Leaves and roots metabolomic signatures underlying rootstock-mediated water stress tolerance in grafted pepper plants

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ABSTRACT

Grafting onto pepper rootstock NIBER® is an effective strategy to mitigate water stress effects on the grafted variety. In this work, we comparatively explored the metabolomic responses to water stress in the pepper variety "Maestral F1" (V) grafted onto NIBER® (V/N) and self-grafted (V/V) by untargeted metabolomics on leaves and roots. Leaf water status was also evaluated by relative water content (RWC) and gas exchange measurements. Under water stress, the V/N water use efficiency (WUE) and leaf RWC were higher than V/V, in agreement with major stomata closure and water retention in leaves. V/N showed a tolerance response, which was manifested in the untargeted metabolomic analysis. NIBER® modulated the grafted variety response to water stress as reflected in the differential metabolomic profiles in leaves and roots. The V/N-enriched metabolic pathways showed that the NIBER® response to water stress involved cutin and suberin biosynthesis, which act as protection layers, and jasmonic acid (JA) and jasmonates biosynthesis to favor signaling pathways. NIBER® did not induce flavonols and chlorophyll b synthesis, but likely promoted anthocyanins biosynthesis and maintained an undisturbed chlorophyll a:chlorophyll b ratio. Moreover, NIBER® increased vitamin B6, anthocyanins and stearic acid concentration in the variety leaves, whereas siroheme content rose in roots to improve nitrogen assimilation. Further studies are required to understand the contribution of secondary metabolites, such as phenylpropanoids, glycoalkaloids, and nitrogen-containing secondary metabolites, to NIBER® water stress tolerance.

1. Introduction

Drought is one of the most threatening natural phenomena to affect agriculture. In fact, for the main crops, yield losses can reach 50% (Wang et al., 2003). Due to climate change, droughts have become more frequent, severe and prolonged (IMF, 2022). According to the FAO (2023), 40% of the world population depends on agriculture to survive. This devastating situation threatens global food security and could result in food scarcity in the near future (Foley et al., 2011).

The final consequence of drought stress for plants is significant productivity and yield losses, but processes, such as nutrient uptake, CO_2 net assimilation and radiation use efficiency, are negatively affected by water deficit (Ullah et al., 2019). The presence of water stress in soil is sensed by roots and information is transmitted through the vascular system from roots to shoots (Takahashi et al., 2020). The communication between both organs is mediated by hormonal signaling, secondary messengers like reactive oxygen species (ROS) and calcium ion, small solutes, mRNAs, small RNAs and proteins (Albacete et al., 2014; Salehi-Lisar et al., 2016; Kaur and Ashtir, 2017; Rouphael et al., 2018).

Grafting onto tolerant rootstocks is a proven strategy to overcome plant water stress (de Oliveira Sousa et al., 2022). Studies have shown promising results when employing tolerant rootstocks under water stress conditions (Schwarz et al., 2010; Kumar et al., 2017) associated with vigorous roots from the rootstock (Yao et al., 2016) and mechanisms such as: active osmotic adjustment (Penella et al., 2014), enhancement of the antioxidant defense system (Liu et al., 2014), the ability to sustain photosynthesis (López-Serrano et al., 2019), regulation of water-stress related hormonal network (Padilla et al., 2023a), among other

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strategies.

In line with all this, "omics" sciences have been recently considered a powerful tool to uncover changes in the rootstock functional properties that affect the scion under environmental stresses and to understand the mechanisms that underlie tolerance (or sensitivity) in grafted plants to a greater extent (Lu et al., 2020). In particular, untargeted metabolomics spots the differential accumulation of metabolites in both the rootstock and scion, including the biochemical changes that occur under water stress (Kumar et al., 2015; Lucini et al., 2020). The metabolomic profile in plants includes a wide diversity of low-molecular-weight compounds, including sugar alcohols, polyamines, phenols, lipids, carbohydrates, amino acids, among others. Many of them are important and essential compounds for plant acclimation under stress conditions (Fraser and Chapple, 2011; Zandalinas et al., 2017). Variations in primary metabolism are easily recognized as a response to abiotic stresses (i.e., sugars, polyols, amino acids), yet secondary metabolism alterations are more specific of stress conditions and species, and should not be left behind (Arbona et al., 2013).

Accordingly, grafting has been reported as a factor for regulating the metabolome of a plant (Aslam et al., 2021). Some studies evaluate the metabolomics of low-temperature-exposed pepper rootstock roots (Aidoo et al., 2019) or the volatile profile of pepper-grafted fruit (Gisbert-Mullor et al., 2020a). Other studies show the effects of drought on the metabolic profile of pepper fruit (Borràs et al., 2021) or pepper roots and shoots (Vílchez et al., 2018). However, information about the metabolic changes that lead to grafted pepper plants' increased tolerance to overcome water stress is lacking, with very little evidence in tomato and citrus (Kalozoumis et al., 2021; de Oliveira Sousa et al., 2022). In our previous works, by a breeding program we obtained a hybrid pepper (NIBER®, F1) with greater vigor and more growth to be used as a rootstock in grafted plants. Then we tested the hybrid under water stress conditions in the field (Gisbert-Mullor et al., 2020b) to demonstrate that grafting a pepper cultivar onto an appropriate rootstock (NIBER®) overcomes the negative effects of sustained water stress conditions. All this was attributed to a less marked reduction in shoot dry weight (DW), which allowed greater whole photosynthesis by maintaining sink activity and higher plant biomass production and fruit yields than ungrafted plants.

By bearing this background in mind, in this study we hypothesize that stress mitigation in grafted plants using a tolerant rootstock (NIBER®) compared to self-grafted plants under water shortage conditions could be ascribed to a distinct modulation of metabolic profiles. Accordingly, the present work aimed to evaluate and identify traits of the tolerant rootstock NIBER® and the scion associated with early metabolic responses, and to elucidate biochemical bases under water stress conditions by an untargeted metabolomics approach.

2. Material and methods

2.1. Plant material

Pepper variety "Maestral F1" (V) (sweet pepper, California-type, Semillas Fitó, Spain) was self-grafted and grafted onto the pepper hybrid rootstock named NIBER® (N) (*Capsicum annuum* x *C. annuum*), which is tolerant to water stress (Gisbert-Mullor et al., 2020b, 2023). Two plant combinations were used: self-grafted V plants (V/V) and V grafted onto N (V/N). Early in March, the seeds of V and N were sown in 104 seedling trays filled with a peat-based substrate for germination. After 2 months, the grafted plant combinations were performed using the tube-grafting method (Penella et al., 2015). Plants were maintained in a chamber with relative humidity above 95% and air temperature around 28-29° C for a 4-6-day period (Penella et al., 2014).

2.2. Hydroponic greenhouse conditions

Three weeks after grafting, seedlings were removed from substrate and their roots were cleaned before being placed in 2 L polyethylene pots covered with aluminum sheets. Pots were filled with a nutrient solution (Sonneveld and Voogt, 2009) and were artificially aerated with an air pump. Electrical conductivity and pH were 2.1 dS m^{-1} and 6.7, respectively. After allowing plants to acclimatize for 7 days, the water stress treatment was started by adding 5% PEG (polyethylene glycol) 8000 (Sigma Co.) to nutrient solution. The osmotic potentials of solutions were measured by a vapor osmometer (Digital osmometer, Wescor, Logan, USA), with -0.55 Mpa for 5% PEG and -0.05 Mpa for the control solution (0% PEG). Plants remained in a Venlo-type greenhouse under natural light conditions (610-870 μmol m^{-2} s^{-1}) for 48 h. Temperature and relative humidity ranges were 21-25°C and 52-72%, respectively. The layout was completely randomized, with 10 plants per combination (V/V and V/N).

2.3. Leaf water status

Gas exchange measurements were performed using a LICOR-6400 infrared gas analyzer (LI-COR, Nebraska, USA) 48 hours after PEG (5%) addition. The net CO_2 assimilation rate (A_N), stomatal conductance (gs) and transpiration rate (E) were determined on fully expanded leaves. Parameters A_N/gs and A_N/E were calculated as intrinsic WUE and instantaneous WUE, respectively.

Relative water content (RWC) was expressed as: RWC (%) = (FW – DW)/(TW – DW) × 100; FW is leaf fresh weight, DW is dry weight after drying leaves at 65°C for 72 h and then weighed; TW is the turgid weight (TW) of leaves submerged in water for 24 h.

Samples were tested by a two-way analysis of variance (ANOVA) with Statgraphics Centurion XVIII (Statgraphics Technologies, Inc., The Plains, Virginia, USA). Fisher's Least Significant Difference test (LSD) was performed at p <0.05.

2.4. Untargeted UHPLC-QTOF-HRMS metabolomics analysis

The samples (leaves and roots) for the metabolomics analysis were taken 48 h after water stress treatment had begun. Measurements were taken with fully expanded mature leaves and 2 cm from distal roots. Samples were frozen in liquid nitrogen immediately after harvest, conserved at -80°C and later freeze-dried. The layout was randomized with five samples of independent plants.

Sample preparation was conducted as previously reported (Formisano et al., 2021). Briefly, the freeze-dried samples were extracted with an Ultra-Turrax (Ika T-25, Staufen, Germany) at a proportion of 1 g of sample: 20 mL of buffer containing methanol/water solution (80:20, v/v) and 0.1% HCOOH. Extracts were centrifuged (12000 x *g*) and filtered through a 0.22 μm cellulose membranes directly into amber vials for the untargeted metabolomics analysis. The metabolomics analysis was performed by an ultrahigh performance liquid chromatography coupled with quadrupole-time-of-flight high-resolution mass spectrometer equipped with an electrospray ionization source (UHPLC-QTOF-HRMS; G6550, Agilent®, Santa Clara, CA, USA). The analysis was run as previously optimized (Formisano et al., 2021). Briefly, reverse-phase chromatographic separation was done in an Agilent pentafluorophenylpropyl (PFP) column (2.0 × 100 mm, 3 μm) (Santa Clara, CA, USA) with a water-acetonitrile gradient elution from 6% to 94% in 33 min. High-resolution mass acquisition was conducted in the full scan mode within the m/z 100-1200 (1 Hz) range with positive polarity and a resolution of 30000 FWHM.

2.5. Metabolomics data processing

Before compound identification, data processing steps were undertaken. Initially, the raw data from UPLC-QTOF-MS were subjected to rigorous processing with specialized software for the qualitative analysis. The metabolomics dataset was computationally processed with the MassHunter Profinder software (v. 10.0, Agilent®) by applying the

"find-by-formula" algorithm. Peak finding was accurately extracted by retention time (RT) and accurate mass alignment within 1-32 min and 100-1200 m/z, respectively. RT tolerance was set at 0.05 min and mass accuracy tolerance at 5 ppm.

Next, a filtration process was implemented to discern and retain only those prevalent compounds across a substantial portion of the dataset to, thus, ensure the selection of the most representative entities. Dataset reduction was applied by considering those features found in at least 80% of replicates in the same group. Feature annotation was performed by accurate mass determination on the full scan MS data when considering the isotope pattern (including monoisotopic accurate mass, isotopic pattern and isotopic accurate spacing) in compliance with Level 2 of identification (putatively annotated compounds) according to the Metabolomics Standard Initiative (MSI) (Salek et al., 2015) and manually curated for the specific organism. For compounds annotation, a specific plant-centered database was used (PlantCyc 12.6 database; Plant Metabolic Network; Release: October 2021).

2.6. Statistics and chemometric interpretation of metabolites

Mass profiler Professional B.12.06 (Agilent Technologies, Santa Clara, CA, USA) was used for chemometric interpretations. The raw dataset (Supplementary Table 1) was employed for the subsequent analysis after transformation processing as previously reported (El-Nakhel et al., 2023). Compound abundance was $\log_2$-transformed, normalized at the 75th percentile and baselined against the median. The unsupervised hierarchical cluster analysis (HCA) was based on fold-change values according to Ward's agglomerative algorithm of Euclidean distances. A supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was performed with the SIMCA 16 software (Umetrics, Sweden). Fitness parameters were also calculated and Hotelling's T2 was applied to exclude outliers. CV-ANOVA (cross-validated ANOVA) ($p < 0.01$) and permutation testing ($n = 100$) were used for model validation and to exclude overfitting, respectively. The differentially accumulated metabolites (DAMs), compared to the control conditions, were identified by a Volcano plot analysis (p-value < 0.05, Bonferroni multiple testing correction; FC > 2).

A Plant Metabolic Network Pathway Enrichment analysis (Hawkins et al., 2021) was conducted on PlantCyc, a specific Tool for plant metabolites, for the DAMs in stress compared to the control conditions using the Fisher Exact test (p<0.05) and the *Capsicum annuum* database for accurate annotation of plant metabolic pathways. The significant pathways for V/V and V/N were classified according to the Pathway Tools pathway ontology (Karp et al., 2010).

3. Results

3.1. Water use efficiency and relative water content

Both WUE and RWC were significantly higher in V/N than in V/V under water stress conditions (Table 1).

3.1.1. Water use efficiency

Under the control conditions, A_N/E and A_N/gs did not show any significant differences between V/V and V/N (Table 1). However, both parameters increased under water stress with significant differences between plant combinations, with the highest values for the V/N plants (Table 1).

3.1.2. Relative water content

The RWC (%) values for the plants under the control conditions varied between 97% and 94% at the end of the experiment with no significant differences among plant combinations (Table 1). Water stress significantly reduced the RWC in both plant combinations, but the V/V plants were more sensitive to water stress (RWC=73%) than the V/N ones (RWC=90%) (Table 1).

3.2. Untargeted metabolomics analysis of leaves and roots

An untargeted metabolomics approach identified 3995 and 4097 chemical features for leaves and roots, respectively (Supplementary Table 1).

The unsupervised fold-change-based hierarchical clustering (HCA) output on the metabolomic profile of leaves and roots (Fig. 1) was used to identify similarities and dissimilarities among treatments and plant combinations. This analysis pointed out that water stress was the main factor in clustering for both leaves (Fig. 1A) and roots (Fig. 1B). In fact two main clusters were observed to separate the control plants from the PEG-treated plants regardless of plant combinations. Nevertheless, V/V and V/N were separated into two subclusters in water stress, and a similar trend was observed for both leaves and roots. This separation was further confirmed by the supervised OPLS model (Supplementary Table 2), where the first latent vector separated the control plants from those treated with PEG, while the second vector separated samples according to rootstock (V and N).

3.3. Water stress impact on plant metabolism

A Volcano analysis was conducted to identify DAMs as a result of water stress exposure for each plant combination (V/V and V/N) and organ (leaves and roots) compared to the control plants (Supplementary Table 3).

3.3.1. Leaves

The Venn diagrams for the DAMs in leaves under water stress compared to the control conditions revealed significant overlaps (44% up- and 15% down-accumulated) and a similar number of specific metabolites in both plant combinations (Fig. 2).

3.3.2. Roots

In roots, the number of both the up- and down-accumulated specific metabolites was larger in V/N (Fig. 2). There were also overlapped metabolites in roots (67% up- and 8% down-accumulated) between both plant combinations under water stress (Fig. 2).

3.4. Metabolic pathways associated with water stress

Several biosynthesis pathways were enriched for DAMs, which indicates significant metabolic changes in response to water stress (Supplementary Table 4). Amino acid biosynthesis pathways (Fig. 3A) were enriched in leaves, including "L-serine biosynthesis I" (V/V and V/N), the "superpathway of L-lysine, L-threonine and L-methionine biosynthesis II" (V/V) and, forming part of the latter, "L-methionine biosynthesis II (plants)" (V/V). The amine- and polyamine biosynthesis-enriched pathways (Fig. 3B) were "phosphatidylethanolamine biosynthesis II" and the "superpathway of choline biosynthesis", both in V/V leaves. Regarding carbohydrate biosynthesis pathways (Fig. 3C), the involved compounds came from the V/V leaves for "UDP-sugar

Table 1
Instantaneous water use efficiency (A_N/E), intrinsic water use efficiency (A_N/gs) and relative water content (RWC) after 48 h of water stress treatment (WS, 5% PEG addition) and the control I conditions in the self-grafted variety (V/V) and the variety grafted onto NIBER® (V/N). Values are the mean of n=6±SE. Different letters indicate statistical differences for the LSD test with a p-value < 0.05.

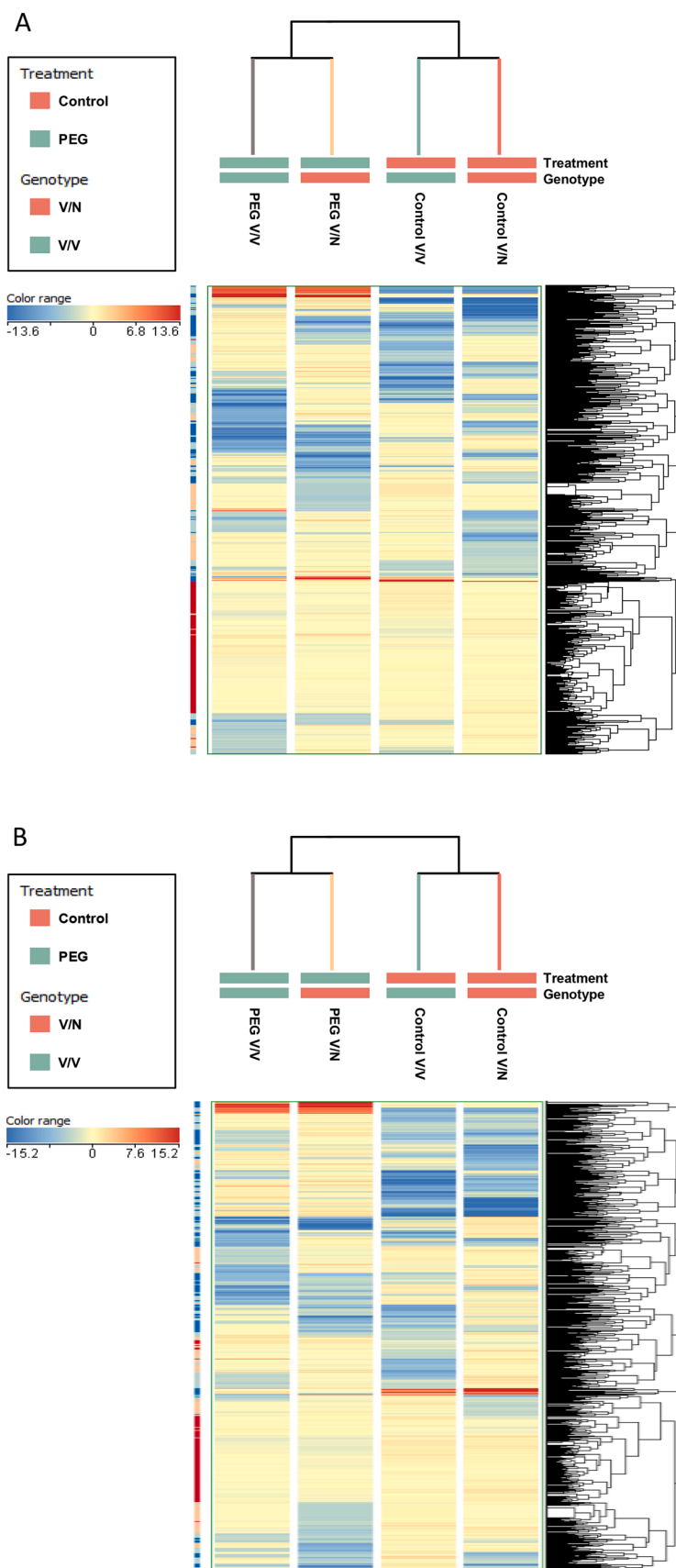|	V / V - C	V / V – WS	V / N – C	V / N – WS
A_N/E	2.1 ± 0.3 c	3.1 ± 0.1 b	2.4 ± 0.27 c	3.5 ± 0.2 a
A_N/gs	24.6 ± 1.8 c	68.2 ± 8.2 b	26.4 ± 2.0 c	109.4 ± 3.5 a
RWC (%)	97.6 a	73,3 c	95 a	90 b

Fig. 1. Unsupervised hierarchical cluster analysis (HCA) (Euclidean similarity; Ward linkage rule) from leaves (A) and roots (B) metabolomic profile of V/V and V/N under water stress (PEG) and the control conditions obtained by UHPLC/QTOF-MS untargeted metabolomics. Clustering was performed on heat maps based on fold change from compound intensity.
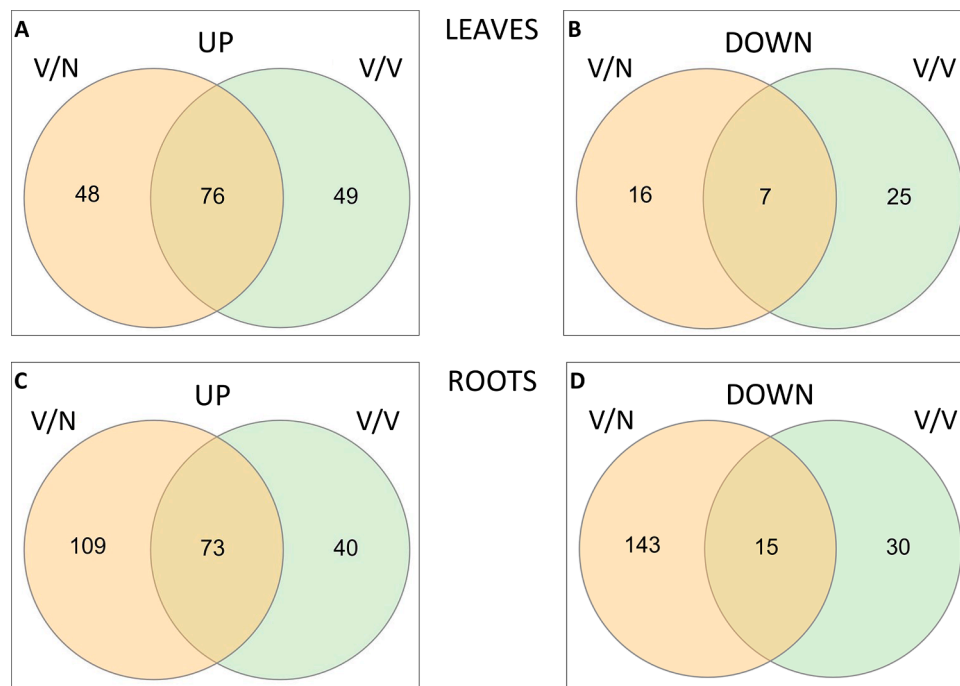
Fig. 2. Venn diagrams for the differential accumulated metabolites (DAMs) under water stress in relation to the control conditions. (A) and (B) show the up-accumulated and down-accumulated DAMs in leaves, respectively. (C) and (D) denote the up-accumulated and down-accumulated DAMs in roots, respectively. Orange depicts the DAMs exclusive for V/N, blue represents the DAMs specific for V/V, and green identifies the DAMs shared by both plant combinations.

biosynthesis" and the V/N leaves for "GDP-L-fucose biosynthesis I (from GDP-D-mannose)". Cell structure biosynthesis pathways (Fig. 3D) "cutin biosynthesis" and "suberin monomers biosynthesis" were enriched in compounds in V/N roots. The pathways related to cofactor, carrier and vitamin biosynthesis (Fig. 3E) were only enriched in leaves, with "NAD metabolism" and "NAD biosynthesis" for both plant combinations and "vitamin B6 biosynthesis" for V/N, specifically "pyridoxal 5'-phosphate biosynthesis II" in leaves. Both the leaves and roots of V/V and V/N were enriched in fatty acid and lipid biosynthesis pathways (Fig. 3F); i.e., the "superpathway of phospholipid biosynthesis II (plants)" was enriched in V/V leaves, whereas "fatty acid biosynthesis" was enriched in the leaves of V/V and V/N and in the roots of V/V. As part of the latter, "very long fatty acid biosynthesis II" was enriched in all the plant combinations and organs, while "stearate biosynthesis II (bacteria and plants)" and "polyunsaturated fatty acid biosynthesis pathways" were enriched only in V/N leaves. Regarding the nucleotide and nucleoside biosynthesis pathways (Fig. 3G), the "superpathway of pyrimidine ribonucleosides salvage" was enriched in the leaves of both plant combinations, the "superpathway of pyrimidine deoxyribonucleoside salvage" in V/V roots, and the "superpathway of purine nucleotides *de novo* biosynthesis I" in V/V leaves and V/N roots. In the last one, "adenosine ribonucleotides *de novo* biosynthesis I" and "inosine-5'-phosphate biosynthesis II" were enriched in V/V leaves and V/N roots. The pathways belonging to the Other metabolic category (Fig. 3H) were enriched in leaves, i.e., the "curcumin glucoside biosynthesis" pathway was enriched in both plant combinations. For polyprenyl biosynthesis (Fig. 3I), enrichment was present only in the V/V leaves for the "superpathway of geranylgeranyl diphosphate biosynthesis II (*via* MEP)". Last, the tetrapyrrole biosynthesis pathways (Fig. 3J) were exclusively enriched in roots; that is, "porphyrin compound biosynthesis" in V/V and "siroheme biosynthesis" in both plant combinations.

Given the DAMs associated with the secondary metabolism, the enriched secondary metabolites biosynthesis pathways are represented independently in Fig. 4. Nitrogen-containing glucoside biosynthesis pathways (Fig. 4A) were associated mainly with glucosinolate biosynthesis and enriched in the leaves of both plant combinations and V/N

roots. Other nitrogen-containing secondary compound biosynthesis (Fig. 4B) only included "alpha-solanine/alpha-chaconine biosynthesis" as an enriched pathway in the roots of both plant combinations and V/V leaves, despite many compounds being differentially accumulated in V/N roots. Regarding hormone biosynthesis pathways (Fig. 4C), "trans-zeatin biosynthesis" was enriched in V/V leaves, "jasmonate biosynthesis" in the leaves of both plant combinations and V/N roots and, as part of the latter, "jasmonic acid biosynthesis" in the leaves of both plant combinations. Terpenoid biosynthesis (Fig. 4D) showed only enriched pathways in V/V, with "methylerythritol phosphate pathway I" and "methylerythritol phosphate pathway II" in leaves, and "olivetol biosynthesis" in roots. Regarding phenylpropanoid derivative biosynthesis pathways, the enriched flavonoid biosynthesis pathways (Fig. 4E) were "flavonol biosynthesis" in V/V leaves, which comprised "quercetin gentiotetraside biosynthesis" in the leaves of both plant combinations. Also belonging to flavonoid biosynthesis (Fig. 4E) was the "anthocyanin biosynthesis pathway", which was enriched in V/N leaves and included "cyanidin 3,7-diglucoside polyacylation biosynthesis" and "shisonin biosynthesis" enriched in V/V roots, and "viodelphin biosynthesis" enriched in the leaves of both plant combinations and V/V roots. Other phenylpropanoid derivative biosynthesis enriched pathways (Fig. 4F) were "stilbene phytoalexin biosynthesis" in V/V roots, "cinnamate biosynthesis" and, forming part of the latter, "chlorogenic acid biosynthesis II" in V/N roots, "hydrolysable tannin biosynthesis" in the leaves of both plant combinations and, pertaining to the latter, "ellagitannin biosynthesis" in both V/V leaves and V/N roots.

4. Discussion

4.1. Better leaf water status is achieved using the NIBER® rootstock

Water stress is an important constraint and, as such, understanding tolerance mechanisms for the development of tolerant crops is crucial. In this study, we performed a water stress experiment on self-grafted pepper plants (V/V) and plants grafted onto the tolerant pepper rootstock NIBER® (V/N) (Gisbert-Mullor et al., 2020b, 2023).
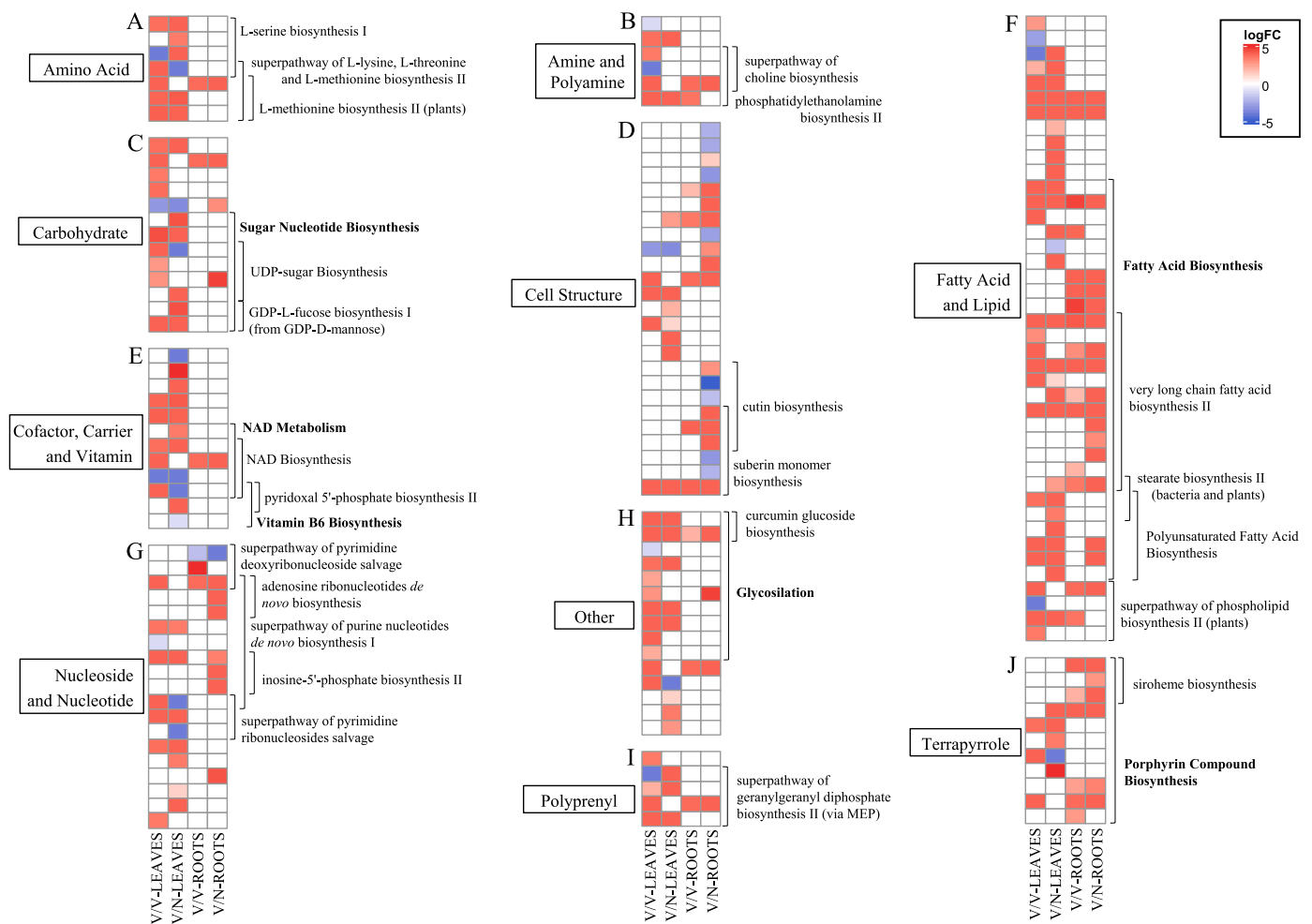
Fig. 3. Enriched biosynthesis metabolic pathways under water stress in relation to the control conditions in V/V and V/N leaves and roots. Heat maps were based on metabolites log-fold change (logFC) in water stress *versus* the control conditions. Red depicts up-accumulation under water stress (positive logFC), blue denotes down-accumulation (negative logFC), and white indicates no differences between treatments.

Upon short-term water stress, tolerance in V/N can be observed in better leaf WUE. A_N/E and A_N/gs increased in V/N under water stress in relation to control conditions, which indicates major stomata closure. This strategy might be related to the phytohormones regulation described in previous experiments (Padilla et al., 2023a), in which ABA increased in V/N leaves with PEG addition. V/N leaves avoid transpiration losses, which allows higher RWC and better plant water economy optimization (Yao et al., 2016), which could facilitate metabolic processes. The differential responses under water stress in V/V and V/N may be associated with the distinct metabolomic profiles observed in both plant combinations, which provide useful information on water stress tolerance mechanisms.

4.2. The metabolic response to water stress is rootstock-dependent

Plant metabolism is entirely affected by water stress, and both primary and secondary metabolisms are disturbed together with metabolic networks, which reprogram to counteract the detrimental effects of stress (Obata and Fernie, 2012; You et al., 2019). During our experiment, a strong impact was noted in the early water stress exposure stage on metabolism because the biggest differences in metabolic profiles were found between treatments (water stress and control conditions) in both plant combinations (V/V and V/N) and organs (leaves and roots) (Fig. 1). These results evidence the importance of studying the metabolic profile to understand the metabolic responses to short-term water stress

and the associated metabolites.

Several authors report many "drought-responsive" metabolites. You et al. (2019) identified more than half the metabolites as drought-responsive metabolites (113 of 221 metabolites), which are common for drought-tolerant and sensitive sesame genotypes. Ma et al. (2021) obtained similar results in the metabolic profile of drought-tolerant and -sensitive alfalfa cultivars under drought stress, with 131/427 metabolites shared by both cultivars. In the present study, we obtained comparable results for the number of common metabolites between genotypes in leaves, but the metabolic response in roots was more genotype-specific. In fact the total number of accumulated metabolites under water stress in relation to the control conditions was significantly bigger in roots than in leaves (322 *vs.* 138), which points out that rootstock influences the metabolic response to water stress. Some authors have highlighted the importance of the rootstock in the metabolic profile of mango plants (Vittal et al., 2023), citrus (Hayat et al., 2022) and grapevine (Chitarra et al., 2017).

4.3. Implications of the enriched metabolic pathways under water stress

4.3.1. Amino acids and amines

The *L-serine biosynthesis I pathway* was enriched in V/V and V/N leaves under water stress and is, thus, a common response. This pathway is crucial for plant development and metabolism, and is responsible for providing serine under abiotic stress conditions (Ho and Saito, 2001).
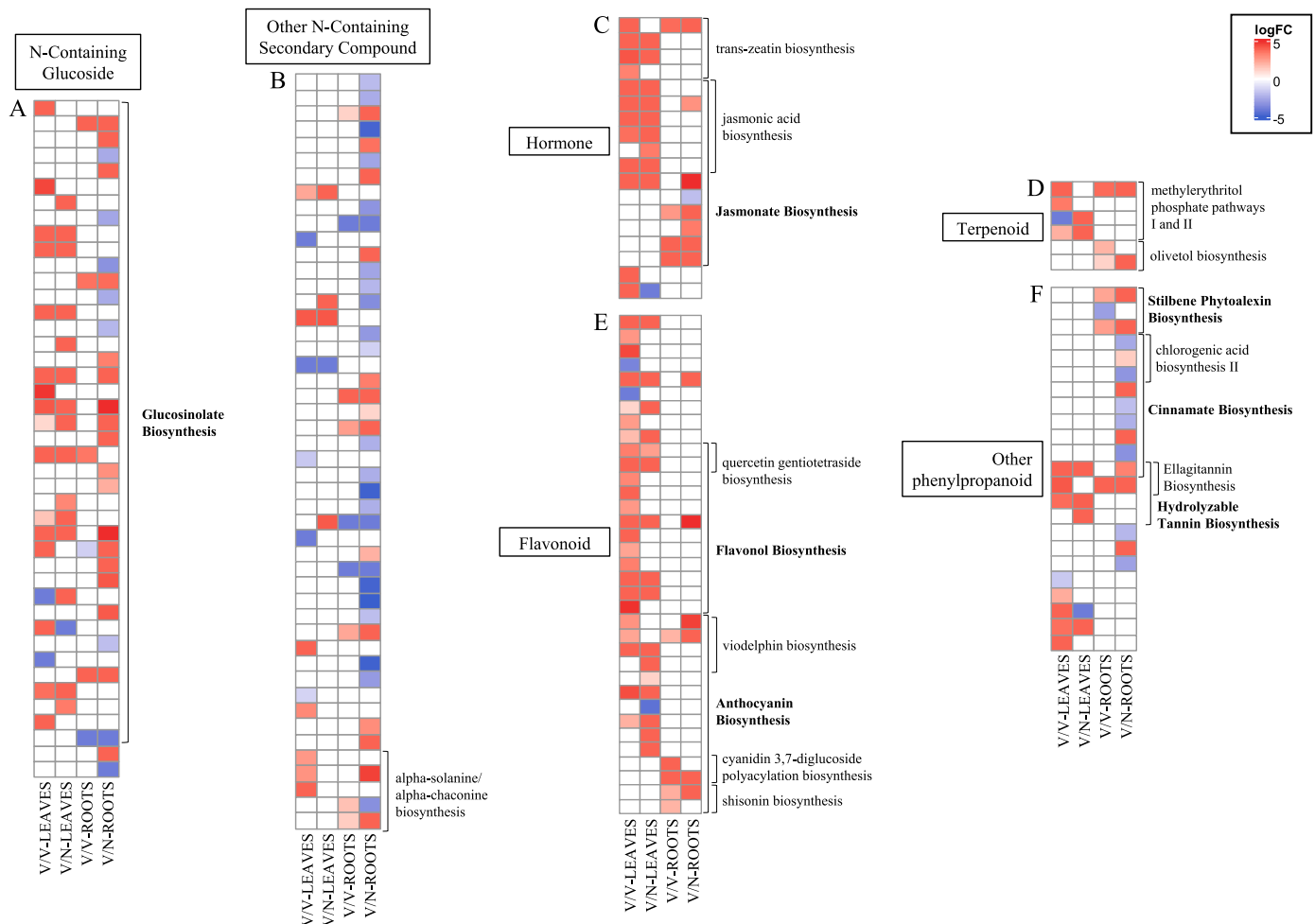
Fig. 4. Enriched secondary metabolites biosynthesis metabolic pathways under water stress in relation to the control conditions in V/V and V/N leaves and roots. Heat maps were based on metabolites log-fold change (logFC) in water stress *versus* the control conditions. Red depicts up-accumulation under water stress (positive logFC), blue refers to down-accumulation (negative logFC), and white denotes no differences between treatments.

Serine is an amino acid used for the biosynthesis of phospholipids and amines, and for signaling and creating reducing power (Kishor et al., 2020).

Of amines, choline has been identified as a precursor for glycine betaine, and its synthesis pathway is enhanced in salt stress for osmoprotection (Summers and Weretilnyk, 1993; Nuccio et al., 2000). However, there is competition between CDP-choline and CDP-ethanolamine to produce either phosphatidylcholine or phosphatidylethanolamine (Dewey et al., 1994). In our experiment, the V/V leaves seemed to prefer phosphatidylethanolamine biosynthesis over phosphatidylcholine. In fact intermediate CDP-ethanolamine was up-accumulated in V/V and V/N leaves. These results indicate that choline may not be involved in the water stress response in pepper grafted plants.

4.3.2. Cofactors and vitamins

The first enzyme phosphatidylethanolamine biosynthesis, named serine decarboxylase, is heavily activity-dependent on pyridoxal 5'-phosphate (PLP) (Rontein et al., 2001). NIBER® is able to specifically induce the synthesis of PLP in V/N leaves, where it is up-accumulated under water stress. PLP is the biochemically active form of vitamin B6, which is used as a cofactor to catalyze numerous reactions (Tambasco-Studart et al., 2005). The vitamin B6 antioxidant role is emerging, and its ROS scavenging has been linked with abiotic stress tolerance (Mooney and Hellmann, 2010).

NAD^+ is another cofactor widely used in many metabolic reactions.

Abiotic stress unbalances the redox system in cells, which depends on the reducing power of NAD^+ and $NADP^+$ to reduce glutathione and ascorbate, among other antioxidant compounds (Gakière et al., 2018). NAD^+ was up-accumulated under water stress for both plant combinations, but NADH increased only in V/N leaves. This NADH in leaves could be an advantage for V/N to regenerate the NAD^+ pool and to increase reducing power under water stress.

4.3.3. Fatty acids (FA) and lipids

Very long-chain fatty acids (VLCFAs) are needed to synthesize a large part of the long-chain FA contained in cellular membranes, but to also produce tissue-specific compounds like sphingolipids, cuticular waxes, suberin, lipids from the pollen coat, among others (Haslam and Kunst, 2013). To do so, plants can start from stearoyl-CoA conversion, which was up-accumulated in V/V roots and in V/N roots and leaves. Gundaraniya et al. (2020) observed similar results in peanut under water stress: up-accumulation of stearic acid in the leaves of a drought-tolerant genotype and the roots of a drought-sensitive genotype. This finding suggests that the NIBER® rootstock caused stearate biosynthesis in the leaves of the grafted variety under water stress for the synthesis of VLCFAs and their derivatives. In fact the synthesis pathways for suberin monomers and cutin were enriched in V/N roots, probably to maintain the stock of extracellular barrier constituents, i.e., cutin and suberin (He and Ding, 2020). These lipids could be involved in the better plant water economy of V/N because they act as barriers that control non stomatal fluxes of water, solutes and gases to protect plants from water stress

(Pollard et al., 2008). These results align with the lipidomic response to drought in the sorghum genotypes studied by Zhang et al. (2021), who reported an increase in cutin and its monomers only in the drought-tolerant genotype.

4.3.4. Hormones

According to He and Ding (2020), apart from being extracellular barrier constituents and membrane components, unsaturated FAs are involved in stress defense as the precursors of bioactive and signaling molecules (i.e., JA, nitroalkenes). During the experiment, the biosynthesis pathways for JA and JA precursors and derivatives were enriched in V/V leaves and V/N leaves and roots. An increase in JA was quantified in the NIBER® roots under water stress in relation to the control conditions in previous experiments (Padilla et al., 2023b), and coincided with the up-regulation of transcription factor *MYC2*, which promotes the gene expression of dehydration resistance-associated genes (Li et al., 2019). Taken together, these results support the involvement of JA in NIBER® tolerance under water stress. However, the other hormone biosynthesis pathway enriched in this analysis (trans-zeatin) displayed contrasting behavior in both V/V and V/N. The pathway and intermediate metabolites were enriched and up-accumulated in V/V leaves, and the opposite was observed in V/N roots, where trans-zeatin and other intermediates were down-accumulated. In previous experiments, cytokinins (CKs) concentration at 24 h under water stress increased in NIBER® roots (Padilla et al., 2023a), following a decrease until the end of the experiment (48 h). This scenario suggests that CKs synthesis is not prevented in the very early response to water stress (24 h), and regulation mechanisms could be later promoted (48 h). Changes in the shoot/root ratio are common under water stress (Kurepa and Smalle, 2022), and CKs regulate this ratio to promote shoot growth over root growth. Past and current results suggest a regulation of CKs synthesis in the NIBER® response to water stress at 48 h to favor root growth and, thus, water and nutrients absorption.

4.3.5. Tetrapyrroles

Of tetrapyrroles, chlorophyll a (Chl a) is usually damaged during drought and the photosynthetic apparatus needs to resynthesize the pigment to avoid an irreversible decline in photosynthetic ability (Kalefetoğlu-Macar and Ekmekçi, 2008). Chl a was up-accumulated in V/N (leaves and roots) and V/V leaves, whereas 7^1-dihydroxychlorophyllide a was up-accumulated in V/V roots. 7^1-dihydroxychlorophyllide a is an intermediate in chlorophyll b (Chl b) biosynthesis, which is performed by chlorophyllide a oxygenase (CAO) (Oster et al., 2000). In previous experiments (Padilla et al., 2023b), the *CAO* gene was down-regulated in NIBER® *vs.* A10 (water stress-sensitive accession) under the control conditions. It may seem that NIBER® does not promote Chl b biosynthesis, perhaps to avoid changes in the Chla:Chlb ratio, which usually lowers under water stress, and this reduction is faster in drought-sensitive genotypes (Majidi et al., 2015).

Siroheme is a modified tetrapyrrole that is similar in structure to chlorophyll. This compound and some intermediates of the synthesis pathway were up-accumulated in roots of both plant combinations under water stress. Siroheme is an iron-containing prosthetic group in sulfite and nitrite reductases and, thus, plays a main role in nitrogen assimilation (Murphy and Siegel, 1973). In preceding experiments, when NIBER® was used as a rootstock under salt stress, its roots showed a slighter decrease in nitrate reductase activity than the self-grafted and ungrafted variety compared to the control conditions (López-Serrano et al., 2020). Moreover, in Padilla et al. (2023b), up to five genes coding for vacuolar iron transporters were down-regulated in NIBER® roots under water stress. By taking together previous and present results, it may seem that NIBER® attempts to perform better nitrogen assimilation by modulating nitrate and nitrite reductases activity, the latter by increasing siroheme synthesis, which is favored by less iron compartmentation in the vacuole.

4.3.6. Phenylpropanoids

When plants are affected by stress, their metabolism can switch between primary and secondary metabolisms to cope with unfavorable conditions (Jia et al., 2020). Secondary metabolites like phenolic compounds are promoted during abiotic stress to help plants to adapt to stress conditions, probably due to their antioxidant properties that derive from hydroxyl groups (Gundaraniya et al., 2020).

Despite chlorogenic acid (CGA) having the potential to play a detoxifying role in drought stress to diminish oxidative damage in plants, two intermediate metabolites were down-accumulated in V/N roots. Indeed Nouraei et al. (2018) observed increases in CGA in drought-stressed artichokes leaves and heads, which coincided with the reduced chlorophyll, RWC, growth and yield produced by oxidative stress (higher lipid peroxidation and H_2O_2). Down-accumulation in NIBER® roots may prevent decreases in growth, chlorophyll and yields. Therefore, the role of CGA in the water stress response should be further investigated.

Regarding flavonoids, flavonol biosynthesis seems to be preferred by V/V, whereas V/n favors anthocyanins biosynthesis. Of flavonols, several quercetin and kaempferol metabolites were up-accumulated mainly in V/V leaves. Quercetin, kaempferol and their related metabolites have been linked with photoprotection under light stress in *Arabidopsis* and *Vitis vinifera* (L.)*,* and increases in these metabolites have been associated with good ROS production and reduced photosynthesis under high irradiation and drought stress (Havaux and Kloppstech, 2001; Griesser et al., 2015). In conclusion, flavonols are important ROS scavengers that can help to reduce ROS in V/V, but such ROS may be a product of enhanced oxidative stress, and these metabolites might be indicators of plants suffering from water stress (Sharma et al., 2022). It would seem that the NIBER® response to water stress does not involve flavonols biosynthesis under the water stress conditions of this experiment. Instead the anthocyanin biosynthesis pathway was enriched in V/N leaves. Anthoncyanins are another class of flavonoids that display powerful antioxidant activity, and their function in ROS scavenging has been proposed because they are usually located in vacuoles, near ROS production sites like chloroplasts and peroxisomes (Naing and Kim, 2021). In fact anthocyanins content increases in pea leaves by UV-B radiation and drought (Nogués et al., 1998), Anthocyanins include viodelphin, which was up-accumulated in the leaves of both plant combinations and V/V roots. Last, malonylshisonin is a cyanidin-type anthocyanin that was up-accumulated in V/V and V/N roots. Interestingly, its synthesis pathway has been described mostly in Perilla frutescens (L.), in which malonylshisonin increased after high-light treatment and this increase is regulated by several transcription factors (*MYBs* and b*ZIPs*) (Xie et al., 2022). Thus it is worth further studying its implications in water stress response.

4.3.7. Nitrogen-containing secondary compounds

Nitrogen-containing secondary compounds derive from amino acids and principally perform a defensive function. During this experiment, nitrogen-containing secondary compounds differentially accumulated in both plant combinations, and organs mainly belong to alkaloids and nitrogen-containing glucosides.

Glycoalkaloids (GAs) are toxic metabolites found in the Solanaceae family (Papathanasiou et al., 1999). Bejarano et al. (2000) observed an increase in GAs in drought-sensitive potatoes, but a slighter increase or no increase at all in drought-tolerant potatoes under drought conditions. During this experiment, alpha-chaconine was up-accumulated in V/V roots and down-accumulated in V/N roots. Alpha-chaconine is more toxic than α-solanine given its greater ability to disrupt cholesterol-containing cell membranes (Friedman, 2006). Apart from the above-mentioned role in drought-stressed potatoes, α-solanine and α-chaconine have not been associated with abiotic stress responses. Other nitrogen-containing compounds are glucosinolates, which contain a considerable amount of the plant's sulfur. Drought stress modulates sulfur metabolism, mainly for ROS detoxification mediated by

glutathione (Chan et al., 2013). Indeed glucosinolates have been linked with drought tolerance through crosstalk with phytohormones, i.e., auxins and stomatal regulation (Salehin et al., 2019). V/V and V/N leaves seemed to promote glucosinolates biosynthesis because many related metabolites, including precursors, were up-accumulated. Nonetheless, V/N roots did not follow a marked trend and showed up- and down-accumulated metabolites. Although glucosinolates are widely distributed compounds in Brassicaceae, their presence in Solanaceae has been isolated (Rawani et al., 2014), which would explain the presence of related compounds in this study.

4.4. Limitations and future research

The results demonstrate that NIBER® rootstock modulates the metabolic responses to water stress in grafted pepper plants. This study highlights the importance of secondary metabolites in water stress tolerance, although further research is needed to elucidate their specific roles.

5. Conclusion

To conclude, by focusing on NIBER® and its tolerance mechanisms, distinct metabolomic signatures can be observed in both roots and leaves. In particular, broad reprogramming was triggered at the metabolome level by the NIBER® rootstock, which indicates specific stress mitigation processes. This rootstock increased the vitamin B6, stearic acid and anthocyanins contents in the leaves of the grafted variety. Moreover, NIBER® roots increased siroheme content for better nitrogen assimilation and decreased trans-zeatin content which rendered a higher root/shoot ratio. Besides, V/N enhanced cutin and suberin biosynthesis, which act as protection barriers, and JA and jasmonates biosynthesis to promote tolerance signaling. Finally, NIBER® did not stimulate flavonols and Chl b biosynthesis to favor anthocyanins biosynthesis and to maintain the Chla:Chlb ratio. The contribution to the water stress response of several secondary metabolites like CGA, shisonin, glycoalkaloids and glucosinolates should be studied in-depth because most have not been previously described in pepper, and information about their role in water stress tolerance is lacking.

This study provides valuable insights into the metabolic responses of grafted pepper plants under water stress. The findings show metabolites that may have significant implications for improving water stress tolerance in crops and might broaden knowledge about metabolic networks in grafted plants.

CRediT authorship contribution statement

Yaiza Gara Padilla: Writing – original draft, Methodology, Investigation, Data curation. **Begoña Miras-Moreno:** Writing – review & editing, Writing – original draft, Data curation. **Ramón Gisbert-Mullor:** Methodology, Investigation. **Luigi Lucini:** Writing – review & editing, Supervision, Resources. **Salvador López-Galarza:** Supervision, Investigation, Funding acquisition, Conceptualization. **Ángeles Calatayud:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available in the Supplementary Material of this article. Therein, the whole list of compounds annotated, with individual abundances and composite mass spectra, is provided.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100542.

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