field experiments should incorporate multiple independently randomized irrigation sectors, together with direct measurements of plant water status and total fruit yield, to determine the generality of the patterns observed here.

5. Conclusions

Our findings show that tomato hormonal and biochemical responses to herbivory differed between well-watered and water-deficit environments, with the magnitude and direction of these differences varying according to herbivore species identity and combination. Water-deficit conditions were associated with marked differences in ABA and proline, whereas herbivore-dependent patterns were particularly evident for JA, SA, IAA, zeatin, and downstream biochemical defenses. Exploratory correlation analyses revealed contrasting associations among hormonal and biochemical variables between irrigation environments, suggesting context-dependent coordination of stress-, growth-, and defense-associated processes. Mixed-herbivore treatments were also associated with distinct biochemical responses, particularly trypsin inhibitory activity (TIA), highlighting the relevance of herbivore community composition. However, because each irrigation regime was established in a single field sector, differences between irrigation environments should not be interpreted as independently replicated causal effects of water availability. Further field studies with independently replicated irrigation treatments are needed to determine the generality of these patterns.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16202028/s1>, Table S1: Insecticides and fungicides used for preventive phytosanitary management before the establishment of the experimental treatments. Figure S1: Drip irrigation and trellising system used in the experimental field. (A) Overview of the experimental tomato plots showing the drip irrigation and plant support system. (B) Detail of the drip irrigation line positioned adjacent to the tomato plants. Figure S2: Gravimetric soil moisture under water-deficit and well-watered irrigation conditions. Soil moisture was calculated as $[(\text{fresh soil mass} - \text{dry soil mass}) / \text{dry soil mass}] \times 100$. Bars represent mean $\pm$ SEM ($n = 5$ soil samples per irrigation environment). Soil moisture was significantly lower in the water-deficit than in the well-watered irrigation environment (Mann–Whitney $U = 0$, exact $p = 0.008$). $** p < 0.01$. Supplementary Methods: Equations used to calculate crop evapotranspiration, phytohormone concentrations, lipoxygenase and polyphenol oxidase activities, and trypsin inhibitory activity.

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