A multi-omics approach to unravel the interaction between heat and drought stress in the Arabidopsis thaliana holobiont

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Abstract

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Abstract

The impact of combined heat and drought stress was investigated in*Arabidopsis thaliana* and compared to individual stresses to reveal additive effects and interactions. A combination of plant metabolomics and root and rhizosphere bacterial metabarcoding were used to unravel effects at the plant holobiont level.

Hierarchical cluster analysis of metabolomics signatures pointed out two main clusters, one including heat and combined heat and drought, and the second cluster that included the control and drought treatments. Overall, phenylpropanoids and nitrogen-containing compounds, hormones and amino acids showed the highest discriminant potential. A decrease in alpha diversity was observed upon stress, with stress-dependent differences in bacterial microbiota composition. The shift in beta-diversity highlighted the pivotal enrichment of *Proteobacteria* , including *Rhizobiales* , *Enterobacteriales* and *Azospirillales* .

The results corroborate the concept of stress interaction, where the combined heat and drought stress is not the mere combination of the single stresses. Intriguingly, multi-omics interpretations evidenced a good correlation between root metabolomics and root bacterial microbiota, indicating an orchestrated modulation of the whole holobiont.

Keywords

Heat stress; drought; multi-omics; phytohormones; Proteobacteria; Rhizobiales.

INTRODUCTION

Heat and drought are two environmental stresses that occur with climate change-associated extreme weather events, which substantially impact plant growth and development (Giordano, Petropoulos & Rouphael 2021). Drought stress occurs due to an imbalance between the evapotranspiration flux and water intake, mainly when the soil water availability and the atmospheric humidity are low and the air temperature is high. Hence, both stresses often occur simultaneously. The plant response to drought stress depends on the species, plant growth stage, and environmental factors (Fahad *et al.* 2017). Heat stress, defined as the rise in soil and air temperature beyond a threshold level for a minimum amount of time (Lamaoui, Jemo, Datla & Bekkaoui 2018), may inactivate enzymes and cause damage to proteins and changes in their synthesis. Moreover, heat stress could have major effects on cell division processing. Most experimental studies have focused on a single stressor due to the challenging biological cross-talk between the multiple plant responses and their interpretations.

Plants are always colonised by complex microorganism communities in their root system (i.e. the plant microbiota) that, together with their plant host, build the holobiont (Lyu *et al.* 2021). It is well known that the rhizosphere is colonised by a broad diversity of microorganisms which may accumulate depending on the stress level of the holobiont (Froncioli *et al.* 2021). Bacteria are one dominant group of rhizomicrobiota, known to directly interact with the host plant by various mechanisms impacting growth and the plant immune system (Rizaludin, Stopnisek, Raaijmakers & Garbeva 2021). Indeed, through roots, plants exude a mixture of small molecules that select specific portions of soil bacteria (Zhang *et al.* 2021). Such a "Cry for help strategy" suggests that stressors lead to changed signaling and substrate release in the root system and rhizosphere to acquire beneficial microbes (Rizaludin *et al.* 2021). This active recruitment of microorganisms improves resilience to abiotic stresses by eliciting physiological, biochemical, and molecular responses in the plant's local and distal parts (Meena *et al.* 2017)(Saeed *et al.* 2021). Noteworthy, single and combined abiotic stresses may indirectly alter plant functions via the modulation of plant- and root-associated microbiota (Rahman, Hamid & Nadarajah 2021), triggering substantial changes in plant development (Froncioli *et al.* 2021; Lewin, Froncioli, Ulrich & Kolb 2021). Despite plant adaptation processes and the microbiota responses to abiotic stresses being known, the effect of combined stressors on holobionts is still poorly investigated (Rivero, Mittler, Blumwald & Zandalinas 2022).

Plant responses to such stresses are complex and may not correspond simply to the sum of the two abiotic stresses applied individually (Zandalinas, Fritschi & Mittler 2020), but they can interact and negatively impact plants even if the effect of each stress is slight (Zandalinas *et al.* 2021). For instance, the response to two different stresses applied simultaneously to two different leaves of the same plant was different and more extensive than the response to two different stresses applied individually (Balfagón *et al.* 2019). Furthermore, increased atmospheric CO_2 concentrations reduce the impact of combined heat and drought stress on *Arabidopsis spp.* , activating antioxidant defence mechanisms and reducing photorespiration (Zinta *et al.* 2018). These complex and interconnected responses involve several molecular and physiological modifications

2

and acclimation to include systemic signaling, accumulation of stress-specific transcripts, and hormones. The gene expression of heat shock proteins (HSPs) (Georgii *et al.* 2017) is altered and occurs in different ways when stresses are combined (Zandalinas, Sales, Beltrán, Gómez-Cadenas & Arbona 2017).

Hence, our work aimed at investigating the impact of heat, drought, and their combination on the plant holobiont, considering plant-specific metabolites and hormones, as well as the root and rhizosphere bacterial microbiota. Recently, a holo-omics approach has been suggested to assess simultaneously in one experimental design both the plant host and its microbiota response to environmental changes to better understand changed interactions and the relevance of enriched microbial taxa for its host plant (Xu *et al.* 2021). Accordingly, a holo-omic approach was applied as an integrated analytical strategy to resolve the coordinated and complex dynamic interactions between the plant and its rhizosphere bacteria, using *Arabidopsis thaliana* as a model plant species.

MATERIALS AND METHODS

Plant growth and morpho-physiological assays

The experiments were carried out on *Arabidopsis thaliana* plants (cv Columbia 0) following the protocol proposed by Rizhsky et al. (2004) with some modifications. A professional potting soil (Orticole alveolo TecnoGrow, Tercomposti, Calvisano BS, Italy) was sterilised before the experiment (120 °C for 20 min). Before sowing, seed germination was synchronised by soaking the seeds in sterile water for 76 hours at 4 °C in dark conditions. Ten seeds were then sown per pot, and after germination, they were thinned, leaving one seedling per pot. A total of 24 pots (5 x 5 x 5 cm) were prepared for each treatment and replicate.

Seedlings were grown in a growth chamber under controlled conditions: 12 h light / 12 h dark photoperiod (long day), 21 ± 1 °C, 100 µmol m^{-2} s^{-1}, and relative humidity of 60-70%. Seedlings were fertilised every other day through sub-irrigation using a half-strength Hoagland solution and grown for 22 days [Arabidopsis growth stage 3.50, Rosette is ˜ 50% final size (Boyes *et al.* 2001).

The following treatments were applied: C (Control watered plants), D (Drought stress), H (heat stress), and D+H (Drought + Heat stress). Drought treatment was achieved by blocking plant irrigation until they reached a relative water content (RWC) of 65 % to 70 % (typically 5–6 d). In contrast, heat stress was applied by raising the temperature gradually (˜ 4 °C per hour) to avoid heat shock, in the growth chamber to 35 °C, and then keeping the plants at this temperature for 12 h.

At the end of the experiments, 1 g of the rhizosphere and root samples were collected for the microbiome analysis. Roots were carefully washed in sterilised distilled water and immediately snap-frozen in liquid nitrogen. Both soil and powdered roots were stored at -80 °C (Thijs*et al.* 2017).

During the experiments, morphological and physiological parameters were monitored. In particular, the changes in biomass were evaluated at the end of the experiments on the fully developed rosette, measuring the changes in fresh weight (FW), dry weight (DW) and the DW/FW ratio. Another indicator of plant stress was the chlorophyll content, carried out at the end of the experiment using a chlorophyll meter SPAD (MC100, Qingdao Tlead International Co. Ltd). Finally, to have an indicator of plant water status and the plant's ability to preserve it, the temperature of the leaves was monitored using a thermocamera (FLIR), and the leaf relative water content (% RWC) as previously reported by Araniti *et al.* , 2020.

Untargeted metabolomics analysis

Arabidopsis roots were extracted using a homogeniser-assisted extraction in 80% methanol solution with 0.1% (v/v) formic acid, centrifuged and filtered through 0.22 µm cellulose filters. The phytochemical profile of roots was investigated through ultra-high-pressure liquid chromatography (UHPLC) coupled with quadrupole-time-of-flight (QTOF) mass spectrometry, as previously reported by Senizza *et al.* , 2021. Briefly, the mobile phase consisted of a mixture of water and acetonitrile (both LC-MS grade, VWR, Milan, Italy) acidified with 0.1% (v/v) formic acid, with a gradient from 6 to 94% of acetonitrile in 35 min. An injection volume of 6 µl and a pentafluorophenylpropyl column (2.0 × 100 mm, 3 µm - Agilent Technologies, Santa

Clara, CA, USA) were used. The mass spectrometer acquired ions in the range 100-1200 m/z in positive scan mode (ESI+) at a rate of 0.8 spectra/s (40,000 FWHM, absolute peak height threshold 5000 counts).

For raw data processing, the software Profinder B.07 (Agilent Technologies) was used, considering monoisotopic mass (5-ppm tolerance for mass accuracy), isotope spacing and ratio according to the "find-by-formula" algorithm. Mass and retention time alignment, as well as compound filtering, were performed before compounds annotation. The database PlantCyc was used as a reference for annotations (Hawkins*et al.* 2021). Only the compounds identified in 100% of replications within at least one condition were retained in the dataset and used further. According to COSMOS Metabolomics Standards Initiative, the annotation process corresponded to a Level 2 of identification (i.e., putatively annotated compounds) (Salek *et al.* 2015).

Analysis of the rhizosphere bacterial microbiota structure by metabarcoding

The DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) was used to extract DNA from roots and soil samples. The amplification of bacterial DNA was carried out by LGC genomics GmbH (Berlin, Germany) using the primers 799f and 1115r, and the amplicons were sequenced on an Illumina MiSeq instrument with 300bp paired-end reads.

Demultiplexing was conducted with Illumina bcl2fastq 2.17.1.14 software following the clipping of barcode and sequencing adapters. Primers were removed using Cutadapt v3.0 (Martin 2011). Sequences were processed in R 4.1 with dada2 version 1.22.0 (Callahan *et al.* 2016). Due to adapter ligation-based library prep, the raw sequences were in mixed orientation. To get the correct final orientation for learning error rates, reads were split into two groups (forwardRead.forwardPrimer - reverseRead.reversePrimer, and reverseRead.forwardPrimer - forwardRead.reversePrimer), denoised separately and merged after chimera removal. Forward and reverse reads were truncated at positions 265 and 210, resulting in 4073 unique Amplicon sequencing variants (ASV). Taxonomic classification was performed using the q2-feature-classifier plugin from Qiime2 version 2021.8.0 with a naïve Bayes classifier trained on the Silva 138.1 NR99 database.

Statistical Analysis

All the experiments were carried out in a completely randomised design with 5 replications. The univariate analysis of morphological and physiological parameters was carried out using XLSTAT 2014.5.03. Data were analysed through one-way ANOVA using Duncan's test as post hoc (P [?] 0.05).

Concerning metabolomics, the post-acquisition data analysis was carried out using the software Mass Profiler Professional 12.6 (Agilent Technologies); the compounds were log2 transformed, normalised at the 75th percentile, and baselined against the median. Afterwards, both unsupervised and supervised multivariate statistics were applied for interpretations. According to Euclidean distance and Ward's linkage, the unsupervised hierarchical cluster analysis was used to underline the relatedness across the different treatments. In addition, the supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) was carried out, and the model parameter (goodness-of-fit R^2Y and goodness-of-prediction Q^2Y) were recorded. Also, the OPLS-DA model was cross-validated (CV-ANOVA), inspected for outliers (Hotelling's T2), and the overfitting was excluded through a permutation test (n = 200). Then, the Variable Importance in Projection (VIP) analysis was used to select the metabolites having the highest discriminant potential (VIP score > 1.2). Finally, the differential compounds obtained from the ANOVA and fold-change analysis (FC) (p < 0.05, Bonferroni multiple testing correction and Fold-Change FC [?] 2) were exported into the Omic Viewer Pathway Tool of PlantCyc (Plant Metabolic Network) (Stanford, CA, USA) software for interpretation (Karp *et al.*2009; Caspi, Dreher & Karp 2013)

Regarding metabarcoding data, downstream analysis was performed using RStudio with R version 4.1.1. Phyloseq v1.38.0 was used to handle ASV sequences and tables. Samples were split into compartments (soil, root) and separately analysed. After outlier removal (one sample from control treatment on root and in soil, one sample from drought treatment and two from Drought-Heat treatment due to small sampling size), ASVs were filtered for mitochondria, and unassigned sequences were removed. Only ASV that (a) occurred in at least 3 samples and (b) occurred >10 times in total were retained for downstream analysis, leading to 1455

ASV in soil samples and 915 ASV in root samples. Alpha diversity indices (number of observed ASVs, Inverse Simpson index) were calculated using rarefied and filtered samples from root and soil datasets and plotted by treatment. Bray-Curtis dissimilarity indices were calculated on rarefied relative abundances and used to perform principal coordinate analysis (PCoA) and permutational analysis of variance (PERMANOVA) to investigate treatments' effect on the bacterial community structure. For PERMANOVA, 999 permutations were carried out per dataset. Linear discriminant analysis of effect size (LEfSe) was applied to the root and soil datasets aggregated to the Genus level to identify keystone taxa that drive the differences between treatments (Segata *et al.* 2011). LEfSe was run with a Wilcoxon and Kruskal-Wallis cut-off value of 0.01. An LDA cut-off value of 2 resulted in 182 and 145 marker genera for root and soil, respectively, and an LDA cut-off of 4 in 43 and 10 marker genera. In addition, analysis of compositions of microbiota with bias correction (ANCOMBC) was used to identify differentially abundant features (Lin & Peddada 2020). The Holm-Bonferroni method was applied to adjust p-values, and features with adjusted P values <0.01 were considered significant, resulting in 193 and 313 genus-level markers for root and soil datasets, respectively.

Combined Discriminant Analysis of Metabarcoding and Metabolomics datasets

Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DI-ABLO) from the package "mixOmics" was used for the integration of metabolomics and root metabarcoding datasets (Rohart, Gautier, Singh & Le Cao 2017). This supervised approach allowed the integrated analysis of multiple datasets and was used to identify discriminant features in both datasets that drive differences between treatment groups. Values of the design matrix were set to 0.1 to prioritise the discriminant ability of the model. Center log ratio (clr) transformation was applied to both datasets, and root metabarcoding data was aggregated at the genus level beforehand. An optimal number of 3 components for "centroid.dist" distance was determined using the function perf() with 6-fold cross-validation and 10 repeats. The number of features selected for sparse PLS-DA was tuned with the function tune.block.splsda() using 4-fold cross-validation with 10 repeats. The features selected for each component were 18, 40, 6 for metabarcoding and 6, 14, 90 for metabolomics. The correlation among components of each dataset was checked with plotArrow().

RESULTS

Morpho-physiological parameters

The experiment lasted until the loss of turgidity in the leaves of the stressed control. Firstly, the chlorophyll content measurement was performed at the end of the trial, randomly measuring SPAD on 5 leaves per plot. The SPAD index was significantly ($p<0.05$) lower in drought-stressed plants, with a decrease of 25% compared to the watered control (C). Also, both fresh and dry weights were significantly ($p<0.05$) reduced in drought conditions (D), being about three times lower than the control.

In parallel, an increase in leaf temperatures was registered. The temperatures after four days (T4) were 10% higher compared to the C+D at the starting point (T0) and +6% compared to C. Similarly, the relative water content (RWC) after four days was reduced, resulting in about half of the control.

Untargeted metabolomics profiling of *Arabidopsis thaliana* roots by UHPLC/QTOF-MS

The untargeted metabolomics approach was used to investigate the effect of heat and drought stresses and their combination on the metabolomic profile of *A. thaliana* roots. The metabolomics raw data are submitted to a public repository (https://www.ebi.ac.uk/metabolights/MTBLS6421). This approach allowed us to putatively annotate more than 1100 compounds, further used to infer the biological processes involved in plant stress responses. The list of the annotated metabolites, with composite mass spectra and abundance, is provided as supplementary material (Supplementary Table S1).

Unsupervised hierarchical cluster analysis and supervised OPLS discriminant analysis were successfully performed to identify patterns between the conditions under investigation. The clusters produced from the heatmap based on fold-change highlighted distinct metabolomic profiles in roots, depending on the stress applied. In fact, two main clusters were generated, one featuring H and H+D and the other including the control and D (Supplementary Figure S1). These findings were corroborated by the supervised OPLS-DA,

5

where all stress conditions separated from the control and underlined a separation of D from the other conditions (control, H and H+D) (Figure 1). The score plot also showed that the combined stress (H+D) influenced metabolic profile closer to the H and far from the control and D. Afterwards, VIP analysis was carried out to find the compounds with the highest contribution to the OPLS-DA discrimination (VIP score > 1.2 , Supplementary Table S2). Overall, phenylpropanoids and nitrogen-containing compounds, hormones, and amino acids exhibited the highest discriminant potential.

Then, Volcano Plot analysis identified 405 differential compounds significantly differing from the control (p-value < 0.05; FC [?] 2). Despite the large number of compounds modulated in response to the stresses, only 65 metabolites overlap for all the H, D and H+D (Supplementary Table S3). Moreover, the combined treatment (H+D) presented several compounds common with H stress, indicating a hierarchical prevalence of the latter. In contrast, D alone provoked a distinct response, as suggested by OPLS-DA. Figure 2 depicts the modulation of the biosynthetic pathways in response to the specific stresses resulting from differential compounds accumulation. Overall, all the individual and combined stress had a detrimental effect on root metabolism, particularly specialised metabolism. In agreement with multivariate analysis, roots exposed to H stress showed a comparatively stronger modulation of specialised metabolism. In general terms, several plant defense mechanisms were activated in a stress-specific manner. Despite the general decrease of specialised metabolites, glucosinolates increased in response to stress (H, D, H+D). In this sense, phytoalexins related to glucosinolates pathways (i.e. indole-3-carboxaldehyde and indole-3-carbinol) and glutathione-related compounds were also modulated under combined stress conditions (H+D).

Phenylpropanoids decreased in response to H and, to a lesser extent, to H+D while increasing in response to D, which promoted the accumulation of flavonoids and anthocyanins. Regarding terpenes, H decreased these compounds while H+D and D alone elicited this pathway, including their precursors. Although the detrimental effect of H on plant metabolism, plants exposed to H increased in phospholipids while sterols accumulated in the two other stress conditions considered (D, H+D).

The stresses also influenced the phytohormone profile, with distinctive modulations as a function of the condition considered (Figure 3). In more detail, cytokinins (CKs) were accumulated by the combined stress (H+D) and D stress. Moreover, in H, CKs and abscisic acid (ABA) synthesis were negatively modulated, while gibberellins (GAs) and jasmonates (JAs) were down accumulated under water deficit conditions (D). Ascorbate-related compounds were also modulated in plants exposed to the stress, with dehydroascorbate accumulated under H and D stress.

Bacterial richness and biodiversity

In soil and root samples, 1455 and 915 bacterial ASVs remained after filtering out sparse ASVs and outliers, respectively. These AVSs were affiliated with 20 phyla, 39 classes, 87 orders, 130 families and 184 genera in root and 27 phyla, 51 classes, 114 orders, 170 Families and 238 Genera in soil.

Both soil and root samples were dominated by Proteobacteria, which made up, on average, 89% of classified reads in root samples and 53% in soil samples (Figure 4A). Taking a more detailed look at the distribution of orders within the Proteobacteria (Figure 4B), pronounced different patterns between the compartments (root, soil) as well as the treatments (C, H, H+D, D). In soil samples, a large fraction of Micropepsales (average of 11.5 % of reads) was detected, which is substantially less frequent in root samples (1.2% of reads). In the roots, treatment differences also refer to proteobacteria, mainly classified as Rhizobiales and Burkolderiales. A large fraction of Pseudomonadales occurs in both the C and D treatments. The combined H+D treatment only was characterised by a substantial increase in Enterobacteriales (26.4% of reads), which were also present in the heat treatment to a lesser extent (mean 1.56%).

A decrease in bacterial alpha diversity was observed from the rhizosphere to the root. Considering the different stress, differences in alpha-diversity were evident under drought stress (with lower values than the not stressed control). Analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test revealed no effect of the treatments on the richness in either compartment. However, a significant interaction between treatment and Inverse Simpson index for the root samples was identified

(p[?]0.05).

The beta-diversity was determined using principal coordinate analysis (PCoA) with Bray-Curtis dissimilarities. The first two axes accounted for over 72% and 58 % of the variance between treatments of root and soil samples, respectively. PERMANOVA analysis on the root and soil datasets indicated a significant effect of all treatments, where drought alone explains 23.2% of the variance, heat 38.7% and the combination 22.2% in root samples. In soil samples, D explains 29.1% of the variance, heat only 18.9 % and combined H+D only 13.6% of the variance (p[?]0.001) (Supplementary Table S4). Then, linear discriminant analysis effect size (LEfSe) was carried out to identify the taxa driving the differences between treatments (Supplementary Figure S2) and the ANCOM-BC differential analysis was used to identify the taxa differentially abundant between the treatments. Both methods perform a similar analysis but look at the problem differently. As expected, the marker taxa established as significant were largely shared between the methods, being 131 in roots and 138 in soil (Supplementary Figure S3). For interpretation, we focused then on LefSE results to identify keystone taxa that explain differences between sample groups.

In root samples, LefSe analysis revealed 21 microbial biomarkers in C, 11 in D, 71 in H and 14 in H+D.The most discriminant taxa in H belonged to the phyla of *Actinobacteriota* and *Proteobacteria* , in particular, *Rhizobiales* , *Xanthomonadales* and*Streptomycetales,* while *Burkholderiales* characterized the D samples. The combined stress (H+D) was characterized by the presence of *Gammaproteobacteria,* being the *Enterobacterales and Azospirillaleles* the most discriminating taxa. However, non-stressed roots (C) were characterized by *Pseudomanadales* and*Caulobacterales* . (Supplementary Figure S4A)

In Soil samples there were 12 microbial biomarkers detected in C, 9 in D, 20 in H and 25 in H+D. Here the most discriminant taxa for H were the*Alphaproteobacterium Azospirillum* and the Phylum*Firmicutes* member of *Bacillales* and *Clostridiales* . At the same time, H+D was most defined by a taxon from*Polyangiales* , followed by *Rhizobiales, Fibrobacterales, Sphingobacteriales* and *Chitinophagales,* and member from the phylum *Elusimicrobiota.* In D differences were driven by*Devosiaceae* and *Cryseobacterium* and C by*Geobacterales* and *Ktedonobacterales* (Supplementary Figure S4B).

MultiOmics integration

Following the holo-omics approach, we integrated the metabolomics and root metabarcoding datasets using the DIABLO framework (Singh *et al.* 2019) with multiblock sparse PLS-DA (partial least squares discriminant analysis). Model tuning helped us to select features from metabarcoding and metabolomics to improve the modelling of differences between groups. Noteworthy, the datasets are highly correlated for all three components (Figure 6, components 1 and 2, supplementary figure S5, components 2 and 3). The agreement between metabarcoding and metabolomics is high for all samples and treatments. The combined drought and heat treatment separates from the other three treatments on the first axis, and heat treatment on the second axis, while the drought and control treatments are not well separated by components 1 and 2, but are separated by component 3 (Supplementary figure S5.

As a follow-up analysis to DIABLO, Pearson correlation network analysis was performed. We aimed to identify positive correlations between features assigned to the same treatment by DIABLO. These pairs likely resolve the underlying interaction between the plant metabolome and the rhizosphere microbiota.

Only bacterial genera and plant metabolites significantly assigned to a feature by DIABLO were included in network construction. Three clustering methods (Optimal, Louvain, fast greedy clustering) reproduced four similar modules. The largest was mainly constituted by correlations between features of the control treatment and several heat or drought metabolites. The latter were likely false positives of the DIABLO analyses.

Besides the control module, only metabolites and genera assigned as features of the H+D treatment by DIABLO co-occurred in a single module. Thus, crosslinks between metabolite and bacterial datasets were characteristic of the H+D treatment appearing as chords within Figure 7 . Additionally, a fraction of drought-associated bacterial genera and heat-associated metabolites formed connections within this ma-

7

jorly H+D-associated module. The module consisted of 24 nodes in total (Table 1). Among them were predominantly Nitrogen-Containing Secondary Compounds for biosynthesis, Phenylpropanoid Derivative biosynthesis, Polyketide biosynthesis and *Proteobacteria* .

In contrast, the majority of drought-associated metabolites or heat-associated bacterial genera formed no crosslinks between the same feature category across the metabolite and metabarcoding datasets. Instead, drought-associated metabolites correlated with other metabolites and heat-associated bacterial genera interacted with other bacteria, as indicated by characteristic arcs within Figure 7.

In conclusion, a small core of *Proteobacteria* was associated with Nitrogen-Containing Secondary Compounds in response to combined H+D stress. The combined treatment appears to further select plant metabolism responsive to heat metabolism, but rhizosphere bacteria responsive to D.

Node	Genus/Metabolite	Phylum/Pathway
CPD-12423	2-sinapoyloxy-3-butenylglucosinolate	Nitrogen-Containing Compounds
CPDQT-406	(E)-1-(L-cysteine-S-yl)-N-hydroxy-omega (methylsulfanyl)pentan-1-imine	Nitrogen-Containing Compounds
GenASV17	Azospirillum	p_Proteobacteria
GenASV128	Noviherbaspirillum	p_Proteobacteria
GenASV58	Delftia	p_Proteobacteria
GenASV100	o_Enterobacterales|f_|g_un	p_Proteobacteria
GenASV5	f_Enterobacteriaceae|g_un	p_Proteobacteria
GenASV176	Pseudonocardia	p_Actinobacteriota
CPD-11566	heptaketide pyrone	Polyketide Biosynthesis

Table 1. Nodes network module H+D

DISCUSSION

Plants are sessile organisms responding to environmental stimuli and stresses with consequent rearrangement of the metabolism and an impact on the root-associated and rhizosphere microbiota. These two aspects are tightly connected and can be considered two perspectives of the same response since specific plant metabolites like phenolics, glucosinolates, and strigolactones can shape soil microbes and their interactions (Hartman *et al.* , 2017; Jacoby *et al.* , 2021).

A distinctive adjustment of the specialised metabolism was observed depending on the stress, with more pronounced effects in roots subjected to thermal stress (H). Under limited water conditions (D), oxidative stress may induce the synthesis of phenolic compounds and flavonoids via the increase in the activity of phenylalanine ammonia-lyase (PAL), the key upstream enzyme of the phenylpropanoids pathway (Jun *et al.*2018). Despite showing a general down accumulation of phenolics during thermal stress, our results indicate some sub-classes being enhanced, thus suggesting a specific modulation of this class of metabolites. Nonetheless, accumulating other low molecular weight antioxidants such as ascorbate and glutathione may help the plant mitigate ROS-mediated damage related to limited transpiration related to drought. Overall, changes in phenolic acids and flavonoids also depend on the species, the intensity of the stress and its duration. Recent work demonstrated that different wheat genotypes under water scarcity did not exhibit any significant change in phenolic acids and flavonoids, while others had higher concentrations than non-stressed ones (Laddomada *et al.*2021). In addition, a higher phenolic acids content was detected under severe drought conditions, while moderate drought and severe heat stress did not lead to their accumulation (Shamloo *et al.* 2017).

A general down accumulation was observed in both the single stress conditions concerning N-containing compounds. However, glucosinolates and related compounds were accumulated. These compounds have been reported to potentially affect the rhizosphere communities (Jacoby*et al.* 2021). Interestingly, the production of aliphatic glucosinolates is induced by drought in *Arabidopsis thaliana* , in parallel with phytoalexins repression (Kliebenstein 2004). Together with glucosinolates, a slight modulation of amino acids synthetic

pathways was observed in response to single stresses. Under abiotic stress, amino acids may accumulate as precursors for synthesising specialised metabolites and signaling molecules or as substrates for protein synthesis to promote rapid plant metabolism recovery from stress. Among them, L-methionine was reported to be an effective regulator of plant growth under environmental stress such as drought (Mehak *et al.*2021).

Several phytohormones such as jasmonic acid (JA), abscisic acid (ABA), brassinosteroids (BRs), cytokinins (CKs), and gibberellic acid (GA) have been shown to enhance abiotic stress tolerance (Sharma *et al.*2019). In fact, under high temperature and/or water deprivation conditions, plant responses are mediated by phytohormones, which coordinate complex stress-adaptive signaling cascades (Vile *et al.* 2012) through a complex cross-talk between the different signaling pathways (Verma, Ravindran & Kumar 2016). Several studies focused on hormonal changes during a combination of high temperatures and salinity or high light intensity, agreeing that a coordinated hormonal response to each specific stress combination is essential to trigger the proper acclimation responses. In our conditions, ABA was involved in the plant response to heat and drought stress. Zandalinas *et al.* , 2016 revealed that although ABA is required for the acclimation of Arabidopsis, stomatal closure may also be regulated by H_2O_2. A cross-talk mechanism between gibberellin and abscisic acid during limited water conditions was also reported, in which ABA biosynthesis and the control of stomatal conductance were regulated by the receptor for gibberellin under water stress (Gaion *et al.* 2018). These latter are involved in the adaptive response to various abiotic stresses such as cold, salinity, heat, flooding, and drought, despite their role in drought stress adaptation is still unclear. A cross-talk between ABA and BRs has also been reported (Ha, Shang & Nam 2016). However, decreasing CKs (negative regulators of plant root growth and branching) under H stress can improve plant survival rate by reducing the expression of stress response genes (Liu, Zhang, Meng, Wang & Chen 2020). Similarly, also jasmonates have a significant role in abiotic stress tolerance because of their linkage with other growth regulators, antioxidants and osmoprotectants, especially its conjugate isoleucine-JA, the most active form of JAs (Sharma *et al.* 2019). Notably, phytohormones may also be related to microbial colonization and play a pivotal role in the assembly of plant microbiota (Eichmann, Richards & Schäfer 2021). In turn, the ability of beneficial microorganisms to directly produce CKs, GAs, and ABA, rather than aminocyclopropane-1-carboxylase (ACC) deaminase that cleaves the ethylene precursor, can support plant growth under stress (Pascale, Proietti, Pantelides & Stringlis 2020; Vishwakarma *et al.* 2020; Eichmann *et al.* 2021).

Plants' acclimation to a particular abiotic stress condition involves tailored responses to their specific environmental conditions. Previous literature indicates that a moderate overlap could be observed among transcriptomic responses to abiotic stressors such as drought (Rizhsky*et al.* 2004), cold (Kreps *et al.* 2002), salinity (Yong*et al.* 2002) and light excess (Rossel, Wilson & Pogson 2002). Similarly, despite being recognised as a common response to abiotic stressors, the shift in gene expression patterns related to ROS-triggered responses was differently modulated by stress treatments (Mittler, Vanderauwera, Gollery & Van Breusegem 2004). As in our case, several studies have shown that combined stress responses are mostly related to non-additive effects. Our findings revealed that H and D stress largely modulated root metabolism and triggered different plant responses, while the combined stress did not imply a sum of both stresses but presented a metabolic profile closer to those plants exposed to heat stress. These findings agree with previous studies and confirm that the combination of abiotic stresses is rarely the sum of the single stresses (Fahad *et al.* 2017; Lamaoui *et al.*2018; Zandalinas, Mittler, Balfagón, Arbona & Gómez-Cadenas 2018; Laddomada *et al.* 2021). Indeed, the review by Mittler on abiotic stress combination reports how unique stresses cannot be used to extrapolate combined stresses (Mittler *et al.* 2004). To date, little information is available on the molecular mechanisms underlying the interaction between abiotic stresses, even though the comprehension of such mechanisms is important to facilitate the development of crops with enhanced stress tolerance (Rizhsky *et al.* 2004).

Regarding the specific stress combination in our experiments, the interaction between drought and high temperature is realistic in the context of a climate change scenario, and it is also relevant in terms of stress interaction. During heat stress, stomata are opened to increase transpiration and cool leaves; however, the combination with drought hampers this heat dissipation mechanism and represents a clear example of positive interaction. Surprisingly, our results indicate the hierarchically prevalent effect of heat while confirming the

9

positive interaction. Consistently with our findings on multivariate modelling of the plant metabolome, the interaction between heat and drought is believed to provide unique responses (Rizhsky, Liang & Mittler 2002) and should be considered an independent stress condition. Indeed, tolerance to combined stresses involves the cross-talk among different signal transduction processes that requires multiple controls. This latter point, reflecting synergistic relationships among stresses, has been defined as "cross-hardening"(Bowler & Fluhr 2000). As highlighted by our data, the integration between stresses is rather complex; it involves signaling processes like hormones, mitogen-activated protein kinases (MAPK), and calcium and ROS species (Bowler & Fluhr 2000). Moreover, plants employ complex "stress sensing" mechanisms to detect stresses, depending on the species, organ, and type of stress (Kranner, Minibayeva, Beckett & Seal 2010) and the cross-talk between receptors may also be involved (Casal 2002).

Together with highlighting the non-additive effect of multiple stresses, our results strengthen the concept of whole holobiont response to stress where a coordinated plant-microbiome modulation could be observed. Accordingly, the combined effect of heat and drought stress on the rhizosphere microbiome produced a different outcome compared to single stressors. Concerning Rhizobacteria, the literature on their role in plant abiotic stress mitigation is vast, as recently reviewed (Navarro*et al.* 2006; Dimkpa, Weinand & Asch 2009). At the molecular level, plant perception of eubacterial flagellins can activate plant responses at the gene expression level. Xu et al., suggested that at the earlier development stage, the roots bacterial community is more susceptible than at older plant stages (Xu *et al.* 2018), although mitigation features are stress-dependent and not a *per se* feature of the strains (Rolli *et al.* 2015). While heat affects the rhizosphere microbiome via the host plant (indirectly), drought shapes the bacteria community, directly promoting the enrichment of bacteria belonging to Firmicutes and Actinobacteria (Simmons *et al.* 2020), which are known to be physiologically adapted to drought conditions and that their abundance is positively correlated to plant drought resistance (Naylor, Degraaf, Purdom & Coleman-Derr 2017; Fitzpatrick*et al.* 2018; Hartman & Tringe 2019). Moreover, in accordance with our results, it has been recently reported that under drought stress, endophytic actinobacteria induced artemisinin biosynthesis, which accumulation is known to be involved in modulating drought tolerance (Li *et al.* 2012).

Plants actively exudate compounds that act as attractants for*Rhizobium* species and that may be used as carbon sources by other species, including *Burkholderia* , and their breakdown products might modify the microbial biodiversity and the species abundance (Schütz *et al.* 2021). These rhizobiales-plants interactions significantly mitigate abiotic stresses (Munir *et al.* 2022). Our results on beta diversity highlighted the involvement of Proteobacteria, known to be the main members of Arabidopsis' root microbiota both in the roots and in the soil, followed by Bacteoroidetes and Actinobacteria, as a function of the stress applied. As expected, the magnitude of bacterial microbiota shifts was consistently lower in soil than in root-associated compartments.

The involvement of the root endophyte *Enterobacteriales* , stimulated by both H and H+D treatments, in mediating plant thermotolerance has been recently described (Shekhawat *et al.*2021). They reported that *Enterobacter* sp. SA187 enhanced the H3K4me3 levels at heat stress memory gene loci, which was mediated by ethylene signaling. Similarly, Proteobacteria such as *Aeromonas*sp., which use flavonoids-mediated signaling for host recognition, have been proven to be an enhancer of plant dehydration resistance (He*et al* ., 2022).

Actinobacteria possess a drought-tolerant nature and, under stress, increase the transcription of specific genes and the production of spores highly tolerant to dehydration (Omae & Tsuda 2022). Consistently, we observed an increase in *Actinobacteria* , especially under H treatment. Among monoderm lineages,*Actinobacteria* have been reported to exhibit the strongest enrichment under abiotic stress and to support plant carbohydrate and amino acid transport and metabolism, as well as to positively modulate plant secondary metabolism (Ngumbi & Kloepper 2016; Xu *et al.*2018). The mechanisms by which *Actinobacteria* mitigate abiotic stress in plants include the production of osmolytes to maintain osmotic balance, the synthesis of plant hormones, and enhanced availability of nutrients (Fitzpatrick *et al.* 2018).

Proteobacteria , given their high ability to utilize root exudates, are fast-growing rhizosphere and root colonizers that respond rapidly to organic carbon sources (Bulgarelli, Schlaeppi, Spaepen, Van Themaat &

Schulze-Lefert 2013). Despite having a relatively superior colonization ability within the root and rhizosphere under well-watered conditions, diderm bacteria are less suited to survive the selective pressures caused by drought (Xu *et al.* 2018). However, significant differences are present at the genus level, where the structure of the peptidoglycan cell wall, rather than the presence or absence of an outer membrane, can determine significant differences among microorganisms (Sutcliffe 2010; Xu *et al.* 2018). Interestingly, the thickness and composition of the cell wall have been linked to a different tolerance to ROS species, one of the mechanisms proposed in the differences observed at the rhizomicrobiota level under abiotic stress (Shade *et al.* 2012; Fitzpatrick *et al.*2018). Consistently, proteobacteria were extensively altered by the different stress conditions, including the genera *Pseudomonas*(decreasing), *Azospirillium* , *Rhizobium* ,*Enterobacter* , and *Burkholderia* (all increasing in both roots and soil). Beneficial bacteria like *Azospirillales* ,*Rhizobiales* and *Burkholderia* (all involved in beta-diversity shifts following H and H+D) also led to the production of osmoprotectants like proline, betaine, trehalose, and glycine (Zulfiqar, Akram & Ashraf 2020). Similarly, *Enterobacter* can promote stress tolerance, likely because of its phosphate-solubilizing ability (Dolkar, Dolkar, Angmo, Chaurasia & Stobdan 2018). In our experiment,*Enterobacterales* were predominant in H+D stressed roots, while*Rhizobiales* characterized H conditions. At the molecular level, these microorganisms can increase plant biomass under abiotic stress by shaping the phytohormone profile and improving the antioxidant machinery (Brilli *et al.* 2019).

Despite the evident coordinated modulation of plant metabolome and metabolome in root-associated compartments, our results must be considered in the framework of a strong niche differentiation during plant development. Indeed, the root and rhizosphere communities undergo an initial period of dynamic recruitment followed by a later period of relative stability. The roots grown under pre-flowering drought treatment showed a more pronounced reshape of microbiota than post-flowering drought treatments (Xu *et al.* 2018; Francioli*et al.* 2021; Lewin *et al.* 2021; Francioli *et al.*2022). While confirming the orchestrated response of the whole holobiont to a combination of abiotic stressors, based on the studies mentioned above and our work, it would be very promising to investigate dynamics occurring as a function of plant stage at root and rhizosphere microbiota level.

Conclusion

Plants adapt to unfavorable environmental conditions by dynamic development at physiological, biochemical, and morphological levels. These complex responses involve several signaling, metabolites, transcription factors, and hormones. Our results strengthen the concept of stress interaction, pointing out as the combined heat and drought stress is not merely the combination of the single stresses but rather the result of a multi-level interaction that involves specialized metabolites and a complex remodelling of phytohormones. Intriguingly, the metabolomics signatures were shaped in a coordinated manner with root and rhizosphere bacterial microbiota, suggesting the complete plant holobiont should be considered when studying the responses of plants to abiotic stresses. In fact, a complex series of stress sensors and stress signaling processes is likely orchestrating the responses at plant and microbiota levels to mediate plant microbiota assembly and promote a harmonized modulation of stress mitigation processes.

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AUTHOR CONTRIBUTIONS

DATA AVAILABILITY

https://www.ebi.ac.uk/metabolights/MTBLS6421.

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TABLES

Node	Genus/Metabolite	Phylum/Pathway
CPD-12423	2-sinapoyloxy-3-butenylglucosinolate	Nitrogen-Containing Compounds
CPDQT-406	(E)-1-(L-cystein-S-yl)-N-hydroxy-omega (methylsulfanyl)pentan-1-imine	Nitrogen-Containing Compounds
GenASV17	Azospirillum	p_Proteobacteria
GenASV128	Noviherbaspirillum	p_Proteobacteria
GenASV58	Delftia	p_Proteobacteria
GenASV100	o_Enterobacterales|f_|g_un	p_Proteobacteria
GenASV5	f_Enterobacteriaceae|g_un	p_Proteobacteria
GenASV176	Pseudonocardia	p_Actinobacteriota
CPD-11566	heptaketide pyrone	Polyketide Biosynthesis

Table 1. Nodes network module for H+D
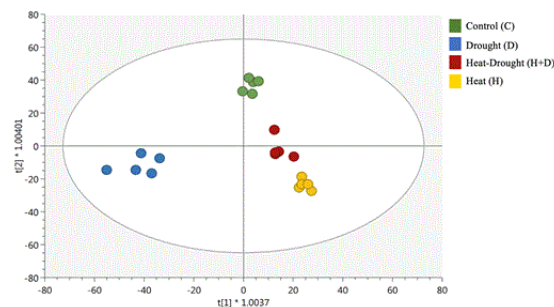
FIGURE LEGEND

16

Figure 1 .Score plot of orthogonal projection to latent structures discriminant analysis (OPLS-DA) supervised modeling carried out on untargeted metabolomics profiles of Arabidopsis roots exposed to heat, drought, and combined stresses ($R^2Y = 0.96$, $Q^2Y = 0.91$).
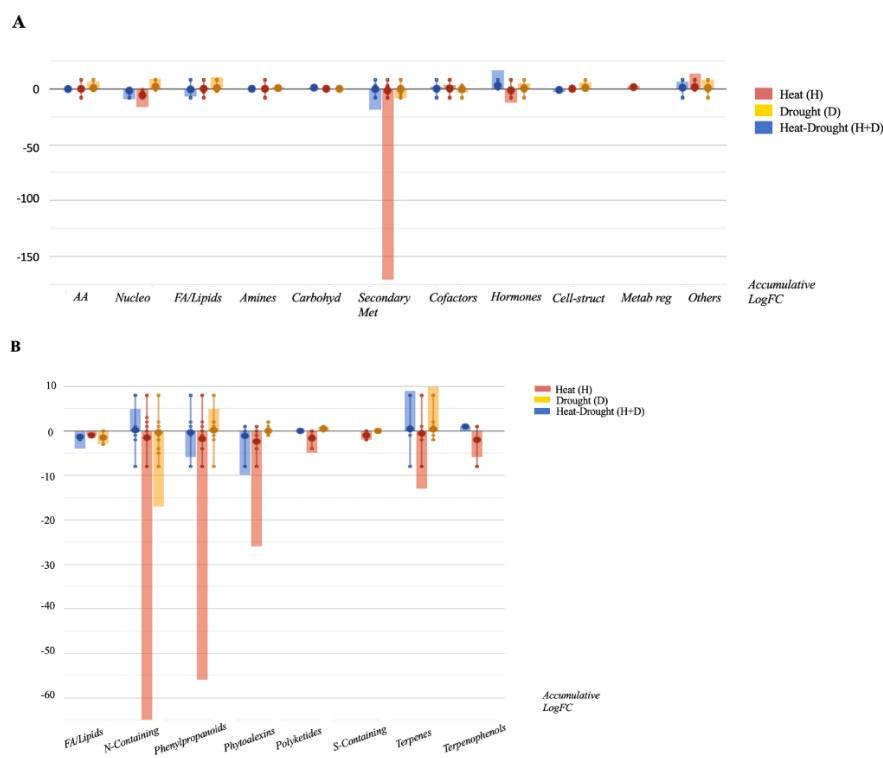

Figure 2. Metabolic processes (A) and sspecialised metabolite biosynthesis (B) modulated Arabidopsis thaliana roots exposed to heat, drought and the combined stress (heat+ drought). The metabolomics dataset produced through UHPLC-ESI/QTOF-MS was subjected to ANOVA and FC analysis ($p < 0.05$, FC [?] 2), and differential metabolites were loaded into the PlantCyc Pathway Tool (https://www.plantcyc.org/). The x axis represents each set of metabolic subcategories, while the y axis corresponds to the accumulative log fold change (FC). The large dots represent the average (mean) of all FCs for the different metabolites in the

17

class, while the small dots represent the individual log FC. The abbreviated subcategory names on the x axis correspond to:AA: amino acids; Nucleo: nucleosides and nucleotides; FA/lipids: fatty acids and lipids; Amines: amines and polyamines; Carbohyd: carbohydrates; Secondary met: secondary metabolism; Cofactors: cofactors, prosthetic groups, electron carriers, and vitamins; Cell-struct: plant cell structures; Metab reg: metabolic regulators; FA/lipids: fatty acid derivatives; N-containing: nitrogen-containing specialised metabolism; S-containing: sulfur-containing specialised metabolism; Terpenophenol: terphenophenolic compounds. The metabolomic dataset produced through UHPLC-ESI/QTOF-MS was subjected to volcano plot analysis (p < 0.05, fold-change > 2) and differential metabolites loaded into PlantCyc Pathway tool.
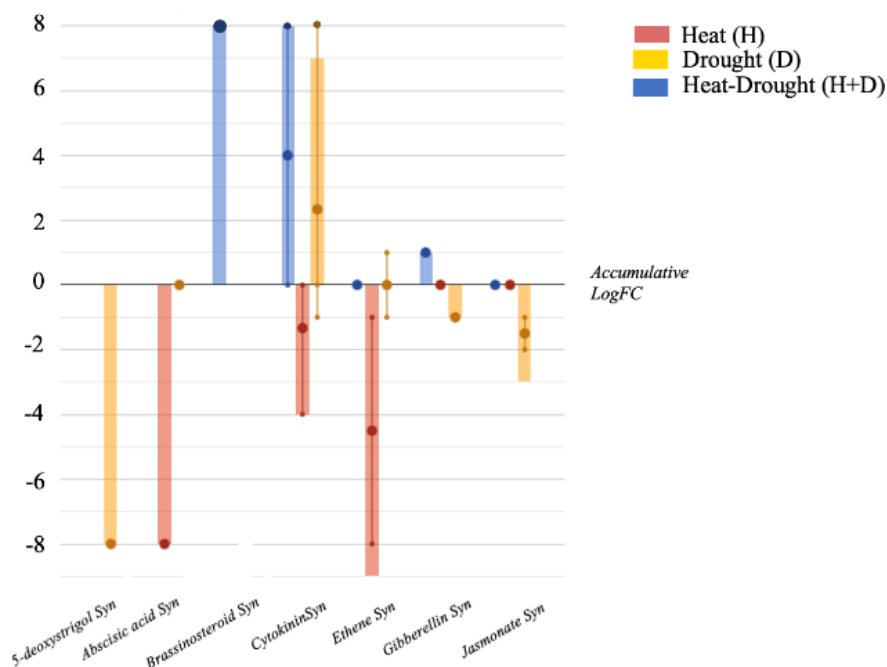

Figure 3. Hormones biosynthesis modulated in Arabidopsis thaliana roots exposed to heat, drought and the combined stress (heat+ drought). The metabolomics dataset produced through UHPLC-ESI/QTOF-MS was subjected to ANOVA and FC analysis (p < 0.05, FC [?] 2), and differential metabolites were loaded into the PlantCyc Pathway Tool (https://www.plantcyc.org/)The x axis represents each set of metabolic subcategories, while the y axis corresponds to the accumulative log fold change (FC). The large dots represent the average (mean) of all FCs for the different metabolites in the class, while the small dots represent the individual log FC.
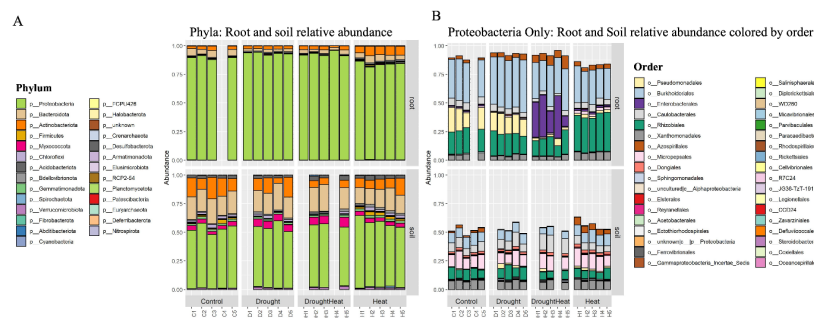
Figure 4 . Taxa barplots for Samples from root and rhizosphere soil. Relative abundances of bacterial taxa identified based on reference to SILVA database v138. Prior to plotting abundance tables were filtered for rare ASVs and outlier samples were removed. A) Phylum level barplot shows relative abundances of identified taxa aggregated on phylum level for root (top) and soil (bottom) compartments for each replicate grouped by treatment. The bacterial microbiota was dominated by Proteobacteria. B) Relative abundances of only Proteobacteria, colored by order level for a detailed resolution of Proteobacteria taxa composition.
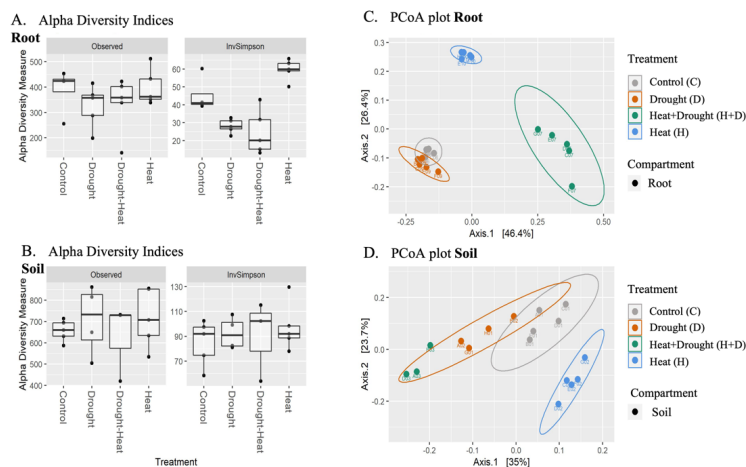

Figure 5. Boxplots of Alpha- and Betadiversity for treatment groups based on observed ASV and Inverse Simpson index for A) root and B) soil. Alpha diversity indices were calculated based on filtered, rarefied data. Bray Curtis dissimilarity indices were calculated for filtered and rarefied root C) and soil D) datasets and plotted using PCoA. Samples groups have a 95% confidence ellipse drawn around them.
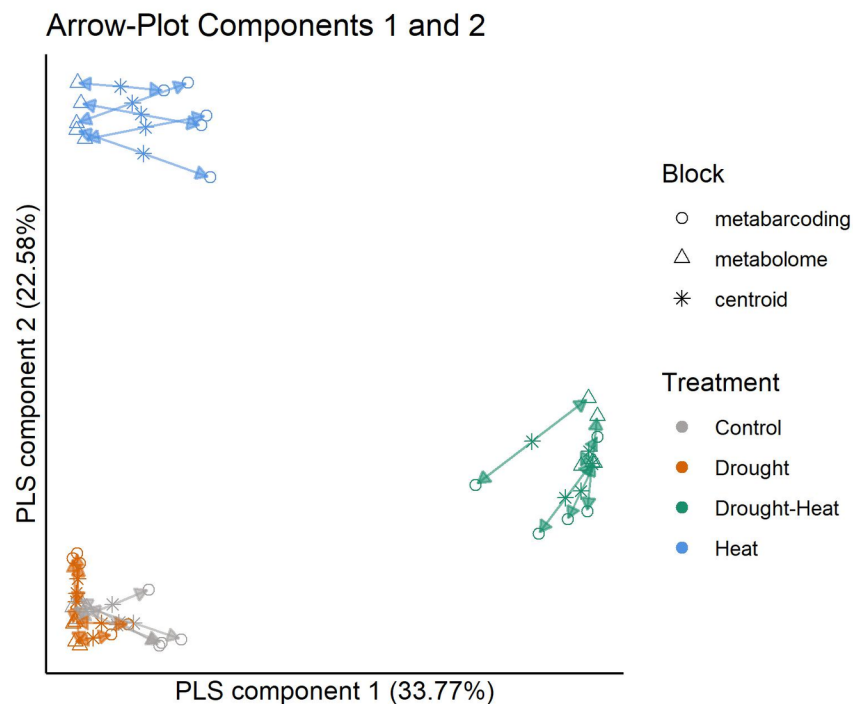
19

Figure 6. Arrow Plot from multiblock sPLS-DA (DIABLO). Samples of the data blocks metabarcoding and metabolomics are plotted into the space spanned by the first two components of the model. The length of the arrows indicates the distance of each sample from the centroids of both datasets. Short distances indicate high levels of agreement between data metabolimics and metbarcoding blocks.
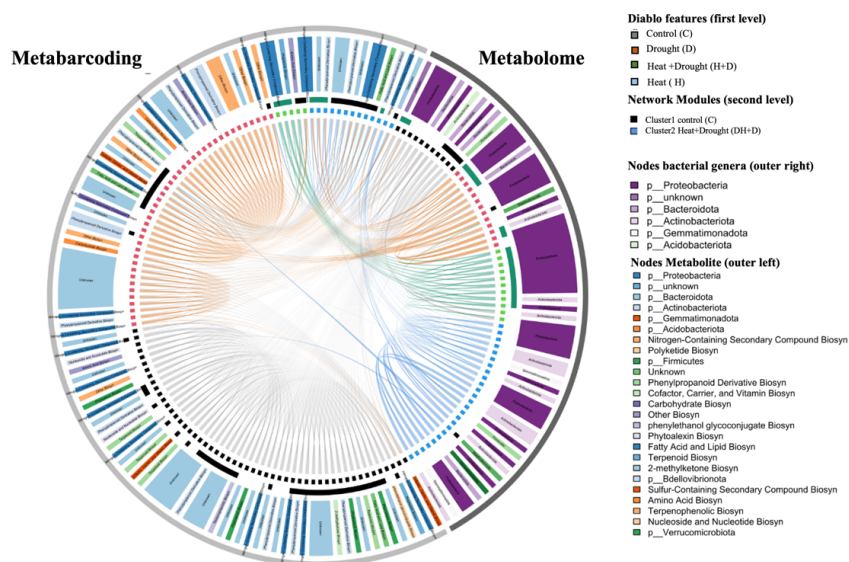

Figure 7: Circular heatmap shows median abundance values of selected features within treatment groups

drought (D), Heat (H), Drought-Heat (H+D) and control (C). Colour track inside shows the feature phylum association for metabarcoding block and functional categories for metabolome features. Chord diagram depicting pearson network analyses of plant metabolites and bacterial genera identified as significant features during DIABLO analyses for treatments. Chords represent correlation large 0.7 with size scaled to r. Inner circle shows network nodes coloured b DIABLO features, second level indicates network modules representative of C and H+D treatment. Outer cirle describes phylum and class of bacterial genera and metabolites.

SUPPLEMENTARY MATERIAL

Supplementary Table S1. Whole dataset produced from untargeted metabolomics carried out in Arabidopsis roots exposed to heat, drought and the combined stress. Compounds are presented with individual intensities and with composite mass spectra (monoisotopic accurate mass/abundance combinations).

Supplementary Table S2. Discriminant metabolites (VIP markers) identified by the OPLS-DA analysis in Arabidopsis roots exposed to heat, drought and combined stress

Supplementary Table S3. Differential metabolites derived from ANOVA and fold-change (FC) analysis (p-value < 0.05, Benjamini multiple testing correction; fold-change threshold FC [?] 2) in roots metabolomic profiles after stress exposure. These compounds were uploaded into the Omic Viewer Pathway Tool of PlantCyc (Plant Metabolic Network, http://www.plantcyc.org/).

Supplementary Figure S1. Unsupervised hierarchical cluster analysis (Euclidean distance; linkage rule: Ward) carried out from root chemical profiles exposed to heat, drought and the combined stress. Metabolites were obtained by UHPLC-ESI/QTOF-MS untargeted analysis, and their intensities used to build up the fold-change heatmap here provided.

Supplementary Figure S2: Cladogram of LEfSe Biomarker analysis in root and soil. Taxonomic nodes that explain the most variance and are indicative for a certain treatment are coloured by treatment. Each ring represents a taxonomic level starting from phylum (p__) over class (c__), order (o__), family (f__) to genus (g__). The described analysis was performed with LDA score cut-off of 3, but only marker with an LDA score > 4 are depicted in this cladogram to avoid an overcrowded image.

Supplementary Figure S3 : Venn diagram showing the number of features shared between LefSe (red) and ANCOMBC (grey) analyses. Filtered soil and root datasets were aggregated at genus level and analysed by discriminant analysis (LEfSe, grey) with LDA cut-off set to 2. For differential analysis p-values were adjusted for multiple testing using holm-Bonferroni method and p-value cut-off = 0.01 was chosen the define significance.

Supplementary Figure S4 : Plotted effect Size of Biomarkers identified by LefSE analysis for A) root and B) soil with an LDA score over 3. Color indicates the treatment they discriminant for size of dot represent p-value. The marker taxonomic lineage is displayed on 3 levels.

Supplementary Figure S5 : multiblock sPLS-DA (DIABLO). Samples from data blocks metabarcoding and metabolomics are plotted into space spanned by the second and third components. Length of the arrows indicate the distance of each sample from the centroids of both datasets. Short distances show a high level of agreement between blocks. For all samples and treatments the agreement between metabarcoding and metabolomics is high.