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Tomato responses to nitrogen, drought and combined stresses: Shared and specific effects on vascular plant anatomy, nutrient partitioning and amino acids profile

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ABSTRACT

Crops are often subjected to various abiotic stresses and interactions between them may occur, but how plants cope with them remains poorly understood. This study explored how combined nitrogen and drought stress impact tomato vascular stem anatomy, nutrient partitioning and amino acids profile. Tomato seedlings were exposed to control (CTR; 100N + 100%W), N stress (N; 50%N), drought stress (W; 50%W), or combined stress (N + W; 50%N+50%W) for 27 days. All treatments similarly reduced the phloem and xylem areas. Plants under N + W stress exhibited increased root synthesis of asparagine and arginine (up to 230% compared to W stress and 66% compared to N stress) and showed a higher reallocation and synthesis of osmolytes such as K^+ and proline, respectively. This, along with the specific increase in other amino acids related to osmoregulation (alanine, tyrosine and phenylalanine), contributed to an enhanced stomatal closure and lower transpiration rate compared to W stressed plants. Conversely, N stressed plants responded mainly through N remobilization from the photosynthetic machinery, leading to decreased chlorophyll content (up to 32%) and photosynthetic rate (up to 57%). Under single W stress, plants invested more in the root system as a strategy to increase W and nutrients' uptake, compared to those grown under N + W stress, and maintained the photosynthetic rate at the level of CTR plants. It is concluded that tomato plants employed distinct mechanisms for reallocating nitrogen and regulating osmosis to withstand either single or combined stresses and that amino acids and nutrients' homeostasis have an important role in these processes.

1. Introduction

Tomato (*Solanum lycopersicum*) stands out as one of the world's most economically important vegetable crop, with an annual production reaching 180 million tons (Wu et al., 2022). Due to its high demand for nitrogen (N) fertilization and water (W) supply, limiting levels of these resources severely impact plant growth (Du et al., 2018; Machado et al., 2022). Different studies have consistently demonstrated that N and drought stresses significantly impact metabolic pathways (Ding et al., 2018; Gonzalez-Dugo et al., 2010), altering vascular tissue anatomy responsible for fulfilling plant W and nutrient requirements (Kapoor et al., 2020; Song et al., 2019). For instance, narrower xylem vessels under lower W potential help to prevent cavitation (Knipfer et al., 2019). However, this may also impair internal W transport when combined with a reduced total xylem area (Hernandez-Espinoza and Barrios-Masias, 2020). Moreover, drought stress negatively affects root nutrient uptake due to reduced soil moisture (Rouphael et al., 2012), further constraining plant growth (Sánchez-Rodríguez et al., 2011a),

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which can be aggravated when plants are facing a combination of both deficits. Additionally, since N is a major macronutrient, its deficit also impacts the accumulation of other nutrients, with its ionic form adjusting the charging balance during the absorption of cations and anions (Borgognone et al., 2013). For example, under NO_3^- deficiency, there is a lower accumulation of cations in the plant tissues (namely calcium, magnesium and potassium), whereas anions (such as phosphate, sulphate and most micronutrients) were reported as having higher accumulation (Machado et al., 2022). Additionally, it has been reported that N uptake diminishes under drought conditions (reviewed by Gonzalez- Dugo et al., 2010), further exacerbating the stress effects. Furthermore, recent findings suggest that a short period of mild N-deficiency, achieved by reducing N fertilizer, might be beneficial in mitigating the negative effects of drought stress (Safavi-Rizi et al., 2021). This highlights the importance of fine-tuning N fertilization to optimize plant resilience under combined stresses.

Amino acids are the structural units of proteins and perform a range of vital functions in plants (Hildebrandt et al., 2015; Kumar et al., 2017). Besides their role in protein synthesis, they act as signalling molecules being involved in several processes such as regulating intracellular pH, generating metabolic energy or redox power, and enhancing tolerance to biotic and abiotic stresses (Guo et al., 2021; Hildebrandt et al., 2015). Interestingly, tomato tolerant cultivars appear to exhibit higher N levels compared to sensitive ones, enabling intensified synthesis of amino acids and proteins, which may contribute to stress tolerance (Sánchez-Rodríguez et al., 2011).

Under stress conditions, such as N deficiency or drought stress, the amino acid pool adjusts through catabolism and synthesis (Yang et al., 2020). For instance, proline can have a relevant osmolyte action, often increasing in plants subjected to drought (Florencio-Ortiz et al., 2018; Živanović et al., 2020). However, it can also act as a metabolic compatible solute, facilitating the storage and transfer of N and carbon (C) following oxidation, with its levels generally decreasing when N is limited (Hayat et al., 2012; Szabados and Savouré, 2010). Additionally, N availability influences C/N ratio thereby impacting the amino acid profile. For instance, higher N availability promotes the production of amino acids with low C/N ratio, such arginine and glutamine (Ruan et al., 2010). Several studies, some of them with tomato plants, have shown that N stress often results in reduced total amino acids content, as N is crucial for their synthesis (Ková ik et al., 2006; Ruan et al., 2010; Sung et al., 2015; Yang et al., 2020). Conversely, under drought stress the free amino acids content tends to increase in tomato plants (Živanović et al., 2020). This suggests that free amino acids may undergo differential adjustments in situations involving combined N and drought stresses. For instance, Jin et al. (2015) found that *Portulaca oleracea* L. plants, exhibited a notable increase in specific amino acids, including glutamine, ornithine, tyrosine, valine, and tryptophan, when exposed to combined water deficit and heat stress. This selective accumulation suggests that the plant is performing cellular osmotic adjustments to sustain leaf turgor under the simultaneous stress condition. Moreover, the metabolite profile of plants grown under combined drought and heat was more comparable to the one found in plants under single heat stress, than to the profiles of control or drought-treated plants (Jin et al., 2015).

Despite the literature on the interactions between N deficiency and drought being limited, recent findings suggest that tomato plants subjected to these combined abiotic stresses employ specific response mechanisms (Machado et al., 2023a, 2023b). For instance, it was found that concerning the roots, plants subjected to combined N and W deficits exhibited similarities to those exposed to single W deficit. These included responses related to osmoregulation, such as higher proline, total soluble protein, and NO_3^- concentration, along with increased nitrate reductase activity and upregulation of *NR* and *GS* genes. Conversely, in the shoots, plants facing the combined deficit were more similar to those exposed to single N deficit. These similarities were primarily related to N remobilization, indicated by the lower proline concentration, higher C/N ratio, GS, and NR activity, as well as increased levels of *rcbS* and *rcbL* transcripts (Machado et al., 2023a). This suggests that in the presence of both stresses, tomato seedlings activate several mechanisms related to nitrogen remobilization and osmoregulation. Nonetheless, more research is needed to further explore additional processes involved in N remobilization and osmoregulatory responses under single and combined nitrogen and W stress – namely the role of the vascular stem anatomy, nutrient partitioning and amino-acids profile. For that, we conducted a comprehensive evaluation encompassing anatomical and morphological traits (including hypocotyl, phloem and xylem area, dry weight of root, stem, leaf and total plant), physiological parameters (chlorophyll content, photosynthetic rate, stomatal conductance and transpiration rate), macronutrient content and partitioning (N, K, P, Ca and Mg), N utilization efficiency and the concentration of 16 free amino acids.

2. Material and methods

2.1. Plant material and growth conditions

The experiment was conducted in a growth chamber under controlled conditions, with the following setpoints: 25/23 °C day/night temperature, 70% relative humidity (RH), 400 μmol mol^{-1} CO_2 concentration, and 150 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The photoperiod was set to 16 h of light [Fluorescent lamps (Osram L58W/840, Lumilux, Cool White, Munich, Germany]).

Tomato cv. Micro-Tom was sown in trays using commercial germination potting substrate (SIRO, Portugal). After approximately three weeks, when the third leaf had emerged, uniform seedlings were selected and individually transplanted into pots (10.5 cm diameter and 10.5 cm height), containing 50 g of vermiculite (0.1–1.5 mm in grade). The seedlings were assigned to one of four treatment groups to induce different abiotic stress conditions: control (CTR; 10.5 mM N; irrigated with nutrient solution to 100% field capacity), nitrogen stress (N; 5.3 mM N), drought stress (W; 50% field capacity) and combined stress (N + W; 5.3 mM N; 50% field capacity). To start introducing a gradual water stress, each pot was first watered until reaching field capacity (FC). Field capacity was determined using the soil gravimetric W content method (Machado et al., 2023a), by adding ~230 mL of the respective nutrient solution as previously described (Machado et al., 2023b, 2024a). Both solutions had a pH of 5.8 and E.C. of 2.1 ± 0.1 dSm^{-1}.

After transplanting the seedlings, each pot was covered with black plastic to minimize evaporation, and no additional nutrient solution was further provided throughout the experimental period. For irrigation management, pots from CTR and N stress treatments were weighed daily, and FC was adjusted to 100% by adding distilled W whenever needed. For W and N + W stress, no additional W was provided until day 10, allowing a gradual reduction in substrate FC. On day 10, three plants per treatment were randomly selected and harvested. Their average fresh weight was subtracted from the total weight of each pot to calculate the remaining volume of W/nutrient solution in the pot, corresponding to 50% FC for W and N + W treatments. Following this, plants were maintained at their respective FC levels (100% or 50%) for 17 days by daily rewatering with distilled W.

2.2. Vascular stem anatomy

At the end of the trial (corresponding to 27-days after transplanting), the roots of six plants per treatment were thoroughly washed. Plants were then promptly dissected into roots and shoots and stored in methanol for later vascular stem anatomy analyses. For anatomical visualization, thin cross-sections were manually prepared from middle part of the hypocotyls. These sections were placed on clean slides with a drop of distilled W. To highlight the lignin, the cross sections of the samples were stained with a solution of 2% phloroglucinol (dissolved in 96% ethanol) and 25% hydrochloric acid. The slides were examined using a Axioskop 2 plus Zeiss epifluorescence microscope connected to an Olympus DP72 camera (Olympus, Japan), and image analysis was conducted using Quick PHOTO MICRO 3.0 software.

2.3. Plant growth analysis and physiological traits

Gas exchange analysis (Infrared gas analyzer, model LI-COR 6400 IRGA; LI-COR, Lincoln, NE, USA) was conducted on the top youngest fully expanded leaf (n = 6 plants), at the end of the experiment. This was performed 2 h after the start of the photoperiod, within a 4-h timeframe, under the following conditions set in the IRGA chamber: light intensity of 300 ± 1 μmol m^{-2} s^{-1}; 400 ± 2 μmol mol^{-1} CO_2 concentration; 25 ± 1 °C leaf temperature and 67 ± 1% RH. The rate of assimilation of CO_2 (A, μmol m^{-2} s^{-1}), stomatal conductance (gs, mol m^{-2} s^{-1}) and transpiration rate (E, mmol m^{-2} s^{-1}). After that, each plant was split into roots, stems, and leaves. The roots were carefully washed, and all samples were placed in a ventilated oven (105 °C for 48-h) for determining their dry weight.

2.4. Macronutrients quantifications

Dried plant tissues were individually ground (n = 3, with each biological replicate including a pool of two plants) and then processed for analysis. For N quantification, the Dumas method was employed. Briefly, approximately 100 mg ground dried plant tissues (roots and shoots) were weighed in an aluminum crucible and analyzed on the Dumatec™ 8000/FOSS equipment with a helium flow rate of 195.0 mL min^{-1} and oxygen flow rate of 300 mL min^{-1}, at a pressure of 1200 mBar. For performing the N calibration curve ethylenediaminetetra-acetic acid was used.

For quantifying the other macronutrients (K, P, Ca and Mg), 250 mg of root and shoot ground dried tissues were mixed with 12.5 mL of 65% nitric acid and 2.5 mL of hydrofluoric acid using a Teflon reaction vessel (MARSXpress, CEM Corporation). The mixture was then heated in a microwave digestor system (Mars ONE, CEM Corporation) applying the following conditions: 175 °C temperature, 15 min of ramp time, and more 15 min of hold time at 800 psi pressure and 900 W of power. After digestion, ultrapure W was added to the resulting clear solution until reaching a volume of 50 mL. The mixture was filtered using 0.45 μm pore size filter and mineral concentration was determined using a ICP-OES (Optima 7000 DV, PerkinElmer, USA) with a radial configuration.

The mineral accumulation (mg/plant; also known as mineral content per plant) was estimated as the coefficient between the total concentration of a given mineral in both plant tissues (mg/g DW) and the total plant dry weight (g). Mineral partitioning was calculated as a ratio of the root or shoot content, of a given mineral, to its total content in the plant, allowing to evaluate how each mineral was distributed among the different plant tissues. N utilization efficiency (NUtE) was calculated by dividing the total plant dry weight by the N concentration quantified in the plant, providing an indication of how efficiently the plants utilized N for growth and development.

2.5. Quantification of free amino acids and proline concentration

Free amino acids were extracted from lyophilized plant tissues frozen at ultra-low temperatures (n = 3, with each biological replicate resulting from a pool of three plants) following a modified protocol from Zheng et al. (2017). 20 mg per sample of lyophilized roots or leaves were re-hydrated with 800 μL of W acidified with trifluoroacetic acid (pH 3.7) and the mixture was sonicated at low temperature for 15 min. The samples were then centrifuged at 15 000 g for 20 min at 4 °C, and the supernatant was filtered through a 0.22-μm PTFE syringe filter into amber autosampler vials. The identification and quantification of individual amino acids was conducted using a high-performance liquid chromatography (HPLC) with Varian ProStar system (Varian Inc., Walnut Creek, CA, USA), that included a ProStar 240 ternary pump, a ProStar 410 autosampler, a ProStar 330 photodiode array detector and a ProStar 363 fluorescence detector. A concentrated stock solution containing each analyzed amino acid in 0.1 M hydrochloric acid was prepared, and calibration standards ranging from 0.8 to 20 mg/L were prepared by diluting the stock solution in 0.1 M hydrochloric acid and filtered with a 0.45 μm filter. An internal standard (IS) stock solution containing homoserine and norvaline at 20 mg/L was also prepared in 0.1 M chloride hydroxide. For the precolumn derivatization, three reagents were prepared: 1) reagent A (120 μl of β-mercaptoethanol mixed with 3 mL of IS stock solution and 500 mg of sodium tetraphenylborate, with the final volume adjusted to 25 mL with 0.1 M borate buffer (pH 9.5)); 2) reagent B (3.5g of indoleacetic acid mixed with 50 mL 0.1 M borate buffer (pH 9.5) and pH adjusted to 9.5 with 4 M sodium hydroxide, and again with 0.1 M borate buffer (pH 9.5) until a final volume of 100 mL); 3) reagent C (225 mg 2-O-phthaldialdehyde diluted in 5 mL of methanol and 500 μl of β-mercaptoethanol until a final volume of 50 mL was reached with 0.1 M borate buffer (pH 9.5)).

For quantification, standards or samples were precolumn derivatized with 2-O-phthaldialdehyde reagent solution. The derivatization reaction took place in the autosampler, where 250 μl of reagent A and 250 μl of reagent B were added to 100 μl of standard or sample. After 3 min of reaction, 250 μl of reagent C were added, with another 3.5 min of waiting before the reaction mixture was injected (10 μL) onto the HPLC. Chromatographic separation occurred on a Chromolith® Performance RP18 encapped column (4.6x100/mm, 5/μm particle size, Merck, Darmstadt, Germany) at room temperature, using a binary mobile-phase gradient elution at a flow rate of 0.8 mL m^{-1} (Table SM1). The fluorescence detector was set at 356 nm excitation wavelength and 445 nm emission wavelength. The column was cleaned with a mixture of 60% methanol and 40% W after each batch of samples to maintain its integrity. The 32 Karat software version 8.0 was used for instrumental control and data acquisition.

Proline quantification was determined spectrophotometrically using 200 mg of ultra-frozen roots or leaf tissues (n = 3), following the method of Bates et al. (1973). In this procedure, samples were analyzed for absorbance at 520 nm. A calibration curve was prepared to calculate proline levels in the samples. The results were expressed in terms of fresh weight (FW).

2.6. Statistical analysis

A complete randomized design with twenty-one plants per treatment was implemented. The number of replicates used for each specific analysis is outlined above in the specific sections. An analysis of variance (ANOVA) was carried out to identify significant differences between treatments, and a Tukey's *post-hoc* test was conducted to determine where these differences occurred ($p = 0.05$). Results are presented as the mean ± standard error of the mean (SEM). The software IBM SPSS Statistics 26 was used to perform all the statistical analyses.

3. Results

3.1. Vascular stem anatomy

Compared to CTR plants, the hypocotyl area on the cross-section decreased in all stress treatments, ranging from 22% lower under N stress to 30% lower under N + W stress (Fig. 1a). The phloem area (Figs. 1b and 2) was only significantly impacted under single W stress and combined N + W stress, displaying a reduction of 34% and 51%, respectively, compared to CTR plants. A decrease in the xylem area (Figs. 1c and 2) relative to CTR plants was observed for all stress treatments, varying between 29% for W stress and 40% for N + W stress.

3.2. Plant growth and physiological traits

All the imposed stresses impacted plant growth (Fig. 3). Root dry

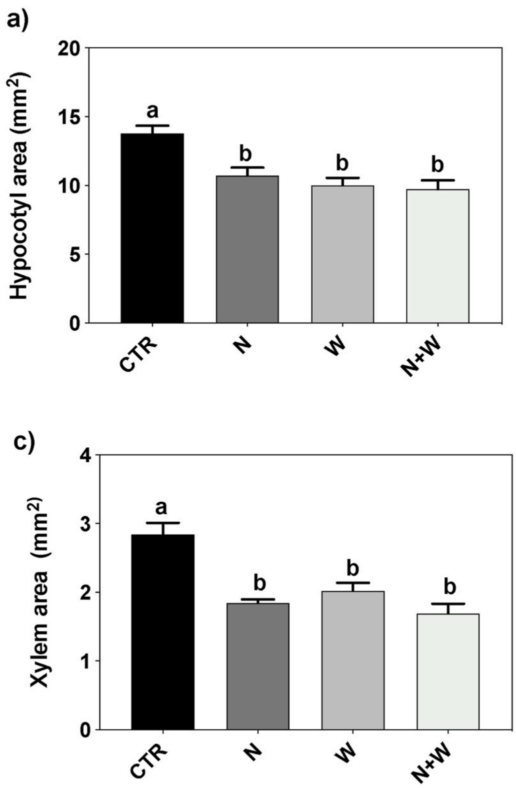

Fig. 1. Hypocotyl area (a) phloem area **(b)** and xylem area **(c)** on cross-section of the hypocotyl of tomato plants cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N + W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to Tukey's HSD test (p = 0.05).

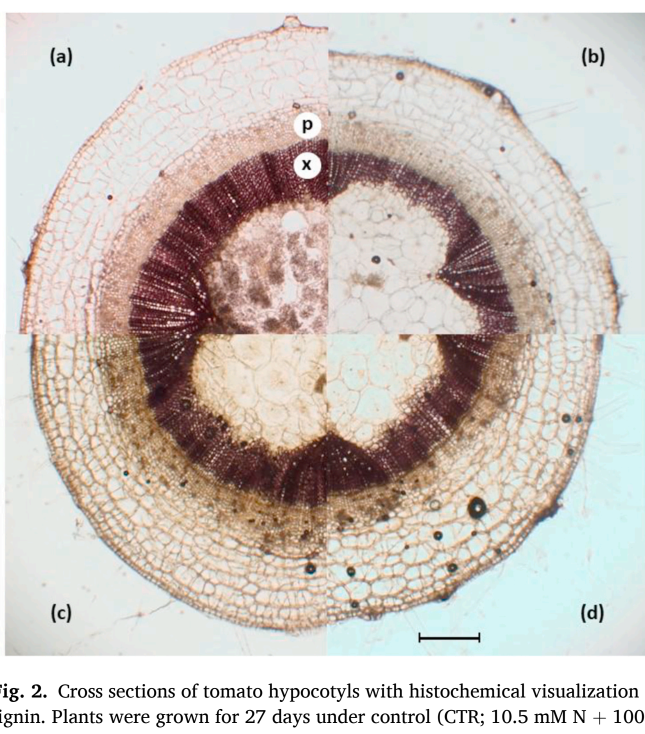

Fig. 2. Cross sections of tomato hypocotyls with histochemical visualization of lignin. Plants were grown for 27 days under control (CTR; 10.5 mM N + 100% W) **(a)**, nitrogen deficit (N; 5.3 mM N + 100%W) **(b)**, water deficit (W; 10.5 mM N + 50% W) **(c)** or combined nitrogen and water deficit (N + W; 5.3 mM N + 50% W) **(d)**. Abbreviations: p – secondary phloem, x – secondary xylem. Scale bar 500 μm.

weight remained unaffected in plants subjected to W stress, but decreased by 25% under N stress and by 50% under N + W stress, compared to CTR plants (Fig. 3a). Both N stress and W stress significantly decreased stem dry weight (by 31%), but this decrease was even more pronounced under combined stress (60% and 42% lower compared to CTR plants and single stress conditions, respectively) (Fig. 3b). Leaf dry weight responded similarly to stem dry weight when compared to CTR plants; however, no significant differences were found between N and N + W stressed plants (Fig. 3c). The root-to-shoot ratio was only significantly affected in plants under drought conditions, leading to a 17–24% higher value compared to the other treatments (Fig. 3d).

Single N stress was the treatment that mostly impacted the chlorophyll content, resulting in a 32% lower SPAD value as compared to CTR plants (Fig. 4a). Interestingly, plants exposed to the combined N + W stress exhibited intermediate chlorophyll content levels of the ones found in plants from the single stresses (Fig. 4a). The photosynthetic rate responded similarly to chlorophyll content when comparing each stress with the CTR plants; however, no significant differences were found between W and N + W stressed plants (Fig. 3c). In contrast, only the plants exposed to combined N + W stress showed significant reductions in stomatal conductance and transpiration rate, which decreased by up to 68% and 70%, respectively. No significant differences were observed among the other experimental groups (Fig. 4c and d).

3.3. Macronutrient accumulation and partitioning

Macronutrient accumulation was significantly impaired under individual or combined N stress (Fig. 5). The impact on potassium (K) and phosphorus (P) accumulation was notably more severe under the combined N + W stress compared to other conditions. However, for the remaining macronutrients, there was no significant difference between the two nitrogen stress treatments (Fig. 5). Moreover, the magnitude of

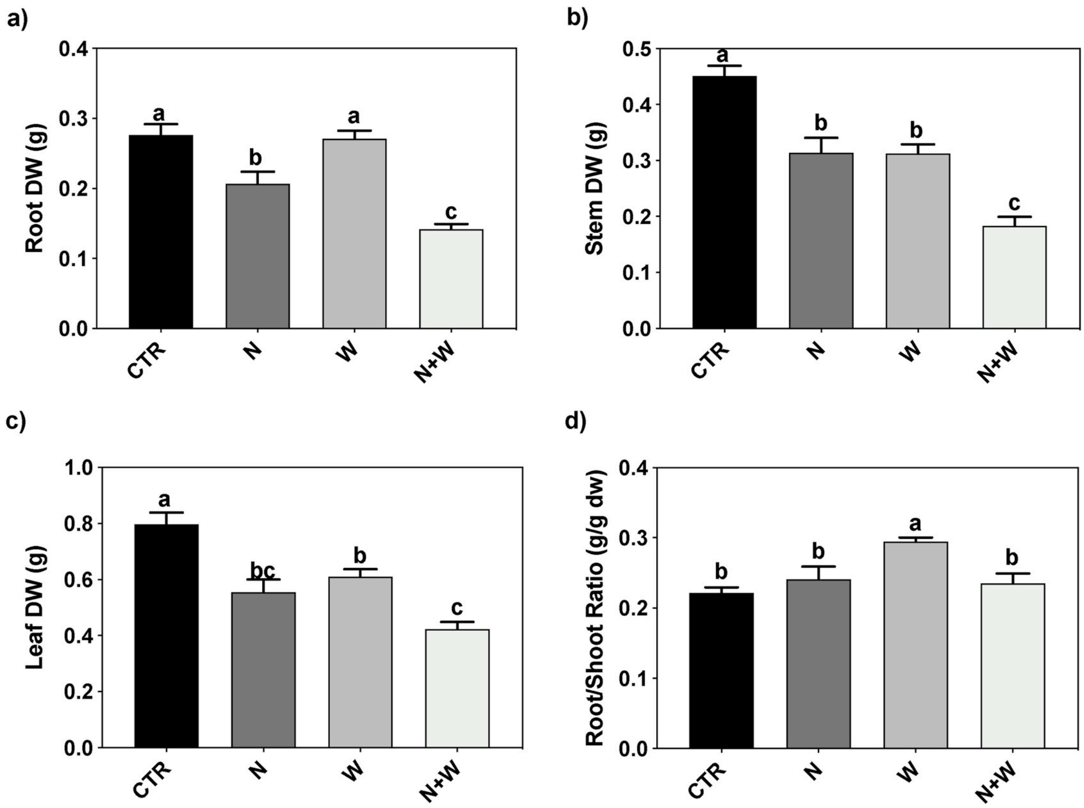

Fig. 3. Root **(a)** stem **(b)** and leaf dry weight **(c)** and root to shoot ratio **(d)** of tomato plants cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N + W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 6). Different letters above bars indicate significant differences according to Tukey's HSD test (p = 0.05).

this effect also depended on the mineral, with Ca and Mg accumulation being the least impacted by N stress (up to 21 % and 43% decrease, respectively). On the other hand, W stress alone only hindered K and P accumulation in the plant tissues, resulting in up to 56% lower levels for both nutrients.

When analyzing the mineral partitioning, among roots and the aerial part of the plant, it was found that (except for N) this was significantly impacted by some of the imposed stress conditions (Fig. 6a). Plants under N + W stress increased K allocation to shoots by up to 23% compared to plants grown under individual stress (Fig. 6b). For P partitioning, an increased allocation to roots (up to 16%) was observed in plants under N stress when compared to the other treatments, while for Ca an increased allocation to shoots (up to 9%) was observed in N stressed plants compared to the other treatments (Fig. 6c and d). On the other hand, plants experiencing W stress showed a notable increase in Mg allocation to roots (up to 85%) compared to the other treatments (Fig. 6e). Finally, it was found that NUtE was significantly reduced in plants grown under W or N + W stresses (up to 22%) (Fig. 7).

3.4. *Free amino acids and proline composition of plant tissue*

In general, the amino acids concentration, determined using HPLC method or spectrophotometry (Figure SM1 and Table 1), was lower in the roots than in the leaves. In the roots, out of the 16 quantified amino acids, only tryptophan was not significantly affected by the imposed stresses (Table 1). Most of them were significantly lower when plants were grown under single N stress than when they were grown under N + W stress [alanine (52%), asparagine (89%), aspartic acid (54%), glutamine (75%), leucine (17%), methionine (29%), phenylalanine (29%), serine (45%), threonine (24%), tyrosine (75%), valine (26%) and proline (78%)]. Interestingly, the roots of the N + W stressed plants had an amino acid profile similar to the one found in the roots of W stressed plants, except for arginine, alanine, tyrosine and phenylalanine, where plants under combined stress showed significantly higher levels of those amino acids (with 25%, 51%, 81% and 23% increase, respectively; Table 1).

In leaves, only five amino acids were not significantly affected by the imposed stresses (isoleucine, leucine, methionine, phenylalanine and threonine; Table 1). When comparing CTR plants to those grown under single or combined N stress, the latter groups exhibited significantly lower levels of asparagine (up to 75%), aspartic acid (up to 31%), glutamine (up to 40%), serine (up to 34%), and valine (up to 35%). Conversely, proline concentration was significantly higher in these stressed plants (up to 77%) (Table 1). On the other hand, single W stress, only proline and tryptophan levels, were significantly higher (96% and 14%, respectively) compared to CTR plants. Contrastingly to roots, the amino acid profile of leaves from plants subjected to N + W stress generally resembled those under single stress conditions, except for asparagine, aspartic acid, arginine, glutamine, and serine, which levels were more similar to those of N-stressed plants (Table 1).

4. Discussion

This study provides novel insights into the processes underlying plant responses to combined N and W stress focusing on the vascular plant anatomy, nutrient partitioning and amino acids profile, both at the root and shoot level. Various anatomical changes, such as reduction in the size of phloem and xylem vessels, decreased thickness of cortical cell layer, as well as changes in surface structures, including the length of epidermal cells, have been observed as a consequence of drought stress in plants (Shafqat et al., 2021). The size reduction of the vascular tissues has been pointed out as a direct response to stress conditions and plays a functional role in acclimation to drought. For instance, plants with decreased xylem vessel diameter showed a higher survival rate in

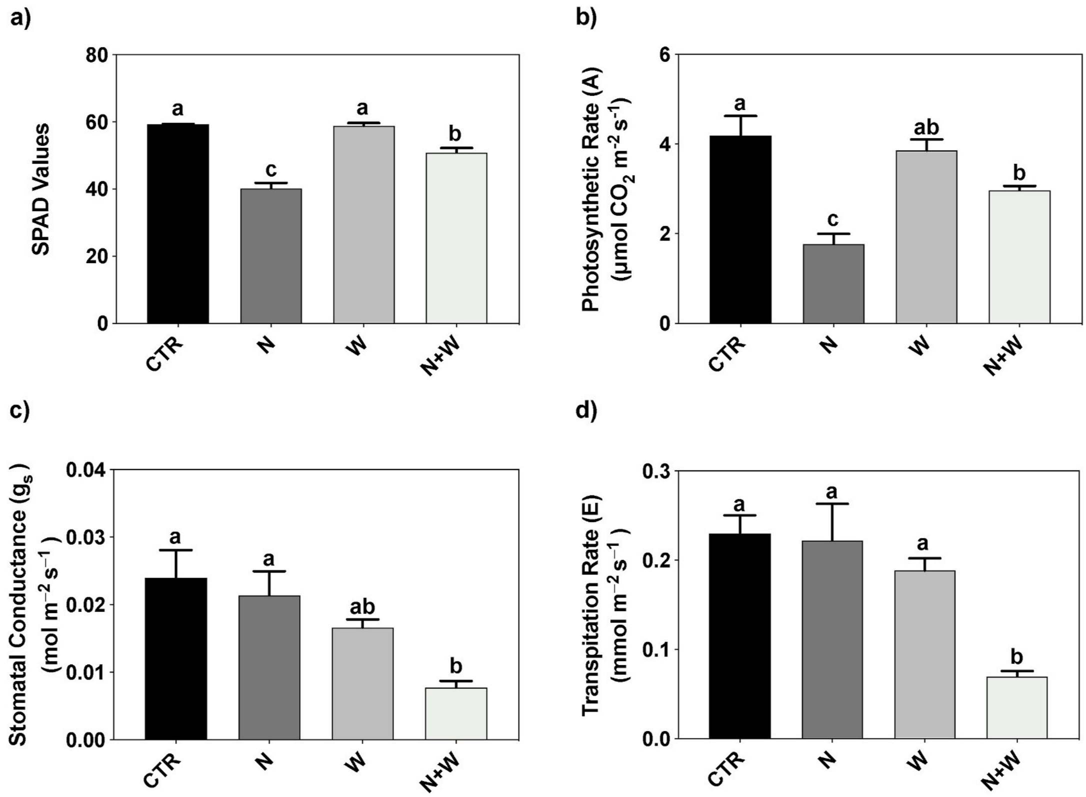

Fig. 4. Chlorophyll content (SPAD values) **(a)** photosynthetic rate (**b**) stomatal conductance **(c)** and transpiration rate (**d**) of tomato plants cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N + W; 5.3 mM N + 50% W).Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to Tukey's HSD test (p = 0.05).

comparison to plants with larger xylem vessel diameter under drought conditions (Qaderi et al., 2019; Shafqat et al., 2021). Furthermore, xylem thickness and xylem cell layers are reduced under low N supply (Song et al., 2019). Moreover, low N supply together with drought stress was shown to cause a larger decrease in xylem thickness and number of xylem cell layers of *Populus alba*, compared to control conditions or to drought stress with adequate N supply (Song et al., 2019). However, in our study the analyzed anatomical traits were affected to the same extent by the three different stresses, all resulting in a significant decrease of the cross-sectional hypocotyl area, as well as reduced xylem and phloem area (Figs. 1 and 2).

Numerous studies have consistently demonstrated that N or drought stresses significantly impact tomato performance, resulting in reduced growth (as recently reviewed by Machado et al., 2022). Recent research has examined the response of various tomato genotypes, including wild relatives, landraces, heirlooms and hybrids to combined N and W stresses. While some variability has been observed, the consensus is that tomato is generally susceptible to these dual stresses (Machado et al., 2024b; Ruggiero et al., 2022). In our study, both stresses, either single or combined, led to a decrease in leaf and stem dry weight, with the combination of both stresses showing a more substantial reduction (Fig. 3b and c). Root dry weight was also decreased in the presence of N and N + W stress, but not under W stress (Fig. 3a). Interestingly, plants subjected to W stress exhibited an increased root-to-shoot ratio, indicating a greater allocation of resources to roots (Fig. 3d). Indeed, since W deficit has a more pronounced impact on shoot growth compared to root growth, the root-to-shoot ratio typically increases under drought conditions (Du et al., 2018; Moles et al., 2018). This response is a common adaptation by plants to cope with W scarcity, and it likely contributed to better plant performance compared to those under N + W stress. This was evidenced by higher chlorophyll content and photosynthetic capacity (Figs. 3a and 4a, b). Conversely, plants grown under N + W stress failed to develop their root system adequately to access W in deeper substrate layers, likely experiencing a more severe W stress. This is reflected in the closure of stomata as a mechanism to reduce transpiration rates (Figs. 3a and 4b, c, d). Indeed, stomatal closure is one of the initial responses to drought stress in most plants, serving as a mechanism to minimize W loss through transpiration pathways (Pirasteh-Anosheh et al., 2016). On the other hand, N-deficient plants exhibited a reduction in photosynthetic capacity due to the dependence of the photosynthetic machinery on N partitioning (Bassi et al., 2018). In our study, plants subjected to N stress displayed the lowest photosynthetic rate, likely attributable to N remobilization, a common process under N deficiency (Sakuraba, 2022), which resulted in lower chlorophyll content (Fig. 4a and b). However, plants subjected to N + W stress employed a different N remobilization strategy as in this case, the decrease in the chlorophyll content was less pronounced, resulting in a greater photosynthetic rate compared to plants experiencing single N stress (Fig. 4a and b).

In general, plants grown under stressful conditions exhibited lower macronutrient accumulation compared to CTR conditions, with this decrease being more pronounced in plants subjected to N stress, whether alone or in combination (Fig. 5). This likely resulted from the underdevelopment of the plant's root system under N limitation in comparison to those grown under CTR or W stress conditions (Fig. 3a). Roots play an important function in W and nutrient uptake, and both N or W stresses are primarily perceived in this structure (Tajima, 2021). Plants subjected to W stress managed to maintain N accumulation in the root tissues at the same level as the CTR plants (Fig. 3a and d and Fig. 5a), as a higher N accumulation may have facilitated coping with drought stress by producing defense molecules such as osmolytes. In fact, the NO_3^- molecule itself is known to have an osmoregulatory function, as

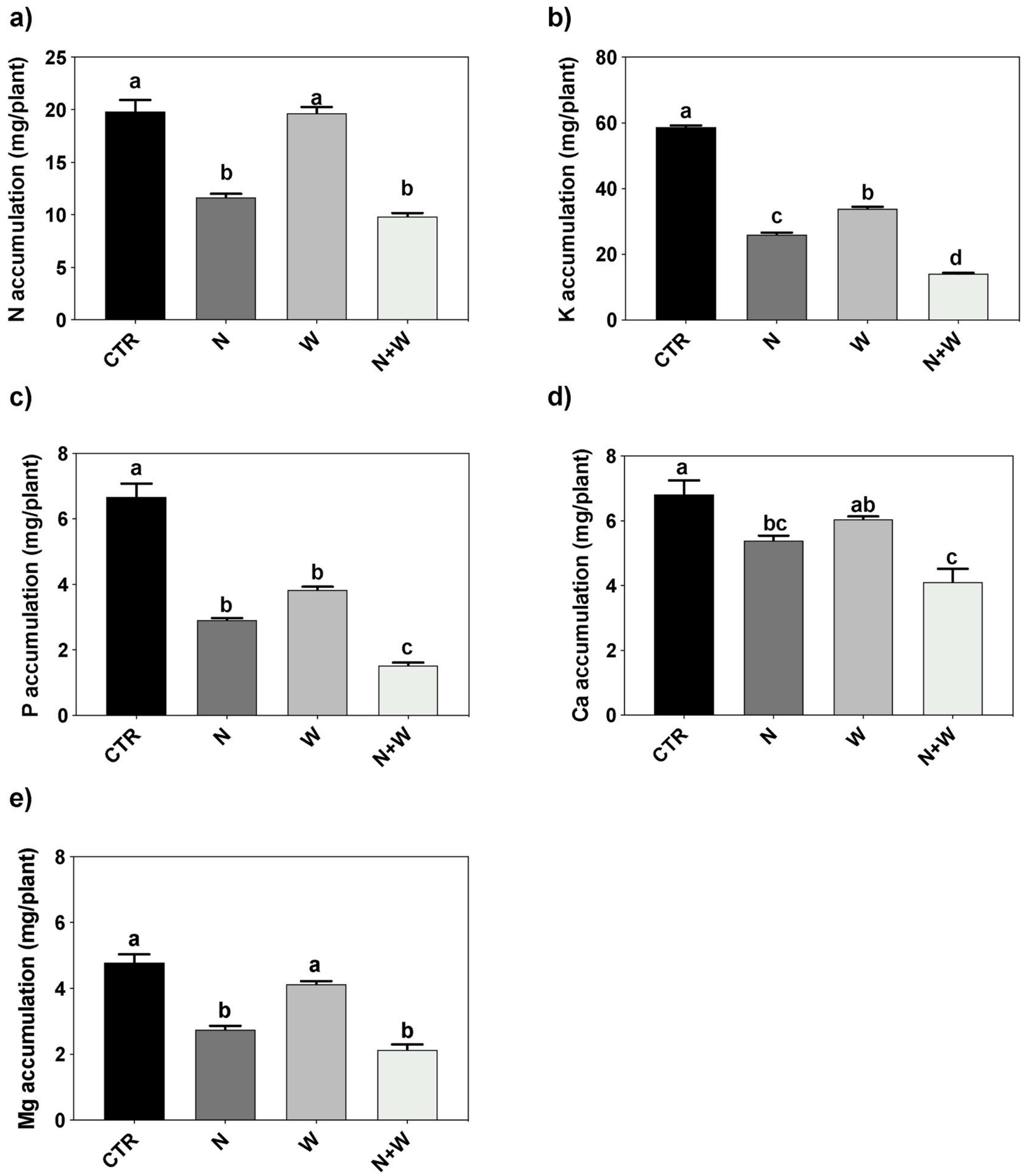

Fig. 5. Nitrogen (N) **(a)** potassium (K) **(b)** phosphorus (P) **(c)** calcium (Ca) **(d)** and magnesium (Mg) **(d)** accumulation per tomato plant cv Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N + W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to Tukey's HSD test (p = 0.05).

demonstrated by Handa et al. (1983), who attributed 4.5% of the total osmotic potential to this molecule in tomato plants. On the other hand, research has demonstrated that NO_3^- uptake can stimulate K^+ uptake, implying that the NO_3^- ion may play a role in facilitating K^+ absorption and transport (Zhang et al., 2010). This hypothesis aligns with our results, as in plants under NO_3^- deficiency (N and N + W stress), K accumulation was significantly decreased (Fig. 5b). However, plants grown under N + W stress altered K partitioning towards the shoots (Fig. 6b). K has a known effect on osmoregulation, being responsive for photosynthesis regulation through osmotic balances and changes in guard cell turgor, affecting stomatal and mesophyll conductance (Tränkner et al., 2018). This corresponds with the decrease in stomatal conductance found in plants grown under combined N + W stress (Fig. 4c). On the other hand, P has poor mobility in soil not being easily accessible by plant roots (Holford, 1997). This lower accessibility is further aggravated by a smaller root system and lower soil moisture, which impairs nutrient uptake (Hu et al., 2007; Wang et al., 2006). P accumulation was lower in all stresses, especially under the N + W stress where plants exhibited lower root dry weight (Figs. 3a and 5c). In response to stress signals Ca serves as a secondary messenger, and its transport to the shoot depends on high transpiration rates (Gilliham et al., 2011; Kumar et al., 2014). In the present study, plants subjected to N deficiency may have activated that defense mechanism. However, when N stress was applied in combination with drought stress, plants were unable to translocate Ca to the shoots due to a lower transpiration rate (Figs. 4d and 6d). Mg is suggested to have a role in increasing root growth and, consequently, root uptake of nutrients and W (Li et al., 2020). Therefore, the lower partitioning of Mg to shoots in plants subjected to W stress may be the cause of the improved root system in these plants, which might have enhanced the W uptake and consequently

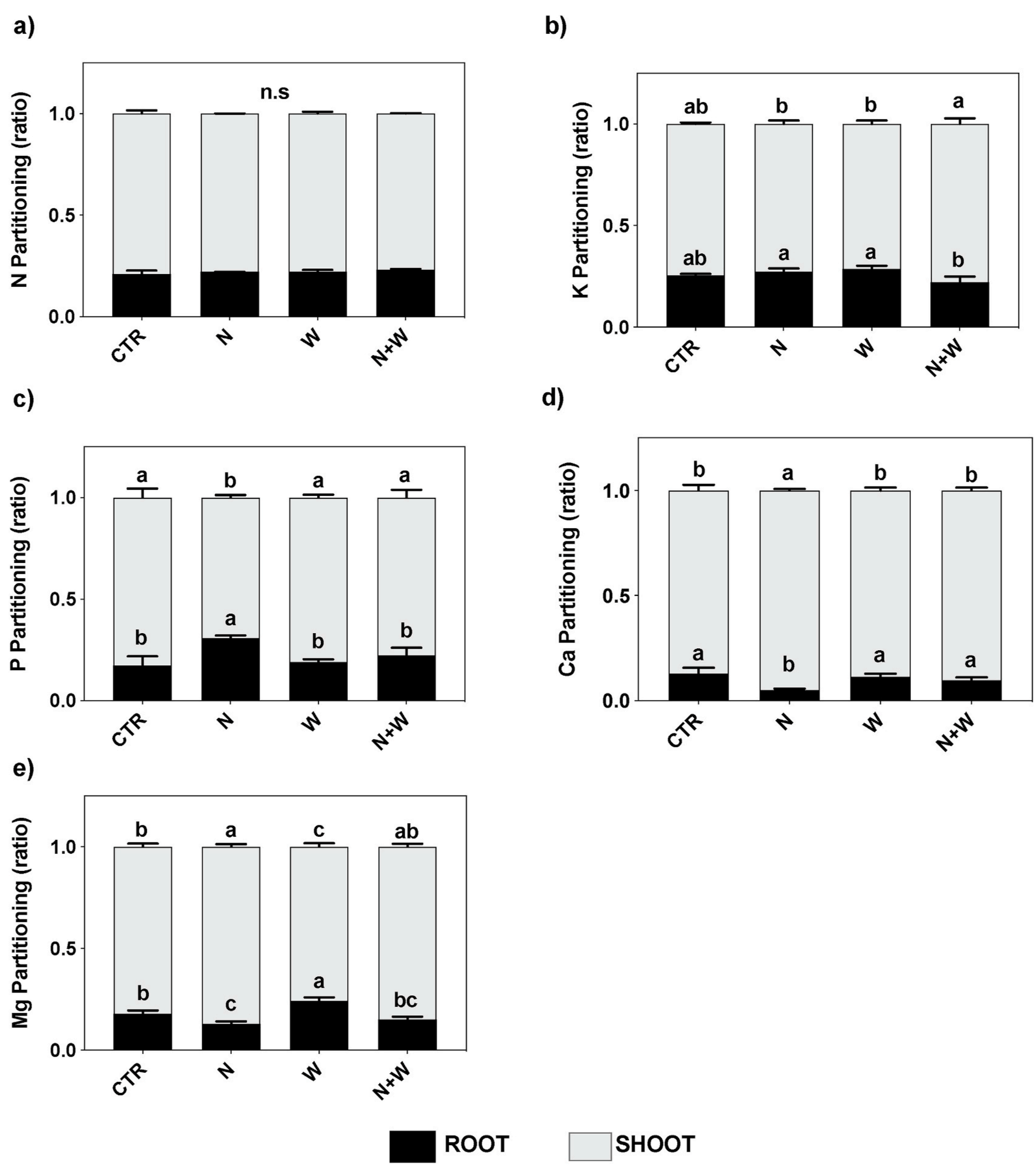

Fig. 6. Nitrogen (N) **(a),** potassium (K) **(b)** phosphorus (P) **(c)** calcium (Ca) **(d)** and magnesium (Mg) **(d)** partitioning among tomato cv. Micro-Tom roots and shoots grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N + W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to Tukey's HSD test (p = 0.05).

mitigated the negative effects of drought (i.e. maintenance of chlorophyll content, photosynthesis, stomatal conductance, and transpiration rate) (Figs. 3a, 2 and 6e). Interestingly, Mg partitioning to shoots is increased in N stress (Fig. 6e). N and Mg might act antagonistically, as it has been found that Mg is boosted by N deficit and decreased by N supply in citrus plants (Heo and Park, 2022).

Understanding how plants accumulate and use N is crucial for comprehending their responses to stress conditions. In this study, we found that plants grown under N stress allocated more N towards growth, leading to a higher NUtE. In contrast, plants subjected to drought (both single and combined) utilized N to produce drought-related compounds (Fig. 7). As the initial stable products of inorganic N assimilation, the amino acids' concentration is often impaired under N deficiency (Nunes-Nesi et al., 2010). Likewise, our study found that plants experiencing N stress generally exhibited lower levels of free amino acids, particularly in the roots (Table 1). Interestingly, when N stress was combined with W stress, this decrease in free amino acids was only observed in the leaves. Free amino acids are known to act as osmolytes helping the plants to increase the osmotic potential under drought stress (Ozturk et al., 2021). A highly abundant amino acid is glutamate which serves as an important precursor for other amino acids such as proline, one of the most conspicuous osmolytes (Alfosea-Simón et al., 2020; Kumar et al., 2017). In our study, we observed an increase in proline synthesis upon drought (both single and combined stress), with the roots exhibiting the most pronounced response. This rise in proline was accompanied by a decrease in glutamate concentration when plants experienced either single stress, but not when both stresses were combined (Table 1). Furthermore, proline has been shown to not only function as an osmoprotectant but also to scavenge reactive oxygen species such as those induced by drought (Sharma et al., 2011). Our results indicated higher proline synthesis under W stress, suggesting a possible response mechanism to cope with oxidative stress, primarily

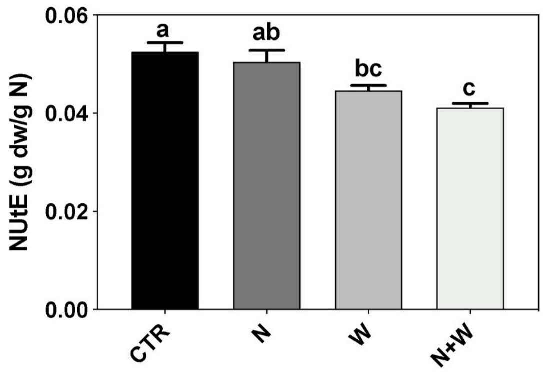

Fig. 7. Nitrogen utilization efficiency (NUtE) of tomato plants cv. Micro-Tom grown for 27 days under control (CTR; 10.5 mM N + 100% W), nitrogen deficit (N; 5.3 mM N + 100%W), water deficit (W; 10.5 mM N + 50% W) or combined nitrogen and water deficit (N + W; 5.3 mM N + 50% W). Data presented are mean ± SEM (n = 3). Different letters above bars indicate significant differences according to Tukey's HSD test (p = 0.05.

occurring in the roots. Furthermore, both W and N + W stresses increased glutamine synthesis, which is in contrast to the single N stress, where a decrease in glutamine was observed. Additionally, arginine showed a specific increase in the roots of plants under combined stress (Table 1). Arginine stands out for having the lowest C:N ratio among amino acids, with glutamate serving as an intermediate product in its biosynthetic pathway (The et al., 2020; Winter et al., 2015). Arginine's function in plant N distribution and recycling is well-established, and it also plays a key role as a precursor in the biosynthesis of polyamines and nitric oxide. These molecules, in turn, are essential messengers involved in various physiological and biochemical processes, enabling plants to acclimate to stressful conditions (Winter et al., 2015). Although plants under single W stress invested more in glutamine and proline biosynthesis to enhance their osmotic potential, those under combined stress utilized glutamate to produce both amino acids and arginine. This suggests a strategic response aimed at improving osmotic potential and enhancing N distribution and recycling (Table 1).

Asparagine is another essential amino acid involved in N transport and storage that has been found to increase under drought conditions (Lea et al., 2007). In the present study, we observed a significant rise in asparagine in the roots of plants subjected to W stress (either single or combined) (Table 1). In the majority of the plant species, N partitioning occurs through amino acids, with root-synthesized amino acids being transported via the xylem to the mature source leaves. Here, these amino acids serve as a N source for constructing the photosynthetic apparatus (Perchlik and Tegeder, 2018). Asparagine has been identified as a major N transport compound in the xylem from the root to the leaves (Lea et al., 2007). The observed increase in root-synthesized arginine and asparagine in plants subjected to N + W stress may explain their ability to mitigate the adverse effects of N deficiency on the photosynthetic machinery (Fig. 4a and b).

In addition to arginine, the levels of alanine, tyrosine and phenylalanine were found to be notably higher in the roots of N + W-stressed plants. These amino acids, along with arginine, have been previously reported to play a role in countering the osmotic stress in both tolerant and sensitive rice cultivars (Matsunami et al., 2020). Muscolo et al. (2015) also showed higher levels of phenylalanine and tyrosine in two drought-sensitive accessions of lentil, which was linked to the initiation of root lignification, a mechanism used to mitigate the damage caused by drought stress. Many secondary metabolites are synthetized as a protective response to oxidative stress damage, with a significant portion derived from the aromatic amino acids phenylalanine and tyrosine through the phenylalanine ammonia-lyase (PAL) pathway. These findings suggest that plants under N + W stress specifically alter their amino acids pool to cope with both abiotic stresses.

5. Conclusions

This study highlights the interplay between mineral partitioning and amino acids profile, at root and shoot level, and their pivotal role in the distinct physiological and biochemical responses of tomato plants' to combined N and W stress, as compared with single stresses. When facing combined N and W stress, plants primarily exhibited an increased root synthesis of several amino acids. Notably, the heightened levels of arginine and asparagine at the root level contributed to lower N remobilization from the photosynthetic machinery, as demonstrated by the lower reduction in chlorophyll content and photosynthetic rate compared to plants experiencing single N stress. Furthermore, plants subjected to combined stress presented an increased reallocation and synthesis of osmolytes such K^+ and proline. This increase played a role in stomatal closure, resulting in a lower transpiration rate when contrasted with plants facing single N stress. Specific amino acids associated with osmoregulation, including alanine, tyrosine and phenylalanine, were also particularly higher in the roots of plants grown under N + W stress. Conversely, when facing single W stress, plants invested relatively more in the root development, stimulating the uptake of W and nutrients compared to plants exposed to combined stress. This allocation strategy

Table 1
Free amino acids and proline concentration of tomato plants, subjected to four growth conditions control (CTR), nitrogen stress (N), drought stress (W) or combined nitrogen and drought stress (N + W) over 27 days after transplanting.

Amino acid (μg/g DW)	ROOT					LEAVES				
	CTR	N	W	N + W	*p*-value	CTR	N	W	N + W	*p*-value
Alanine	73.0 ± 5.4^{a}	28.3 ± 6.8^{c}	39.2 ± 6.0^{b}	59.3 ± 7.5^{a}	**0.0051**	263 ± 25^{ab}	144 ± 24^{b}	317 ± 36^{a}	196 ± 29^{ab}	**0.0130**
Asparagine	135 ± 12^{b}	47.4 ± 4.1^{c}	406 ± 5.0^{a}	446 ± 46^{a}	**<0.0001**	66.3 ± 6.3^{a}	16.3 ± 1.2^{b}	63.2 ± 6.3^{a}	22.6 ± 5.9^{b}	**0.0002**
Aspartic Acid	412 ± 44^{a}	204 ± 21^{b}	359 ± 14^{a}	440 ± 15^{a}	**0.0009**	858 ± 23^{a}	595 ± 34^{b}	875 ± 59 ^{a}	595 ± 20 ^{b}	**0.0007**
Arginine	38.6 ± 5.4^{bc}	29.1 ± 3.3^{c}	51.1 ± 5.7^{b}	63.8 ± 3.4^{a}	**0.0035**	87.3 ± 9.3^{ab}	49.0 ± 7.9^{b}	112 ± 8.7^{a}	61.2 ± 9.5^{b}	**0.0042**
Glutamate	369 ± 27^{a}	206 ± 20^{b}	214 ± 5.7^{b}	304 ± 43^{ab}	**0.0083**	469 ± 31^{ab}	346 ± 35^{a}	557 ± 44^{b}	383 ± 47^{ab}	**0.0236**
Glutamine	541 ± 40^{b}	228 ± 23.6^{c}	911 ± 3.3^{a}	906 ± 63^{a}	**<0.0001**	428 ± 21^{a}	260 ± 14^{b}	507 ± 23^{a}	257.0 ± 25^{b}	**<0.0001**
Isoleucine	77.9 ± 4.2^{a}	45.10 ± 6.8^{b}	76.0 ± 4.5^{a}	57.3 ± 3.5^{ab}	**0.0041**	107 ± 9.9	78.2 ± 8.1	100 ± 0.8	93.6 ± 5.8	0.0958
Leucine	102 ± 9.8^{a}	65.6 ± 5.8^{b}	94.8 ± 7.0^{a}	78.8 ± 5.1^{a}	**0.0269**	116 ± 20	123 ± 7.9	175 ± 17	149 ± 10	0.0749
Methionine	45.4 ± 2.2^{ab}	36.5 ± 1.8^{b}	50.8 ± 1.7^{a}	51.4 ± 3.3^{a}	**0.0068**	39.6 ± 3.0	38.0 ± 2.2	40.2 ± 0.8	39.3 ± 3.2	0.9299
Phenylalanine	72.4 ± 2.2^{ab}	59.1 ± 1.6^{c}	68.1 ± 0.7^{bc}	83.5 ± 4.2^{a}	**0.0009**	144 ± 13	133 ± 7.5	155 ± 20	137 ± 16	0.7404
Serine	126 ± 7.1^{a}	68.1 ± 5.8^{c}	102 ± 3.9^{b}	123 ± 1.6^{ab}	**0.0001**	173 ± 6.9^{a}	121 ± 7.1^{b}	200 ± 3.5^{a}	115 ± 10^{b}	**<0.0001**
Threonine	110 ± 7.3^{a}	75.3 ± 5.4^{b}	97.9 ± 2.5 ^{a}	98.7 ± 4.7 ^{a}	**0.0104**	121 ± 7.4	93.3 ± 9.5	108 ± 14	110 ± 13	0.4546
Tyrosine	18.5 ± 1.1^{a}	5.24 ± 0.5^{c}	11.8 ± 2.0^{b}	21.3 ± 0.2^{a}	**<0.0001**	147 ± 14^{ab}	96.2 ± 10^{b}	166 ± 12^{a}	116 ± 15^{ab}	**0.0226**
Tryptophane	99.2 ± 4.2	106 ± 3.0	104 ± 4.1	104 ± 8.9	0.8656	298 ± 10^{b}	304 ± 3.7^{ab}	341 ± 1.4^{a}	274 ± 13^{b}	**0.0038**
Valine	91.2 ± 0.9^{a}	60.9 ± 3.4^{b}	83.3 ± 4.7^{a}	82.4 ± 5.1^{a}	**0.0032**	155 ± 14^{a}	98.6 ± 4.6^{b}	142 ± 4.7^{ab}	106 ± 13.7^{b}	**0.0111**
Proline	69.5 ± 12^{b}	76.7 ± 20^{b}	427 ± 35^{a}	341 ± 18^{a}	**<0.0001**	118 ± 12^{c}	175 ± 4.1^{b}	232 ± 2.4^{a}	209 ± 21^{ab}	**0.0009**

Data presented are mean ± SEM (n = 3). Different letters indicate significant differences according to Tukey's HSD test (p = 0.05).

allowed them to maintain essential physiological processes, such as photosynthesis. Hence, it is concluded that tomato plants employed distinct mechanisms for N remobilization and osmoregulation in response to single or combined stresses. Additionally, maintaining amino acid and nutrient balance plays a crucial role in these acclimation processes.

Author contribution statement

The experimental design and conception of the study were led by SMPC, MWV and EH. JM was responsible for seed germination, plant growth and sampling, as well as the evaluation of morphological and physiological attributes. APG collaborated with JM in quantifying nutrients and amino acids. BB, MV, DK and AK contributed to the anatomical analysis. All authors were involved in data analysis and interpretation. JM drafted the manuscript, and all authors provided critical revisions and approved its final version. As the supervisor and main coordinator of this research line, SMPC assumes the overall responsibility of this study, from inception to publication.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joana Machado reports financial support was provided by Foundation for Science and Technology. Ana Patricia Goncalves reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2025.109649.

Data availability

Data will be made available on request.

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