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Research article

The combined effects of plant density, chitosan, and amino acids enhance the sunflower growth and yield under water deficit conditions

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Abstract: Integrating plant density management with chitosan and amino acid -based biostimulant represents a promising strategy to enhance crop performance under water-limited conditions. In this study, we evaluated the combined effects of plant density, foliar chitosan, and amino acid-based biostimulant on sunflower growth, morphophysiological traits, and yield under water deficit conditions. A field experiment was conducted using a split-split-plot design with four replicates, where plant density (25,000 and 50,000 plants ha^{-1}) was assigned to the main plots, chitosan application (0 and 200 mg L^{-1}) to the subplots, and amino acid-based biostimulant (0 and 1.2 mL L^{-1} as VIUSID® Agro) to the sub-subplots. Our results indicated that increasing plant density from 25,000 to 50,000 plants ha^{-1} significantly enhanced crop performance under water-deficit conditions, improving growth indices and seed yield. The combined treatment $D_{50,000}Q_{200}A_{1.2}$ (50,000 plants ha^{-1} + 200 mg L^{-1} chitosan + 1.2 mL L^{-1} amino acids) produced the strongest responses and the highest seed yield when compared with $D_{25,000}Q_0A_0$ (25,000 plants ha^{-1} without chitosan or amino acid application). Specifically, $D_{50,000}Q_{200}A_{1.2}$ increased plant height by 66%–70%, leaf area by 56%–71%, leaf area index by 111%–127%, dry biomass by 92%–169%, and relative chlorophyll content by 39%–47%, indicating enhanced

photosynthetic capacity under water-deficit conditions. These improvements translated into substantial yield gains, with seed yield increasing by up to 3.1-fold relative to the $D_{25,000}Q_0A_0$ treatment. Overall, these findings demonstrated that the synergistic integration of optimized plant density with foliar chitosan and amino acid biostimulants enhances sunflower growth, physiological performance, and productivity under water-deficit conditions, providing a robust and sustainable strategy for improving crop resilience and yield stability.

Keywords: amino acid biostimulants; chlorophyll content; crop growth rate, drought tolerance; morphophysiological traits; photosynthetic efficiency

1. Introduction

Sunflower is an important crop primarily known as the raw material for producing oil, which is widely used in cooking and food processing, but also for its nutrient-rich seeds [1,2]. After oil extraction, the remaining meal and hulls serve as important by-products: They are rich in proteins, minerals, and bioactive phenolic compounds and are widely used in animal feed and as functional ingredients in food, making sunflower a remarkably versatile crop [3], and research underscores its potential for upcycling into human nutrition [4]. However, it can face challenges from the increasing occurrence of climate change, like salinity, drought, and heat [5]. Although sunflowers are considered moderately tolerant to water stress, severe water deficits reduce seed yield and oil production [6].

Drought is one of the most severe abiotic stresses in agriculture, negatively affecting plant morphology, physiology, and metabolism and consequently reducing growth and productivity [7–9]. Approximately one-third of the world's agricultural land is vulnerable to drought, which can substantially limit crop growth and yield [7]. In sunflowers, critical growth stages such as germination, flowering, and achene development are sensitive to water deficit, and insufficient soil moisture during these periods may result in empty achenes and yield reductions of up to 50% [1,6]. Water stress impairs growth in sunflowers by reducing germination, seedling establishment, stem elongation, leaf expansion, biomass accumulation, and relative water content. It also affects physiological processes, including stomatal conductance, transpiration, photosynthetic pigments, and overall carbon assimilation [8–10]. Photosynthesis is constrained through two major mechanisms: Stomatal closure, which limits CO_2 diffusion within the leaf, and direct inhibition of CO_2 metabolism, leading to reduced growth and yield under water-deficit conditions [7,11,12].

Strategies to mitigate the effects of drought stress include superabsorbent hydrogels, film farming, biostimulants, nanoparticles, biochar, plant density management, and the use of drought-resistant plant varieties [9,13]. Plant density is utilized to increase yield potential per unit area by creating a more favorable physical environment [14,15]. For example, low plant density can attenuate drought stress by lowering inter-plant competition for soil water [16]. Conversely, high density can exacerbate stress by rapidly depleting soil moisture, resulting in intense competition for resources and a decrease in the yield per plant [17]. Furthermore, the optimal plant density achieves a balance that maximizes water use efficiency and grain yield in water-limited conditions while avoiding excessive competition among plants [18]. Therefore, adjusting crop density is a crucial aspect of sustainable agricultural management aimed at enhancing resilience against unpredictable drought conditions.

Plant growth regulators can be considered an economic and ecofriendly strategy to attenuate the

adverse effects of drought stress. They are composed of several substances like amino acids, nutrients, polysaccharides, plant extracts, humic and fulvic acids, protein hydrolysates, vitamins, minerals, phytohormones, and microorganisms [19,20]. The application of the plant growth promoter can attenuate drought stress primarily by stimulating root development and water-uptake capacity, enhancing osmotic adjustment through osmolyte accumulation, and activating antioxidant and stress-signaling pathways that protect photosynthetic machinery and improve water-use efficiency [7,8]. This coordinated regulation helps maintain cellular integrity, osmotic adjustment, and tissue protection, effectively attenuating the negative impacts of water scarcity, such as oxidative damage and loss of turgor [21].

Chitosan is a linear, cationic polysaccharide derived from the partial N-deacetylation of chitin, consisting of β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine units, and its structure confers solubility and bioactivity that enhance plant tolerance to biotic and abiotic stresses [22]. Exogenous applications of chitosan have been reported to enhance plant resistance to drought stress in different plant species [23–25]. One of the major mechanisms underlying this effect is the stimulation of cellular antioxidant components, which reduces oxidative stress caused by reactive oxygen species and consequently preserves membrane integrity under water deficit conditions [24–27]. In addition, chitosan treatment has been demonstrated to increase the accumulation of osmolytes, including total soluble sugars, soluble proteins, and proline, thereby contributing to osmotic adjustment and maintenance of cellular homeostasis during drought stress [24,25,27]. Chitosan has also been shown to enhance the concentration of photosynthetic pigments, which may indicate improved photosynthetic efficiency [24,25], as well as to up-regulate genes involved in stress responses [26,27]. Furthermore, experimental evidence has associated chitosan application with increased levels of endogenous indole acetic acid and enhanced root and shoot biomass under drought conditions, highlighting its capacity to sustain growth and root development even under water deficit [24,26].

Amino acids are increasingly used in modern agriculture as biostimulants to improve crop resilience by enhancing photosynthesis and bolstering antioxidant defenses, thereby reducing the impact of abiotic stresses like drought, salinity, and heat [28]. For example, foliar application of amino acid mixtures has been shown to activate antioxidant enzymes and stabilize redox balance in plants under water stress, improving yield and stress tolerance [1,29]. A blend of aspartic acid, arginine, glycine, and tryptophan, a commercial biostimulant known as VIUSID® agro, has gained attention for its capacity to strengthen crop growth and resilience diverse abiotic stress conditions [30,31]. In rice, foliar application under drought-simulated conditions, with irrigation every eight days, increases grain yield by 23%, with concurrent improvements in panicle number and grain weight [32]. These effects arise from the coordinated modulation of key physiological pathways, wherein aspartic acid enhances proline-mediated osmotic adjustment, arginine regulates nitric oxide and polyamine synthesis to improve photosynthetic and antioxidant responses, glycine facilitates K^+ uptake and ionic homeostasis, and tryptophan supports osmoregulation, photosynthetic efficiency, and oxidative stress mitigation [33–35].

Collectively, amino acids and chitosan can operate through multiple, complementary physiological and biochemical pathways to enhance stress tolerance and strengthen plant resilience under water-deficit conditions. In this study, we aimed to investigate how plant density, foliar-applied chitosan, and an amino-acid-based biostimulant interact to enhance resilience under water-deficit in sunflower by examining their combined effects on growth, morphophysiological traits, and yield under water-deficit conditions. We hypothesize that optimizing plant density spatial configurations (specifically evaluating 25,000 vs. 50,000 plants ha^{-1}), combined with the exogenous application of chitosan and amino acid-based biostimulant complexes, enhances sunflower resilience under water-deficit conditions by

stimulating leaf area index development, preserving chlorophyll content, accelerating vegetative biomass accumulation, and maximizing seed yield traits.

2. Materials and methods

2.1. Local description, growth conditions, and soil characteristics

The field experiment was conducted during the dry season (December 2024–April 2025) at the research site of the Agro-industrial Sugar Company "Melanio Hernández" in Sancti Spíritus, Cuba (22°00′56″ N, 79°24′49″ W). The region is characterized by a tropical climate with a well-defined dry period and is widely used for agricultural experimentation. All field operations and crop management practices were performed under standard agronomic conditions for sunflower cultivation, without fertilization and irrigation, as indicated by Hurtado et al. [1].

Plants were grown under natural, open-field conditions, and environmental variables (temperature and relative humidity) were continuously recorded using an on-site meteorological station. Mean day/night temperatures (26.2/21.8°C) and relative humidity (65%–85%) represent averaged values over the experimental period. The approximately 12 h light/12 h dark photoperiod reflects the natural daylength typical of the tropical latitude of the study area, rather than controlled conditions. Water scarcity conditions were achieved under natural rainfed conditions during the dry season, with a total precipitation of only 15.32 mm throughout the experimental period, and no supplemental irrigation was applied, ensuring continuous water-deficit conditions.

The soil at the experimental site is classified as a Sialitic Brown soil with moderately acidic properties (pH = 5.9), consistent with the edaphic conditions commonly observed in agricultural soils of the Sancti Spíritus region.

2.2. Plan material

Sunflower seeds cv. 'Caburé-15' were obtained from the Sancti Spiritus Seed Enterprise. Prior to sowing, seeds of uniform size were surface-sterilized in a 10% (v/v) sodium hypochlorite solution for 10 minutes, rinsed thoroughly with distilled water, and air-dried on sterile paper towels. Seeds were then sown at a row spacing of 0.80 m and intra-row spacings of 0.50 m and 0.25 m to establish plant densities of 25,000 and 50,000 plants ha^{-1}, respectively.

2.3. Experimental design and treatments

The experiment was arranged in a split–split plot design with three factors. Plant density (D) was allocated to the major plots at two levels: $D_{25,000}$ (25,000 plants ha^{-1}) and $D_{50,000}$ (50,000 plants ha^{-1}). Subplots received foliar applications of chitosan (Q) at two concentrations: Q_0 (0 mg L^{-1}, water only) and Q_{200} (200 mg L^{-1}). Sub-subplots were assigned foliar applications of an amino acid–based biostimulant (A) at two levels: A_0 (0 mg L^{-1}, water only) and $A_{1.2}$ (1.2 mg L^{-1}).

2.4. Chitosan and amino acid treatments

Sunflower plants were sprayed with Q at 200 mg L^{-1} [36] and A at 1.20 mg L^{-1} [33] once every

seven days between 8:30 and 9:30 a.m. when dew had evaporated and wind drift was minimized. Foliar spraying began in the vegetative stage V2 and finished at V8, in accordance with the vegetative stages proposed by Schneiter and Miller [37]. Q and A treatments were administered using a 16 L backpack sprayer (Matabi, Goizper Group, Antzuola, Spain).

2.5. Chitosan and amino acid compositions

The National Institute of Agricultural Sciences of Cuba (INCA by the Spanish abbreviation) supplied the QuitoMax® product as the source of Q. This liquid product contains the chitosan polymers as the active ingredient and is obtained via deacetylation from chitin extracted from lobster exoskeletons [36]. The amino acid-based growth biostimulant (commercialized as VIUSID® Agro) has the following stated composition: aspartic acid (1.6% m/m), arginine (2.5% m/m), glycine (2.4% m/m), and tryptophan (0.5% m/m), with a pH of 6.80 and a net mass of 1.14 kg [33].

2.6. Cultural practices

The agricultural techniques were carried out in accordance with the guidelines issued by the Ministry of Agriculture in Cuba, with the exception of irrigation, fertilization, and pest control, which were avoided. Prior to seed preparation, all agronomic activities were carried out on the property, including soil preparation, weeding, and furrowing. Seeding was accomplished manually, placing two to three seeds in each area. Ten days after sowing, the plant in excess were physically eradicated, leaving a uniform plant in each location. Weed plants were eradicated using a hoe at 20, 35, and 50 days after sowing (DAS).

2.7. Data recorded

At the vegetative growth state (30 and 60 DAS), 10 plants were randomly selected from the two middle rows of each sub-subplot to record the growth parameters, such as plant height (PH, cm), which was measured from the stem base to the apical tip with a millimeter tape. Leaf area (LA, cm²) was determined according to the Kemp [38] method using Eq (1).

$$LA = \sum (\ l \times w) \times f \tag{1}$$

where l represents the length of each leaf, w denotes the width of each leaf, and f is the constant factor, equal to 0.73, which was determined by de la Vega & Hall [39]. Additionally, leaf area index (LAI) was calculated using equation 2 proposed by Watson [40].,where D is plant density (plants m^{-2}):

$$LAI = LA \ \times D \tag{2}$$

A portable chlorophyll meter (model TYS-B, Lab Equipment–Hinotek, Shanghai, China) was used to measure the relative chlorophyll content (RCC, SPAD values) in five leaves from top to bottom between 10:00 and 11:00 a.m. Aboveground biomass (AB) of the aerial part was measured at 30 and 60 DAS. During both periods, the stems and leaves of ten plants per plot were picked and put in paper bags. Then they were dried at 60°C in a forced ventilation oven (MJW WS 100, Memmert, Berlin, Germany) until a consistent dry mass was achieved. The DM was then weighed using a digital

microscale (0.1 mg; CPA224S, Sartorius, Göttingen, Germany).

The growth indices evaluated were leaf area ratio (LAR, $cm^2\ g^{-1}$), net assimilation rate (NAR, $g\ cm^2\ week^{-1}$), relative growth rate (RGR, $g^{-1}\ week^{-1}$), and crop growth rate (CGR, $g\ m^2\ day^{-1}$). LAR was determined by the method of Watson [40] using Eq (3).

$$LAR_{30-60} = {\Delta LAI}/{\Delta DM} \tag{3}$$

where ΔLAI and ΔDM were determining using the following equations $\Delta LAI = LAI_{30} + \ LAI_{60}\_60$ and $\Delta DM = DM_{30} + \ DM_{60}$, respectively. The NAR and RGR were calculated using the method of Kozlowski and Pallardy [41] and the Eqs (4) and (5), respectively.

$$NAR_{30-60} = {\Delta DM}/{(LA\ \times \Delta t} \tag{4}$$

where LA is the mean leaf area for the interval [(LAI30 + LAI60) / 2] and Δt is the time interval in days.

$$RGR = NAR\ \times LAR \tag{5}$$

CGR was calculated using equation 6 according to the Radford [42] methods.

$$CGR = \left(\frac{DM_{60} - DM_{30}}{\Delta t}\right) \times D \tag{6}$$

The following productive variables were determined during harvest time: Head diameter (HD, cm), which was determined using a graduated rule, number of seeds per head (NSH), which was determined by direct counting, mass of seeds per head (MSH, g), which was determined by weighing all the seeds on a microscale (model BS 124S, accuracy ± 0.01 g), and seed yield (SY, $t\ ha^{-1}$), which was calculated using the MSH by both plant densities (D1 and D2), respectively.

2.8. Statistical analysis

The data were analyzed using a linear mixed-effects model (LMM) suitable for a split-split-plot design. Plant density (D), chitosan (Q), and amino acids (A) were considered fixed factors, while replication and their associated hierarchical error terms were included as random effects, using the lme4 and lmerTest packages. Fixed effects were interpreted by computing estimated marginal means (EMMs) via the emmeans package. Additionally, to evaluate statistical differences among treatment means under the three-way interaction framework, we employed Fisher's Protected Least Significant Difference (LSD) test at the 0.05 probability level. In the figures, error bars represent the calculated LSD ($\alpha = 0.05$), providing a precise visual threshold for statistical significance between treatments. Furthermore, statistical associations between growth, physiological, and yield parameters were evaluated using Spearman's rank correlation. The resulting matrix integrates pairwise correlation coefficients (ρ), significance levels (p-values), and bivariate scatterplots, with diagonal elements representing the kernel density estimation for each parameter. All analyses were performed using the R software version 4.4 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Morphological traits and biomass accumulation

The LMM analysis revealed significant majo effects of plant density, chitosan, and amino acid applications on all measured variables ($p < 0.05$). Significant D × Q × A interactions were detected for PH60, LA30, LAI30, AB60, RCC30, LAR, CGR, HD, NSH, MSH, and SY ($p < 0.001$); for LA60, LAI60, and NAR ($p < 0.01$); and for AB30, RCC60, and RGR ($p < 0.05$; Table S1). Plant height (PH30 and PH60) showed significant variations in response to the D × Q × A interaction (Table S1; $p < 0.001$; Figure 1(a),(b)). The $D_{50,000}Q_{200}A_{1.2}$ treatment resulted in the highest PH30 and PH60 values among the combined treatments, increasing PH30 by 70% and 48% and PH60 by 66% and 28% relative to $D_{25,000}Q_0A_0$ and $D_{50,000}Q_0A_0$, respectively (Figure 1(a),(b)).

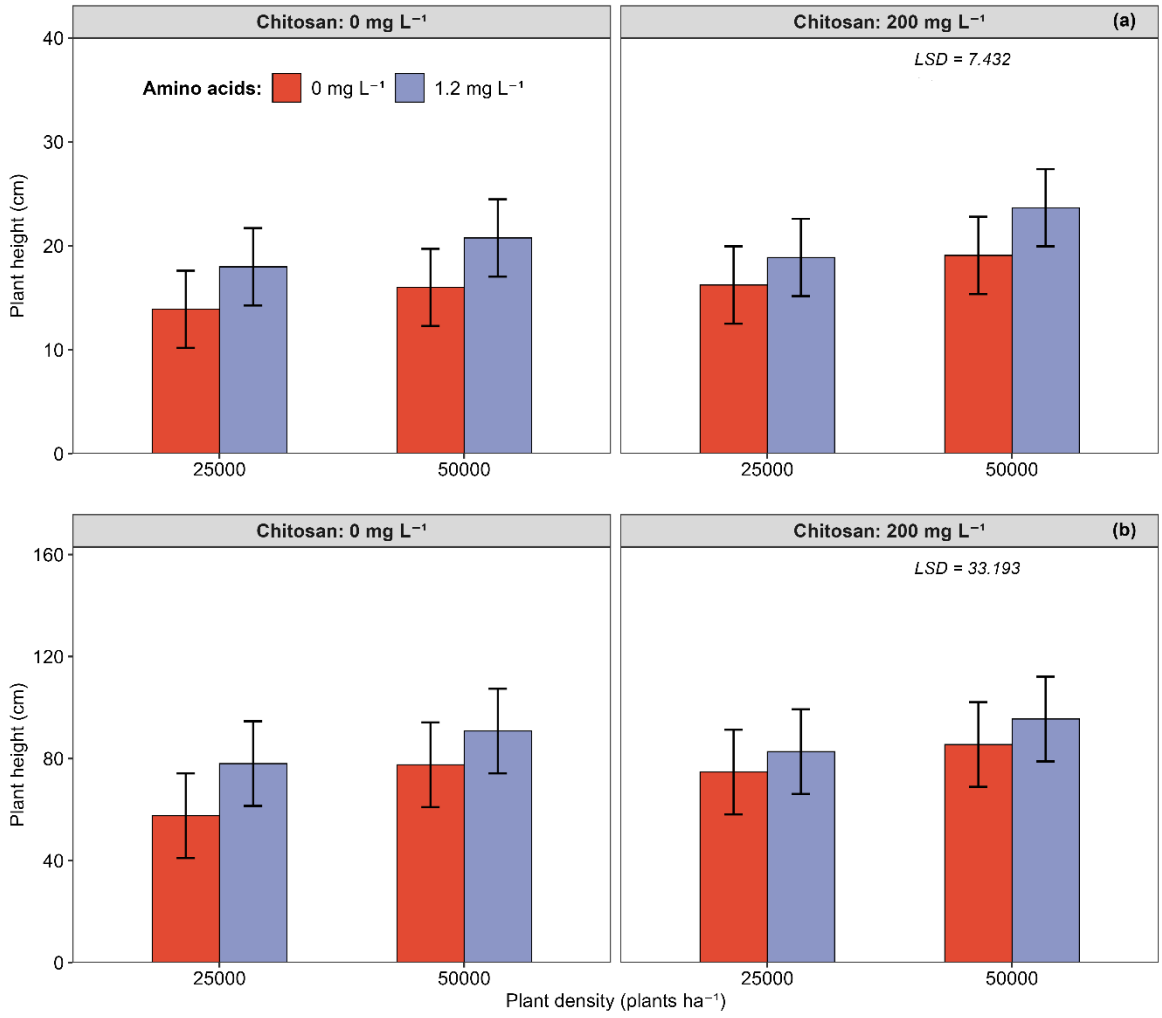

Figure 1. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on sunflower plant height at 30 DAS (a) and at 60 DAS (b) under water deficit conditions. The columns (histograms) represent treatment means ($n = 4$), whereas the error bars represent the LSD values. Treatment means separated by more than the LSD value are considered significantly different.

The three-way interaction (D × Q × A) significantly affected leaf area at 30 DAS (LA30; Figure 2(a)) and 60 DAS (LA60; Figure 2(b)) under water-deficit conditions. At 30 DAS, the combined treatment $D_{25,000}Q_{200}A_{1.2}$ produced the highest LA30 values, exceeding those observed in $D_{50,000}Q_0A_0$ and $D_{50,000}Q_{200}A_0$ by 71% and 40%, respectively (Figure 2(a)). Similarly, at 60 DAS, the $D_{50,000}Q_{200}A_{1.2}$ treatment resulted in the greatest LA60 values, representing increases of 56% and 46% relative to $D_{50,000}Q_0A_0$ and $D_{25,000}Q_0A_0$, respectively (Figure 2(b)).

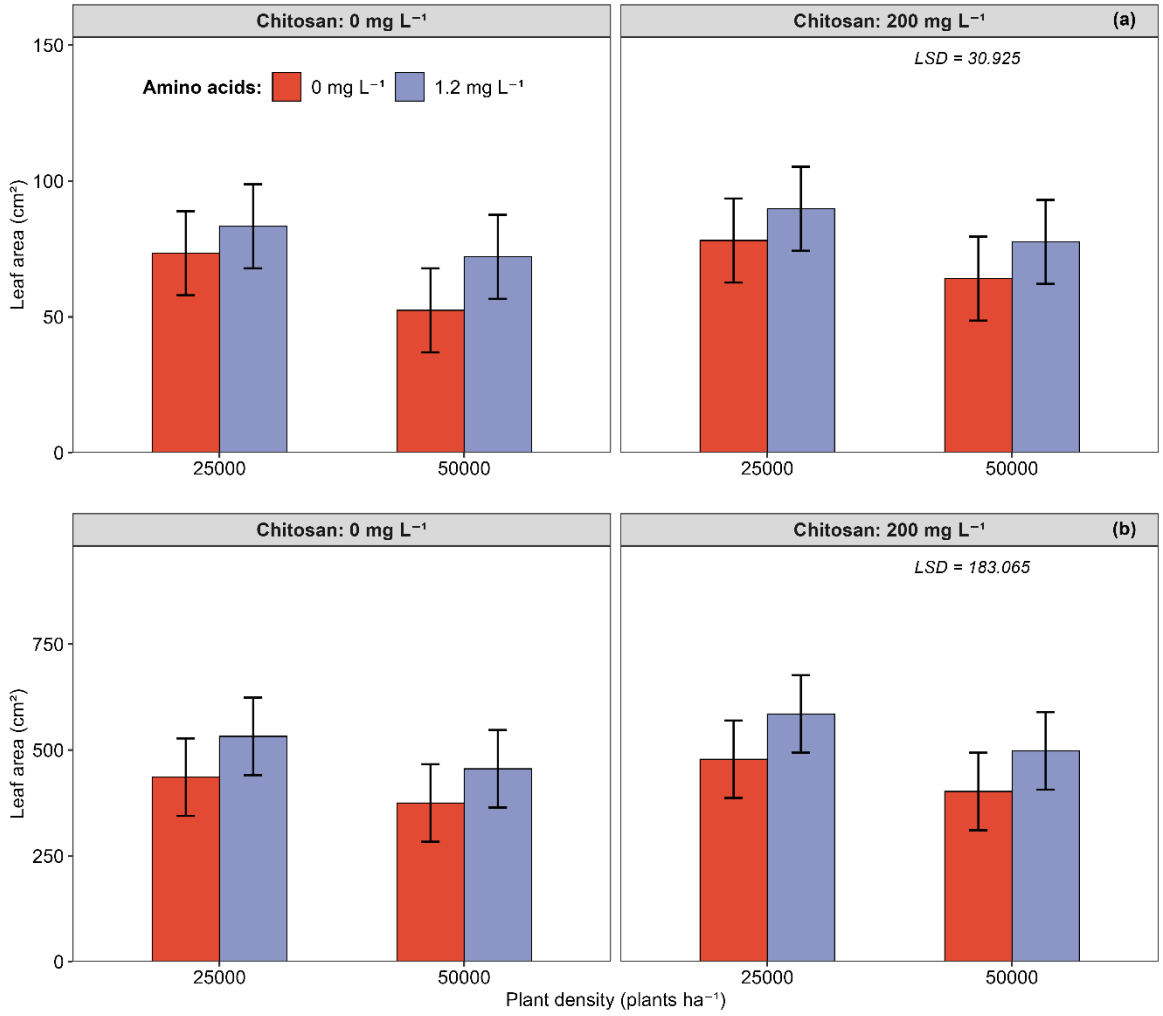

Figure 2. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on sunflower leaf area at 30 DAS (a) and at 60 DAS (b) under water deficit conditions. The columns (histograms) represent treatment means (n = 4), whereas the error bars represent the LSD values. Treatment means separated by more than the LSD value are considered significantly different.

The three-way interaction (D × Q × A) significantly influenced the leaf area index (LAI) of sunflower plants grown under water-deficit conditions at 30 and 60 DAS (Figure 3(a),(b)). The $D_{50,000}Q_{200}A_{1.2}$ treatment produced the highest LAI values, increasing LAI30 and LAI60 by 112% and 127%, respectively, relative to $D_{25,000}Q_0A_0$ treatment (Figure 3(a),(b)).

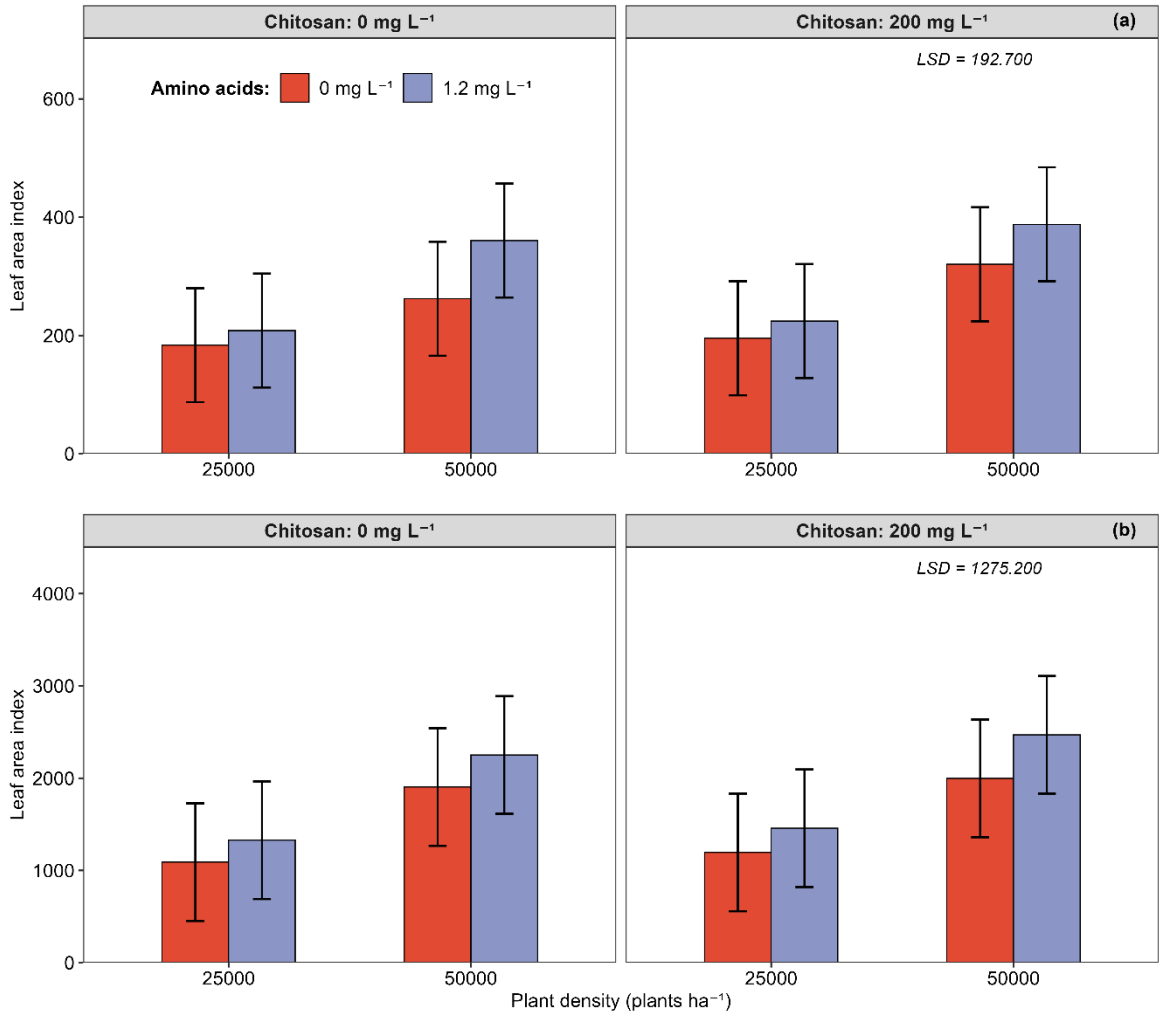

Figure 3. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on sunflower leaf area index at 30 DAS (a) and at 60 DAS (b) under water deficit conditions. The columns (histograms) represent treatment means (n = 4), whereas the error bars represent the LSD values. Treatment means separated by more than the LSD value are considered significantly different.

3.2. Morphophysiological traits

A significant D × Q × A interaction was observed for aboveground biomass accumulation at 30 DAS (AB30; Table S1; Figure 4(a)) and 60 DAS (AB60; Table S1; Figure 4(b)). Among the treatment combinations, $D_{50,000}Q_{200}A_{1.2}$ promoted the highest biomass accumulation, resulting in increases of 169% in AB30 and 92% in AB60 relative to $D_{25,000}Q_0A_0$ (Figure 4(a),(b)).

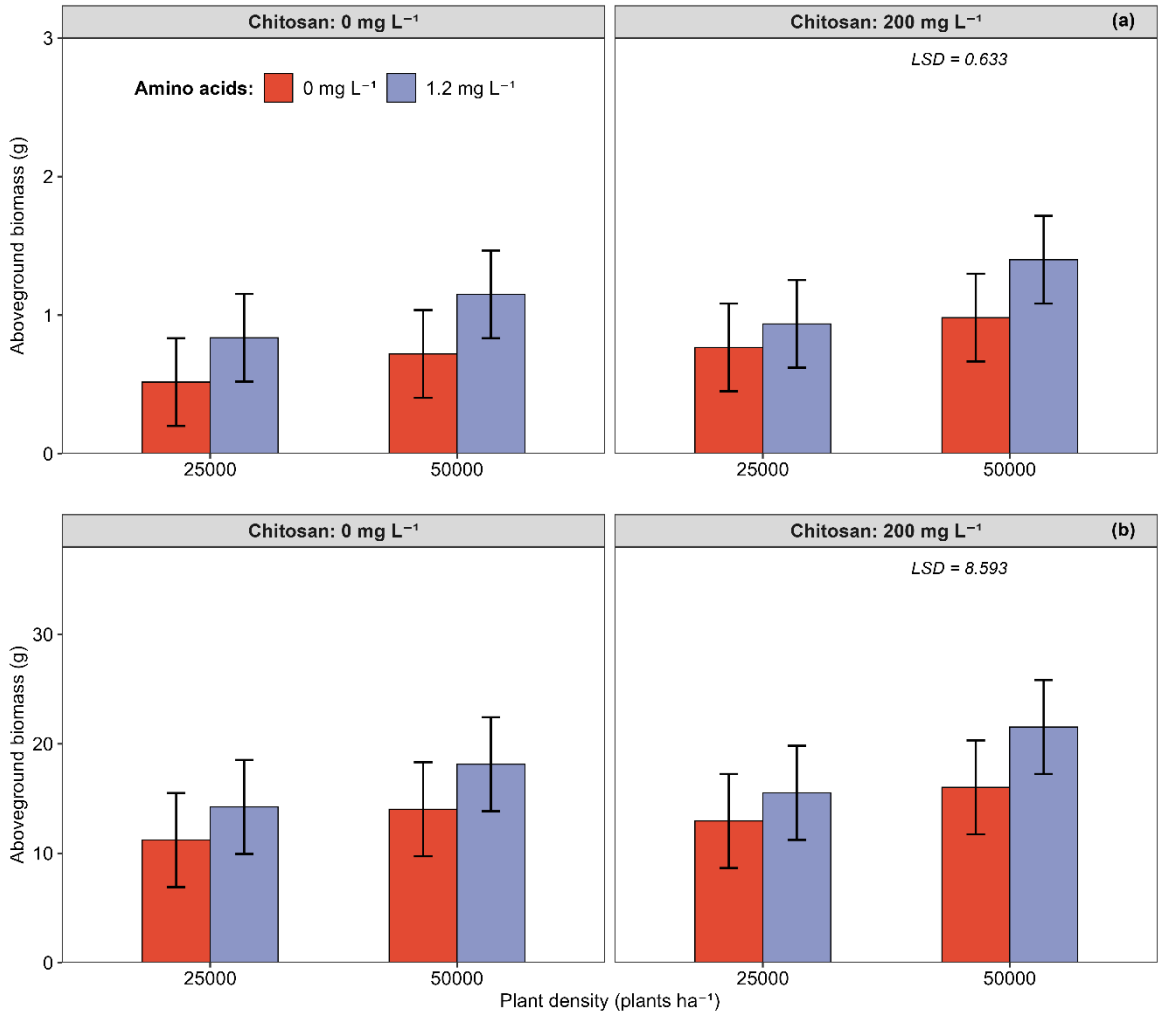

Figure 4. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on sunflower aboveground biomass accumulation at 30 DAS (a) and at 60 DAS (b) under water deficit conditions. The columns (histograms) represent treatment means (n = 4), whereas the error bars represent the LSD values. Treatment means separated by more than the LSD value are considered significantly different.

The three-way interaction (D × Q × A) significantly influenced the growth indices of sunflower plants under water-deficit conditions (Table 1). The $D_{25,000}Q_0A_0$ treatment exhibited the highest leaf area ratio (LAR) and relative growth rate (RGR), increasing these indices by 67% and 11%, respectively, compared with the $D_{50,000}Q_{200}A_0$ treatment. In contrast, the combined $D_{50,000}Q_{200}A_{1.2}$ treatment consistently promoted the highest net assimilation rate (NAR) and crop growth rate (CGR), representing increases of 74% and 3.91-fold, respectively, relative to the $D_{25,000}Q_0A_0$ treatment. Furthermore, the relatively small 2 × SEM values observed for LAR (0.091; $p < 0.001$), NAR (0.000; $p < 0.01$), RGR (0.001; $p < 0.01$), and CGR (0.039; $p < 0.001$) indicated high precision of the estimated means and corroborated the treatment differences detected by the LMM analysis (Table 1).

Table 1. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on the sunflower growth index between 30 and at 60 DAS under water deficit conditions.

	Plant density 25,000 ($D_{25,000}$)				Plant density 50,000 ($D_{50,000}$)			
	Chitosan: 0 (Q_0)		Chitosan: 200 (Q_{200})		Chitosan: 0 (Q_0)		Chitosan: 200 (Q_{200})	
	A_0	$A_{1.2}$	A_0	$A_{1.2}$	A_0	$A_{1.2}$	A_0	$A_{1.2}$
LAR	7.22 ± 0.045	4.506 ± 0.045	6.64 ± 0.045	4.71 ± 0.045	6.474 ± 0.045	4.679 ± 0.045	6.401 ± 0.045	4.322 ± 0.045
NAR	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.003 ± 0.001
RGR	0.103 ± 0.001	0.099 ± 0.001	0.094 ± 0.001	0.093 ± 0.001	0.094 ± 0.001	0.092 ± 0.001	0.094 ± 0.001	0.091 ± 0.001
CGR	0.977 ± 0.020	2.458 ± 0.020	1.143 ± 0.020	2.835 ± 0.020	1.256 ± 0.020	3.215 ± 0.020	1.372 ± 0.020	3.823 ± 0.020

*Note: Data are expressed as means ± LSD values. Treatment means separated by more than the LSD values are considered significantly different.

A significant three-way interaction (D × Q × A) was observed for relative chlorophyll content at both evaluation times (Table S1, Figure 5). Among the treatment combinations, $D_{50,000}Q_{200}A_{1.2}$ produced the highest RCC30 and RCC60 values, exceeding those recorded under $D_{25,000}Q_0A_0$ by approximately 39% and 47%, respectively.

A significant three-way interaction (D × Q × A) was observed for relative chlorophyll content at both evaluation times (Table S1, Figure 5). Among the treatment combinations, $D_{50,000}Q_{200}A_{1.2}$ produced the highest RCC30 and RCC60 values, exceeding those recorded under $D_{25,000}Q_0A_0$ by approximately 39% and 47%, respectively (Figure 5(a),(b)).

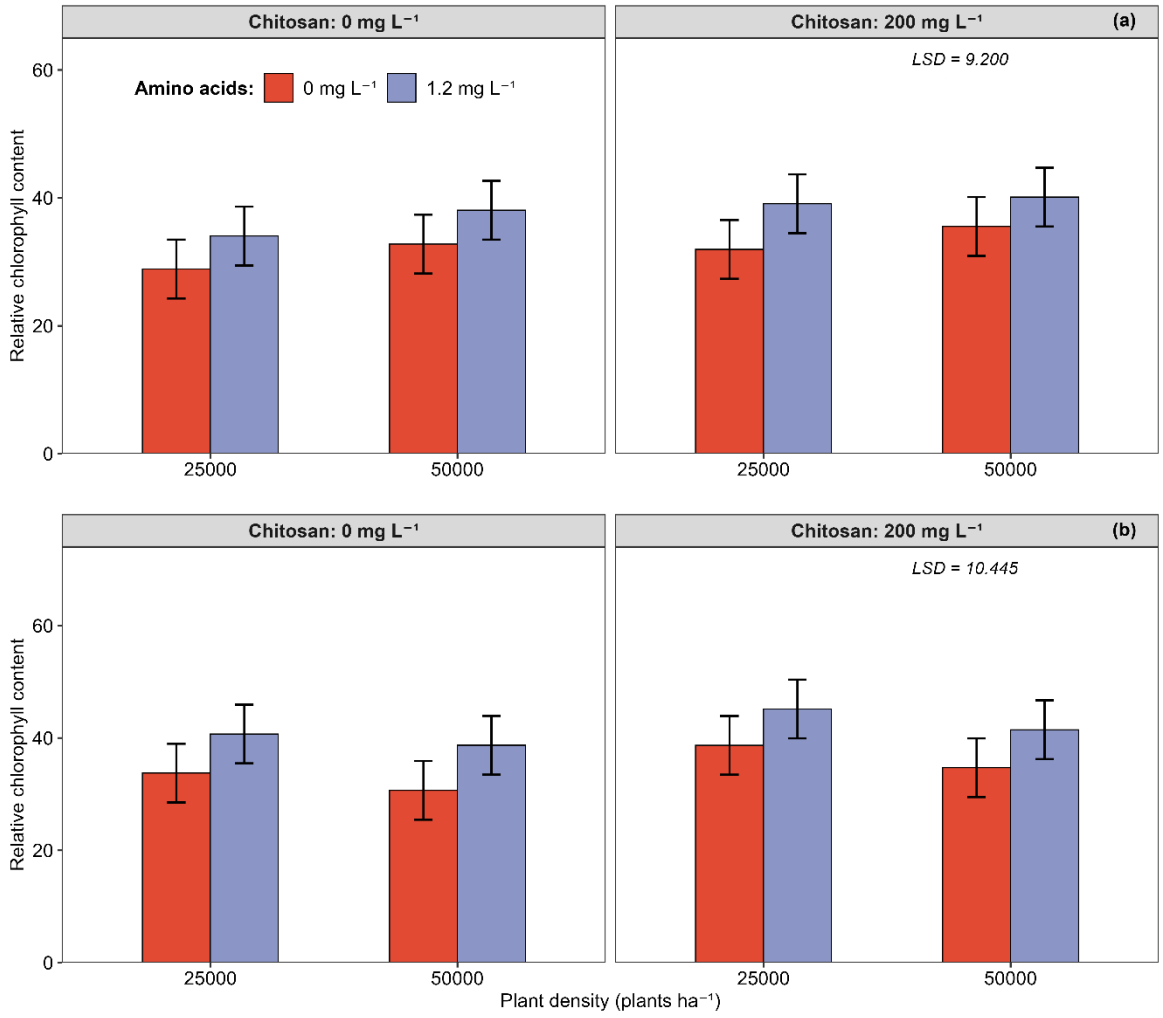

Figure 5. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on sunflower relative chlorophyll content at 30 DAS (a) and at 60 DAS (b) under water deficit conditions. The columns (histograms) represent treatment means (n = 4), whereas the error bars represent the LSD values. Treatment means separated by more than the LSD value are considered significantly different.

3.3. Productivity traits

A significant D × Q × A interaction was detected for head diameter (HD; Figure 6(a)) and number of seeds per head (NSH; Figure 6(b)) under water-deficit conditions. Among the treatment combinations, $D_{25,000}Q_{200}A_{1.2}$ produced the greatest HD and NSH, representing increases of 112% and 35%, respectively, relative to $D_{50,000}Q_0A_0$ treatment (Figure 6(a),(b)).

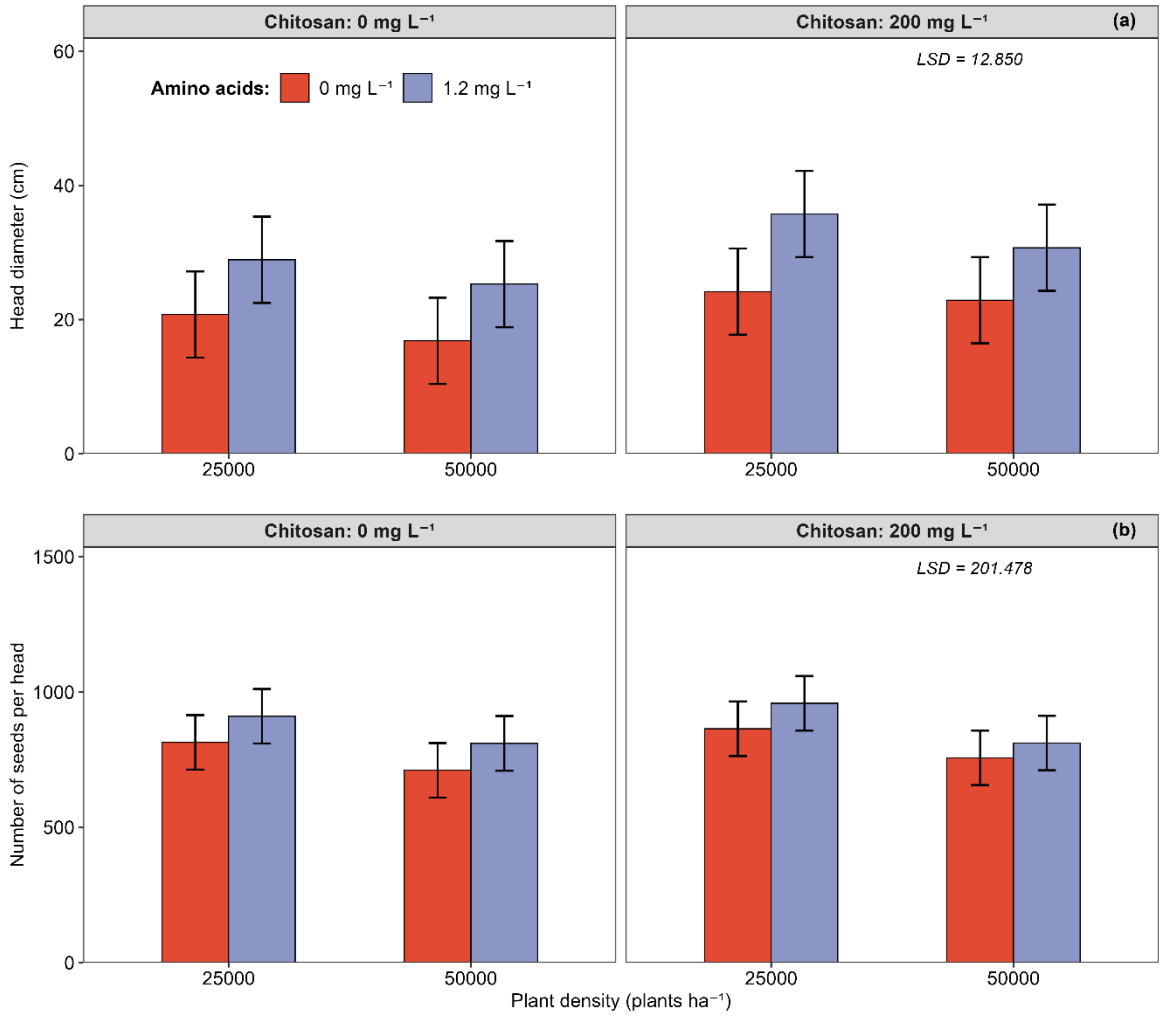

Figure 6. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on sunflower head diameter (a) and number of seeds per head (b) under water deficit conditions. The columns (histograms) represent treatment means (n = 4), whereas the error bars represent the LSD values. Treatment means separated by more than the LSD value are considered significantly different.

A significant D × Q × A interaction affected MSH (Figure 7(a)) and SY (Figure 7(b)) under water-deficit conditions. The combined treatment $D_{25,000}Q_{200}A_{1.2}$ produced the highest MSH, resulting in a 72% increase compared with $D_{50,000}Q_0A_0$ (Figure 7(a)). Conversely, the highest SY was observed under $D_{50,000}Q_{200}A_{1.2}$, which exceeded that of $D_{25,000}Q_0A_0$ by 162% (Figure 7(b)).

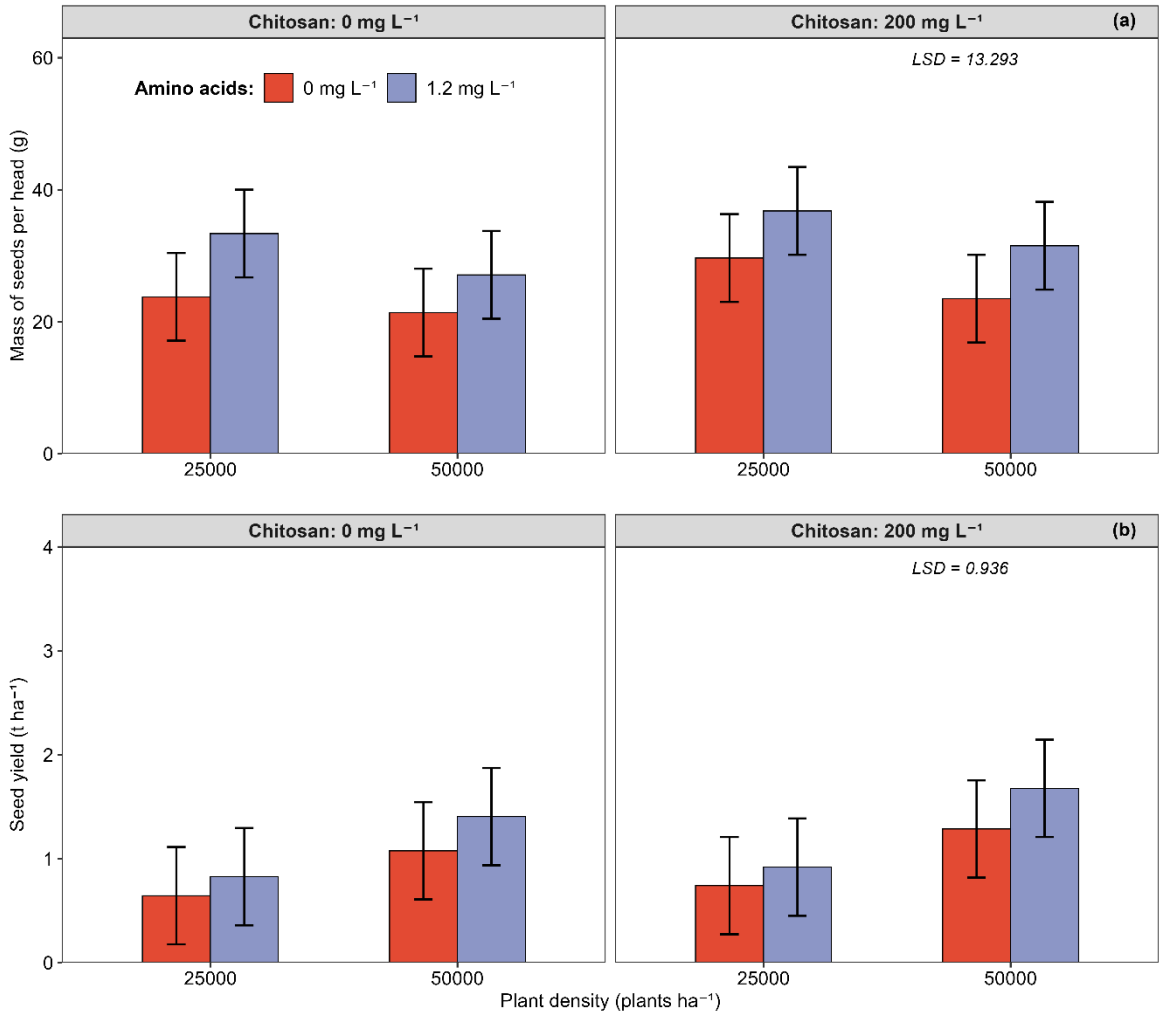

Figure 7. Effects of plant density (25,000 and 50,000 plants ha^{-1}), chitosan application (0 and 200 mg L^{-1}), and amino acid spraying (0 and 1.2 mL L^{-1}) on sunflower mass of seeds per head (a) and seed yield (b) under water deficit conditions. The columns (histograms) represent treatment means ($n = 4$), whereas the error bars represent the LSD values. Treatment means separated by more than the LSD value are considered significantly different.

3.4. Correlation analysis of growth, physiological, and yield parameters

The correlation analysis revealed an integrated network of physiological responses, with several distinct clusters of positive associations (Figure 8). Notably, PH and LA across developmental stages (e.g., PH30, PH60, LA30, LA60) exhibited positive correlations ($\rho = 0.92$ to 0.98; $p < 0.05$). Furthermore, AB30, and AB60 showed a robust positive relationship with LAR and NAR ($\rho > 0.85$), highlighting the critical role of leaf development efficiency in AB accumulation. In contrast, D displayed significant negative correlations with several growth parameters, most notably with LAI30, LAI60, and MSH (ρ = -0.87). Interestingly, while Q application showed weak or non-significant direct correlations with final yield components, supplying A demonstrated positive correlations with RGR and CGR ($\rho = 0.60$; Figure 8).

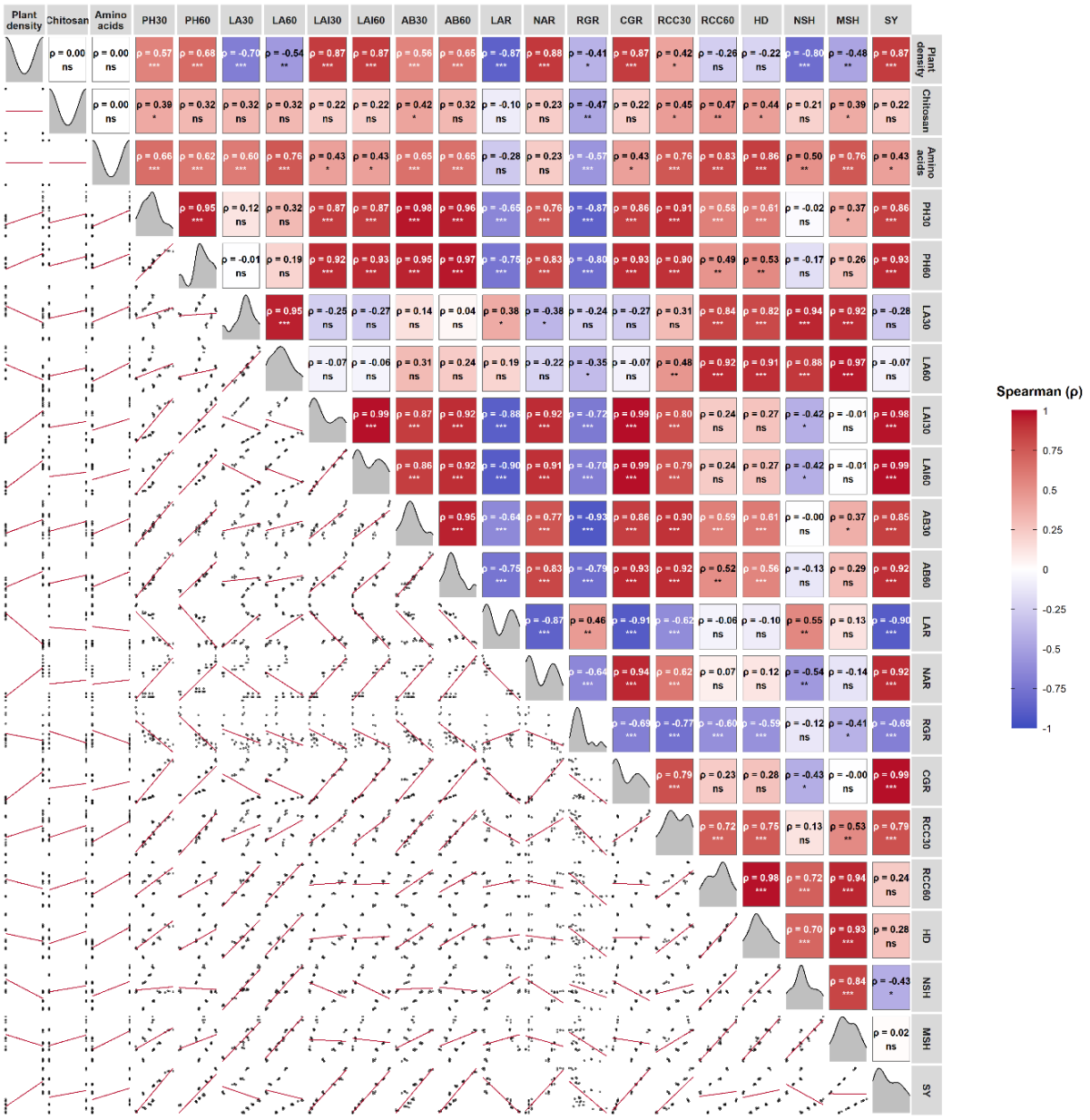

Figure 8. Spearman correlation matrix of plant morphological and physiological traits. The upper triangle displays Spearman's rank correlation coefficients (ρ) with associated significance levels (ns: non-significant; $p < 0.05$ for numerical values). The color scale indicates the strength and direction of the relationship, ranging from deep red (positive correlation, $\rho = 0.01$) to deep blue (negative correlation, $\rho = -0.01$). The diagonal shows the density distribution for each individual variable. The lower triangle presents bivariate scatter plots with fitted linear regression lines, illustrating the pairwise relationships between parameters including plant density, chitosan, amino acids.

The analysis reveals that SY is positively correlated with HD ($\rho = 0.96$; $p < 0.001$) and MSH ($\rho = 0.99$; $p < 0.001$), identifying these as the primary phenotypic determinants of productivity. Conversely, D exhibited a negative correlation with HD ($\rho = -0.80$) and MSH ($\rho = -0.48$). Interestingly, the NSH showed significant, positive correlation with SY ($\rho = 0.43$). Applying A demonstrated a positive influence on HD ($\rho = 0.50$) and NSH ($\rho = 0.76$), while Q showed non-significant or weak associations

with final yield components (ρ = 0.22; Figure 8).

4. Discussion

Spatial variations in sunflower production systems reflect the combined influence of terrain characteristics, vegetation dynamics, land management practices, soil development duration, soil–water–fertilization interactions, cultivars, and climate shifts. Our hypothesis presumes that optimizing plant density can attenuate water-deficit stress by modulating canopy structure and maximizing resource-use efficiency. Our findings validate this hypothesis increasing plant density to 50,000 plants ha^{-1} enhanced structural traits, specifically plant height, LA, and LAI, alongside physiological performance, including chlorophyll content, which sustained higher seed yield under water-deficit conditions. This response indicates that elevated plant populations stimulate efficient light interception and rapid canopy closure, thereby compensating for the restricted phenotypic performance of individual plants [7,35]. Similar results have been reported in studies where optimized planting configurations enhanced water-use efficiency and yield stability within water-limited environments [16]. Consequently, these data substantiate the agronomic principle that crop yield per unit area under environmental stress depends more on collective canopy population dynamics than on individual isolated plant productivity.

Beyond density-dependent effects, our data empirical support the starting hypothesis that the co-application of chitosan and amino acid–based biostimulants synergistically bolsters water-deficit tolerance in sunflower. The significant improvements observed in plant height, biomass accumulation, and chlorophyll content under combined treatments demonstrate a robust synergistic interplay between agronomic and physiological factors. This response is governed by complementary, multi-level physiological mechanisms. Chitosan operates as a potent elicitor that upregulates antioxidant defense systems, triggers osmotic adjustment, and modulates stress-responsive gene expression, thereby strengthening cellular tolerance to water stress [23,24,43,44]. Concurrently, chitosan promotes leaf gas exchange, safeguards chlorophyll pigments, and induces the accumulation of compatible solutes, such as free proline and soluble proteins, under water-deficit conditions [25,45,46]. Furthermore, co-applied amino acid–based biostimulants mitigate osmotic stress by serving as direct metabolic precursors and intracellular osmolytes, which accelerate protein synthesis, stabilize cell membranes, and optimize N assimilation pathways [47,48,49,50]. Parallel studies demonstrate that the exogenous application of chitosan-amino acid matrices improves crop resilience by fortifying biochemical and physiological frameworks under drought conditions [26,34,51]. This defensive baseline is mediated by the functional roles of exogenous amino acids in maintaining cellular homeostasis, maintaining turgor pressure, and preserving metabolic enzyme configurations during drought stress. Crucially, the superior efficacy of the integrated $D_{50,000}Q_{200}A_{1.2}$ treatment is attributed to a definitive cross-talk between structural canopy density modifications and exogenous biochemical regulation (34,51). This multifaceted integration operates through five distinct organizational axes: (i) Canopy-level architectural optimization, (ii) physiological enhancement of photosynthetic apparatuses, (iii) targeted metabolic substrate support, (iv) synergistic hormonal signaling and stress priming, and (v) integrated resource-use efficiencies [52,53]. These interconnected systems explain why the $D_{50,000}Q_{200}A_{1.2}$ treatment exhibited the highest performance thresholds across all recorded morphophysiological variables and final yields. This outcome corroborates the literature where multi-component biostimulant strategies enhanced crop yield performance under water shortages via coordinated physiological and biochemical responses [44,53]. This structural reinforcement of the sunflower stem under $D_{50,000}Q_{200}A_{1.2}$ application enhanced mechanical

lodging resistance and reinforced cell wall structural integrity, thereby minimizing lodging risks and maintaining unhindered vascular transport dynamics under severe water stress [34,51]. Consequently, this phenomenon highlights that integrating modern agronomic strategies with targeted biochemical biostimulation generates compounding field benefits that far exceed the impact of individual isolated treatments, reinforcing our core hypothesis.

The physiological responses of individual LA and population-level LAI provide further mechanistic supports for this interpretation. Under water stress, sharp declines in LA and LAI reflect turgor-loss limitations in cell expansion and drought-induced premature leaf senescence. Conversely, their substantial retention and enhancement under the combined $D_{50,000}Q_{200}A_{1.2}$ framework indicate enhanced canopy growth dynamics and sustained carbon assimilation capacities. This preservation is governed by improved tissue water relations, stricter osmotic regulation, and delayed functional senescence induced by the protective chitosan-amino acid coating. Furthermore, managing the crop through the $D_{50,000}Q_{200}A_{1.2}$ treatment coordinates resource allocation patterns, optimizes light interception efficiency, and fosters cellular physiological resilience [34,52]. Furthermore, when $D_{50,000}Q_{200}A_{1.2}$ treatments are tuned together (timing, concentration, and phenological stage), the combined effect is synergistic: Fewer leaves are lost to shading and stress, individual leaf expansion is maximized, and population-level leaf area per m² is optimized for light capture and C gain, which translates into robust yield stability across variable production environments [34,51,53]. This aligns with earlier findings where targeted biostimulants stimulated leaf expansion and preserved a highly active photosynthetic surface area under abiotic stress conditions [54]. For example, past work noted that a synergy among organic filter cake, beneficial microbial consortia, and free amino acids directly correlated with elevated chlorophyll content, which promoted robust reproductive organ development despite severe water constraints [55,56].

The final seed yield results incorporate all underlying vegetative and metabolic processes, providing strong validation for our central hypothesis. However, the optimized integrated $D_{50,000}Q_{200}A_{1.2}$ treatment increased total seed yield up to 3.1-fold compared to control settings. This substantial gain is attributable to heightened photoassimilates production, optimized source-to-sink source transport dynamics, and preferential dry biomass partitioning into reproductive organs [36]. These observations correspond with literature demonstrating that integrated agronomic management combined with biostimulant improves final yield under water-deficit conditions by enhancing whole-plant physiological efficiency and cellular stress tolerance [1,34]. However, the structural and biochemical efficacy of combined $D_{50,000}Q_{200}A_{1.2}$ treatment in bolstering sunflower yield aligns with historical evidence indicating that these compounds activate complementary metabolic pathways [44,51]. In our trial, this biochemical and molecular synergy empirical is validated by improvements in vegetative growth indices, high relative chlorophyll fractions, enhanced aboveground biomass accumulation, and optimized yield components. Similar observations were observed following the foliar application of chitosan (Q) mixed with amino acids (A), which significantly upregulated growth dynamics and preserved photosynthetic pigments in sunflower plants [30,34].

Our integrated correlation matrix reveals a clear synergistic interplay between exogenous biostimulants and canopy dynamics, where the co-application of chitosan and amino acids serves as a metabolic primer for accelerated plant growth. The strong positive correlation ($\rho < 0.99$; $p < 0.001$) observed between chitosan-functionalized amino acid treatments and the NAR implies a systemic optimization of the of the crop's photosynthetic apparatus. This observation is consistent with findings indicating that chitosan-based nanoconjugates enhance stomatal conductance and chlorophyll

biosynthesis by triggering stress-transduction pathways and upregulating key nitrogen-metabolism enzymes [28,29,57]. Specifically, the film-forming properties of the foliar-applied chitosan matrix as a highly biocompatible carrier prolongs the contact and retention time of the applied solution on the hydrophobic leaf cuticle. This structural layer facilitates a steady, uniform trans-cuticular absorption of the co-applied amino acids, thereby preventing the transient metabolic congestion or sudden localized osmotic imbalances commonly caused by high-concentration, conventional chemical nitrogen sprays [43,54].

Furthermore, the significant coupling discovered between planting density and the relative growth rate in our trials highlights an adaptive compensatory mechanism governing resource competition [58]. Although elevated plant density typically intensifies aggressive intraspecific competition for available light and soil waters and nutrients, its positive correlation with secondary metabolite accumulation points toward a density-dependent elicitation response. Studies on high-density cultivation systems suggest that modified light quality and red to far-red (R:FR) ratios inside crowded canopies trigger a protective biochemical shift, upregulating the phenylpropanoid pathway to enhance defensive compound synthesis [54]. These outcomes demonstrate that the strategic integration of chitosan-amino acid complexes can effectively deployment of chitosan–amino acid complexes directly optimize morphophysiological traits and enhances final yield responses within intensive agricultural systems.

Mechanistically, our empirical data validate the central hypothesis, highlighting the importance of integrating optimized agronomic configurations with targeted biochemical biostimulation to secure sustainable crop production within moisture-deficient environments. This dual-action framework induces coordinated morpho-structural and physiological modifications that collectively maximize whole-plant phenotypic plasticity and yield stability under stress. Consequently, these insights align with current paradigms in sustainable agriculture, emphasizing that combining precise crop management with modern biostimulant technologies is essential to mitigate the compounding threats of climate change on global crop productivity.

5. Conclusions

Importantly, our data directly support the initial hypothesis, demonstrating that the strategic integration of agronomic and biostimulant-based approaches can effectively mitigate these adverse effects. Specifically, the combined application of an optimized plant density (50,000 plants ha^{-1}) with foliar chitosan (200 mg L^{-1}) and an amino acid–based biostimulant (1.2 mL L^{-1}) resulted in synergistic improvements in morphophysiological traits, including leaf area index, chlorophyll content, and biomass production, which collectively translated into substantial gains in yield components and final seed yield under water-deficit conditions. From an agronomic perspective, our findings highlight that optimizing plant density in combination with targeted foliar biostimulant applications represents a practical and environmentally sustainable strategy to enhance sunflower resilience and yield stability under water-deficit conditions. In future studies, researchers should focus on validating these results across agroecological zones, exploring long-term soil-plant interactions, and elucidating the underlying molecular and biochemical mechanisms to further refine this integrated management approach.

Author contributions

Alexander Calero Hurtado: Conceptualization, methodology, software, validation, formal

analysis, investigation, writing–original draft preparation, writing–review and editing, visualization, supervision, project administration, funding acquisition; Felipe Augusto Queiroz de Almeida: software; Formal analysis, investigation, writing–original draft preparation; Daniel Gonçalves da Silva Pinheiro: Software; formal analysis, investigation, writing–original draft preparation, writing–review and editing; Thainá Pereira da Silva Cabral: Visualization, investigation, writing–original draft preparation, writing–review and editing; José Vitor Botter Fasoli: Visualization, investigation, writing–original draft preparation, writing–review and editing; Bruna Wilke Rampassi: Investigation, writing–original draft preparation, writing–review and editing; Kolima Peña Calzada: Methodology, investigation, writing–original draft preparation, writing–review and editing, supervision, funding acquisition. All authors have read and approved the final version of the manuscript for publication.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflicts of interest.

Supplementary materials

Supplementary data associated with this article can be found, in the online version as Table S1. Summary of fixed effects from the Linear Mixed-Effects Model (LMM). Values represent numerator degrees of freedom (NumDF); denominator degrees of freedom (estimated via Kenward-Roger approximation, DenDF), F-statistics and significance levels for the source of variation across all variables.

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