Drought, heat, and their combination impact the root exudation patterns and rhizosphere microbiome in maize roots

*Original*
Drought, heat, and their combination impact the root exudation patterns and rhizosphere microbiome in maize roots / Tiziani, R., Miras-Moreno, B., Malacrinò, A., Vescio, R., Lucini, L., Mimmo, T., Cesco, S., Sorgona', A.. - In: ENVIRONMENTAL AND EXPERIMENTAL BOTANY. - ISSN 0098-8472. - 203:(2022), p. 105071. [10.1016/j.envexpbot.2022.105071]

*Published*
DOI: http://doi.org/10.1016/j.envexpbot.2022.105071

The final published version is available online at:https://www.sciencedirect.

# Drought, heat, and their combination impact the root exudation patterns and rhizosphere microbiome in maize rootsAbiotic stress affects the maize root exudation

Raphael Tiziani, Begoña Miras-Moreno, Antonino Malacrinò, Rosa Vescio, Luigi Lucini, Tanja Mimmo, Stefano Cesco, Agostino Sorgonà

PII: S0098-8472(22)00293-3
DOI: https://doi.org/10.1016/j.envexpbot.2022.105071
Reference: EEB105071

To appear in: *Environmental and Experimental Botany*

Received date: 13 April 2022
Revised date: 25 July 2022
Accepted date: 13 September 2022

Please cite this article as: Raphael Tiziani, Begoña Miras-Moreno, Antonino Malacrinò, Rosa Vescio, Luigi Lucini, Tanja Mimmo, Stefano Cesco and Agostino Sorgonà, Drought, heat, and their combination impact the root exudation patterns and rhizosphere microbiome in maize rootsAbiotic stress affects the maize root exudation, *Environmental and Experimental Botany,* (2022) doi:https://doi.org/10.1016/j.envexpbot.2022.105071

# Drought, heat, and their combination impact the root exudation patterns and rhizosphere microbiome in maize roots

## Running title: Abiotic stress affects the maize root exudation

Raphael Tiziani[1,†], Begoña Miras-Moreno[2,†], Antonino Malacrinò[3,4], Rosa Vescio[4], Luigi Lucini[2], Tanja Mimmo[1], Stefano Cesco[1], Agostino Sorgonà[4,*]

[1] Faculty of Science and Technology, Free University of Bolzano, 39100 Bolzano, Italy

[2] Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

[3] Institute for Evolution and Biodiversity, Westfälische Wilhelms-Universität Münster, Münster, Germany

[4] Dipartimento Agraria, Università Mediterranea di Reggio Calabria, Località Feo di Vito, 89122 Reggio Calabria

***Corresponding author:** asorgona@unirc.it

[†]Equal contributing first-co-authorship

## Abstract

Root exudates play an essential role in helping plants to cope with abiotic stress. However, the modulation of root exudation profiles under multiple stress conditions is still poorly understood. Using targeted and untargeted metabolomics, here we tested the effect of drought, heat stress, and their combination on maize root exudates, also considering the differences that might exist between root types (seminal and primary) and root zones (apical and sub-apical). In addition, we built an analytical framework that relates the root exudation profile with the rhizosphere bacterial community, enabling us to dissect the interactions between specific root exudates and bacterial taxa. The composition of root exudates undergoes distinct modulation according to the single or combined stress and to the root zone, but not according to the root type. In addition, we found that stress-specific exudates can influence the relative abundance of specific bacterial taxa, some of which are known to be beneficial microorganisms. Our results contribute to the understanding of plant-soil interactions under the influence of abiotic stressors, which is key in paving the way towards an increased resilience to abiotic stresses, representing a powerful tool to craft the next generation of agricultural practices.

**Keywords**:

root exudation profile, microbiota, metabolomics, combined stress, root types, *Zea mays* L.

## Introduction

Reduced water availability and the increase in global temperature cause every year substantial agronomic and economic losses worldwide (Jägermeyr *et al.* 2021), and are among the major challenges we are currently facing (IPCC 2018). The effect of single abiotic stresses on plants has been widely dissected over the years. However, plants are rarely exposed to single stresses but rather to a combination of multiple stresses. Several studies have shown that combined stress responses produce non-additive effects (*i.e.*, synergistic or antagonistic). Therefore, the outcome cannot be predicted based on studies on single stressors (Rivero *et al.* 2014; Pandey *et al.* 2015). Hence, it is important to understand how plants respond to the combination of abiotic stress.

The modulation of root exudation is one of plants' most important physiological traits when interacting with the environment (Vives-Peris *et al.* 2019). Root exudates include primary and secondary metabolites which directly influence the rhizosphere's chemical, physical, and biological characteristics (Oburger and Jones 2018; R. Tiziani *et al.* 2020; R Tiziani *et al.* 2020). Despite the large interest in drought- and heat-tolerant crops, only a few studies focused on the root exudation responses to these abiotic stresses (Vives-Peris et al., 2017; Williams & de Vries 2020). Canarini *et al.* (2016) found that sunflower plants exposed to drought increased the amounts of exudates released but the composition remained unaltered, while soybean plants altered the profile of root exudates when exposed to stress without modifying the exudation rate. Under drought stress, *Quercus ilex* plants mainly exuded secondary metabolites, whereas under water recovery conditions, the exudation was shifted to primary metabolites (Gargallo-Garriga *et al.* 2018). Research on the effects of heat stress on root exudation is even more scarce and focused on specific metabolites rather than the whole metabolome. For example, under heat stress, citrus plants have been shown to increase the release of proline, salicylic acid, indole acetic acid, abscisic acid, and jasmonic acid (Vives-Peris *et al.* 2017). While previous studies contributed to the knowledge on the effects of either drought or heat stress on plant root exudates, we still know little about the combination of these abiotic stressors on this essential physiological process, despite the common co-occurrence of drought and high temperatures in field conditions.

Most studies on root exudates do not consider different root classes or types (e.g., primary, secondary, nodal, lateral), which are characterized by distinct developmental, physiological, and functional signatures (Waisel, Y.; Eshel, A. and Kafkafi 2003). For example, several studies reported different morpho-physiological responses among root types to drought stress (Zhan et al., 2015), allelochemicals (Lupini *et al.* 2016), phosphorus deficiency (R. Tiziani *et al.* 2020), and the combination of nitrogen deficiency and drought stress (Lynch 2013). In addition roots also show diversification along the root axis (Sorgonà *et al.* 2011; R. Tiziani *et al.* 2020). Still, few studies considered the within-root variability of the exudation processes, and no studies explored the metabolic profiles of root exudation within different root types and zones. Thus, understanding the variation of exudate profiles among and along the diverse root types will allow to better predict the impact of the root architecture on plant-soil interactions.

Root exudation mediates the interaction between plants and the microbial communities, impacting plant growth and fitness (Sasse *et al.* 2018; Sun *et al.* 2021; Kawasaki *et al.* 2021). The changes in root exudates driven by abiotic stress might impact plant-microbiome interactions in the rhizosphere. This has been suggested by several studies focusing on drought (Christian *et al.* 2021), salinity (Yuan *et al.* 2019) and nitrogen deficiency (Wu *et al.* 2021), including our previous work on drought, heat stress and their combination (Vescio, Malacrinò, *et al.* 2021a). However, we still know little about the effects of single and combined stresses on root exudates, and how this reflects on the rhizosphere microbiome. Here, we tested the effect of drought, heat stress, and their combination on the root exudation profile of maize plants, also considering the differences that might exist between root types (seminal and primary) and root zones (apical and sub-apical). We hypothesize that (i) drought and heat stress will induce changes in the composition of root exudates; (ii) the combination of the two stresses will generate a different exudation profile than each single stress; (iii) these responses will vary according to different root types and zones. In addition, here we also test the effects of changes in root exudates on the rhizosphere microbiome, leveraging the data generated by our previous study (Vescio *et al.* 2021a). Furthermore, according to previous studies suggesting a role of root exudates in recruiting beneficial microbes, we hypothesize that (iv) changes in stress-specific root exudates will correlate with changes in the relative abundance of bacterial taxa that might have a beneficial effect on maize plants.

## MATERIAL AND METHODS

## Experimental setup

The experiment was conducted using maize plants (*Zea mays* L., genotype KXB7554 provided by KWS Italia) as a model. Plants were grown in pots filled with a mix of collected field soil and quartz sand (1:2, v:v). The soil physicochemical parameters are reported in Gelsomino et al. (2010). Quartz sand (Ø1-2 mm, Croci Trading Company s.r.l., Italy) was previously autoclaved for 3 hours at 121 °C, allowed to cool overnight, and then autoclaved for a further 3 hours at 121 °C. Plants were initially grown for 2 weeks at 25 °C, 70% relative humidity and at 14h:10h light:dark photoperiod. Then, plants were exposed to two levels of water availability (30% and 80% of soil water holding capacity, corresponding to a severe drought and no drought) and air temperature (25 °C and 32 °C) for one week. Each treatment group included 5 plants. From each plant, we then collected samples from both the apical and subapical root zones. In total, the experiment yielded 4 (treatments, 2 water availability treatments × 2 air temperature treatments) × 2 (root types, primary and seminal roots) × 2 (root zones, apical and subapical) × 5 (replicates) = 80 samples.

## Root exudates sampling

After 7 days of exposure to treatments, root exudates were collected by a soil-hydroponic hybrid approach (Oburger and Jones 2018) using a compartmented chamber (Fig. 1). This type of chamber has been widely used for root exudate collection in previous studies (Mariano and Keltjen 2003; Liao *et al.* 2006; R. Tiziani *et al.* 2020), but also to study the phosphorus (R. Tiziani *et al.* 2020) and nitrate uptake (Sorgonà *et al.* 2011; Schneider *et al.* 2017b), and water transport (Schneider *et al.* 2017a). Maize plants were sampled and the whole root system was carefully washed after removing the soil. Following this, the single primary and seminal roots attached to the shoot were placed on two different compartmented chambers (2×10×2 cm) with five cells, each 1.8 cm deep and 1.8 cm wide (Fig. 1A). Each cell was connected to the next one by a shallow bridge so that a root could be laid out across the block traversing all the cells. The intact primary and seminal root axes were inserted in separate chambers. The area starting from the root tip to the first visible lateral root (apical root zone) was isolated from the remaining root portion (subapical root zone) by silicon grease (Dow Corning, Midland, MI, USA) (Fig. 1A and 1B). This procedure allowed us to collect exudates from different root types and zones: seminal-apical, seminal-subapical, primary-apical, and primary-subapical.

Before beginning the exudate collection, we submerged the apical root zone with the trap solution (0.5 mM $CaCl_2$) to detect possible leaks between the cells hosting the apical and sub-apical root zones. During the exudates collection, the roots were continuously aerated. To collect root exudates, cells were filled with 6 mL of the trap solution, and every 30 minutes the trap solution was collected for up to 4 hours, yielding ~48 mL from each root zone. This solution was then centrifuged (15 min at 13,000 rpm at 4 °C), the supernatant was freeze-lyophilized, then resuspended in 5 mL 1:1 $H_2O/CH_3OH$ solution, filtered with 0.45 µm syringe filters (Phenex-RC 0.45 µm, Phenomenex), and then used for the targeted and untargeted metabolomics analyses.

## Targeted characterization of root exudates

### *Total phenols*

Total phenol content was estimated following the Folin-Ciocalteau method (Atanassova *et al.* 2011). Briefly, 200 µL of each sample or gallic acid standard solution was mixed with 1.4 mL deionized water and 100 µL Folin-Ciocalteau's phenol reagent (2 mol $L^{-1}$, Sigma Aldrich, Italy). Total phenol content was estimated using a spectrophotometer (Agilent Technologies, Cary Series 100 UV-VIS Spectrophotometer, Italy) by measuring the absorbance at 765 nm, then expressing the results as µmol of gallic acid equivalent.

### *Total flavonoids*

Total flavonoids content was estimated by a pharmacopeia method (Miliauskas *et al.* 2004). Rutin hydrate (purity ≥ 94%, Sigma Aldrich, Italy) was used as the reference compound. Briefly, 160 µL of each sample or a rutin standard solution was mixed with 160 µL $AlCl_3$ (20 g $L^{-1}$ in ethanol, Sigma Aldrich, Italy) and 3.68 mL ethanol (Sigma Aldrich, Italy). Samples and standards were incubated at 20°C for 40 min, and absorbance was read at 415 nm using a spectrophotometer (Agilent Technologies, Cary Series 100 UV-VIS Spectrophotometer, Italy). Results were expressed µmol rutin equivalent.

### *Total flavonols*

The content of total flavonols was estimated following the Yermakov method (Mickelsen and Yamamoto 1958). Rutin hydrate (Sigma Aldrich, purity ≥ 94%) was used as reference standard. Briefly, 200 µL of sample or rutin standard was mixed with 200 µL $AlCl_3$ (20 g $L^{-1}$ in ethanol, Sigma Aldrich) and 600 µL sodium acetate (50 g $L^{-1}$, Sigma Aldrich). Samples and standards were incubated at 20 °C for 2.5h, and absorbance was measured at 440 nm using a spectrophotometer (Agilent Technologies, Cary Series 100 UV-VIS Spectrophotometer, Italy). Results were expressed as µmol rutin equivalent.

### *Organic acids*

Organic acids were estimated by ionic chromatography with photodiode array detection (Waters 2998, Waters Corporation, Italy). Separation was achieved on a cation exchange column (Aminex 87-H column, 300 × 7.8 mm, 9 µm, Bio-Rad) using isocratic elution with 10 mM $H_2SO_4$ as mobile phase, at a flow rate of 0.6 mL $min^{-1}$. Detection was carried out at 210 nm. Standard carboxylates were prepared as individual stock solutions using Sigma Aldrich (Italy) reagent grade compounds, and then combined to produce diluted working standards to be used for retention time comparison and for quantification.

### *Amino acids*

Amino acids analysis was performed by liquid chromatography prior to pre-column derivatization with a commercial kit (AccQ Tag, Waters Corporation, Italy) according to the manufacturer's instructions and using a Nova-PakTMC18 column (Waters Corporation, Italy). Gradient elution with a flow rate of 1.0 mL min$^{-1}$ used a mixture of: A = AccQ Tag Eluent; B = 100% acetonitrile (HPLC grade), C = HPLC grade I water (Waters application note for amino acids). Gradient summary: 100:0 A/B to 83:17 A/B from min 0 to 38. Then 60:40 B/C from min 38.01 to 55. The derivatized amino acids were detected by fluorescence ($\lambda_{ex}$ = 250 nm, $\lambda_{ems}$ = 395 nm; 2475 Multi λ Fluorescence Detector, Waters Corporation, Italy) and quantified by the external standard method using a pure amino acids standard mix.

**Untargeted profiling of root exudates**

Root exudate composition was investigated through ultra-high-pressure liquid chromatography (UHPLC) coupled to a quadrupole-time-of-flight (QTOF) mass spectrometry as reported by Astolfi et al. (2020) with minor modifications. We used a 1290 series LC system equipped with a binary pump, a JetStream Electrospray source, and a G6550 iFunnel QTOF mass spectrometer (Agilent technologies, Santa Clara, CA, USA) working in positive SCAN mode (100–1200 m/z range). Briefly, the chromatographic separation was achieved by reverse phase Agilent PFP column (2.0 × 100 mm, 3 μm) using a gradient of water and methanol (from 6% to 94% organic phase in 33 min, flow rate 200 μl min$^{-1}$). Quality control pooled samples were also analyzed in MS/MS mode (data-dependent, 12 precursors per cycle at 1 Hz, 50–1000 m/z, positive polarity, active exclusion after 2 spectra), with collision energies of 10, 20, and 40 eV (García-Pérez *et al.* 2021).

Compounds were annotated from raw mass features according to Astolfi et al. (2020), using the software Profinder B.07 (from Agilent Technologies, Santa Clara, CA, USA) according to the 'find-by-formula' algorithm following mass and retention time alignment. To this aim, the whole isotope pattern (monoisotopic mass, isotopic spacing and isotopic ratio) was used. A custom database produced by combining compounds exported from PlantCyc 9.6 (Plant Metabolic Network, http://www.plantcyc.org; accessed April 2017), Phenol-Explorer 3.6 (http://www.phenol-explorer.eu; accessed April 2017), as well as information on compounds extracted from the literature and those that might be present in root exudates was used as a reference for annotation (5-ppm mass accuracy tolerance). The annotation level corresponded to Level 2 of accuracy (putatively annotated compounds) as established by the COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi). Only those compounds identified in at least 75% of replications and in at least one treatment were retained. The MS-DIAL 4.24 software, together with publicly available built-in MS/MS experimental spectra (Mass Bank of North America), was used to confirm features obtained using Profinder B.07. (Tsugawa *et al.* 2015).

### Data analysis

Data from targeted analyses were corrected using the dry biomass of each root sample, tested for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Leven median test). Thereafter, three-way ANOVA was used to investigate the effect of treatments (control, drought, heat, and combined), root type (primary and seminal), root zones (apical and sub-apical) and their interactions. The Tukey’s HSD was used to test pairwise contrasts.

The untargeted metabolomics analyses were performed according to García Pérez *et al.* (2021). Briefly, raw mass features were elaborated using the software Agilent Mass Profiler Professional B.12.06 for normalization and baselining, and then similarities among treatments were visualized using the multivariate unsupervised hierarchical cluster analysis (HCA, Euclidean distance, Ward’s linkage rule). A Volcano plot ($\alpha = 0.05$; fold change $\geq 1.2$) was generated from the differential abundance analysis to help identifying compounds varying significantly between treatments and control. In addition, supervised modeling by Orthogonal Projection to Latent Structure Discriminant Analysis (OPLS-DA) was performed using the SIMCA 16 software tool (Umetrics, Sweden). The Variable Importance in Projection (VIP) analysis was used to further identify markers responsible for the discrimination between groups. The multivariate model was then cross-validated by Cross Validation-Analysis of Variance (CV-ANOVA, $\alpha < 0.01$).

## Integration of metabolome and microbiota datasets

The rhizosphere microbiota dataset was obtained from our previous study (Vescio, Malacrinò, *et al.* 2021a). Plants from the present and the previous experiment were grown together (same pot) and were then harvested to test the influence of abiotic stresses on the rhizosphere bacterial community (Vescio, Malacrinò, *et al.* 2021a) and root exudates (this study). Thus, we are confident that both datasets can be safely integrated to test for interactions between root exudates and rhizosphere microbiota. In our previous work, libraries targeting the V4 region of the bacterial 16S rRNA were obtained from DNA extracted from rhizosphere soil (McPherson *et al.* 2018), and sequenced using an Illumina MiSeq platform (San Diego, CA, USA) using the 300PE chemistry. De-multiplexed forward and reverse reads were merged using the PEAR 0.9.1 algorithm using default parameters (Zhang *et al.* 2014). Data processing was carried out using VSEARCH 2.14.2 (Rognes *et al.* 2016) to quality-filter reads, discard chimeric sequences, bin OTUs (97% cutoff), and assign taxonomy by querying the SILVA database (v. 132) (Quast *et al.* 2013).

Data analysis was performed using R statistical software 3.5 (R Core Team 2013). The microbiota dataset was processed to remove singletons and OTUs generated from the amplification of plastidial rRNA. This dataset was then normalized using DESeq2 (Love *et al.* 2014). The metabolome dataset was also processed to remove those metabolites that were not found in at least 25% of the samples and normalized using a log transformation. Then, a framework that considers both metabolomic and metabarcoding datasets was built to finely investigate the interaction between root exudates and the rhizosphere bacterial community. This framework was built using MOFA+ (Argelaguet *et al.* 2018), a factor analysis that infers the latent signal from heterogeneous datasets (i.e. microbiota and metabolome) to identify driving sources of variation across different datasets and factors. In this study, MOFA+ captured three factors explaining a high proportion of variation in both molecules and taxa in the three treatments (Supplementary material 1). This helped us to organize both metabolites and bacterial OTUs according to their contribution to explain variation within each treatment. We used this information to select the top 10% molecules and 0.1% bacterial taxa that mostly contribute to explaining the variation due to the treatments. We selected these cut-offs as they capture, on average, the metabolites/OTUs that explain most of the variation within each treatment, and are thus more likely to have a biological role. Then, we tested the correlation of the relative abundance of each OTU/metabolite combination within each treatment (two-tailed Pearson's correlation), selecting those correlations with $p<0.05$.

## RESULTS

### Targeted characterization

The targeted analysis successfully detected and quantified aconitic acid, total phenols, flavonoids and flavonols, glycine, threonine and alanine in all the samples (Fig. 2). Formic and lactic acid were also detected, yet only in a few samples. Furthermore, we could detect but not quantify serine, histidine, proline, tyrosine and phenylalanine.

Aconitic acid amount was significantly influenced by treatment (Fig. 2a and Supplementary material 2). Plants exposed to heat stress exuded 107% more aconitic acid than those exposed to drought stress, while control plants and those exposed to combined stress showed intermediate values. Also, the exudation of aconitic acid was influenced by the root zone, with the apical zone having exuded 151%

more aconitic acid than the sub-apical one. Similarly, the total phenol content in root exudates was higher in plants exposed to heat than those exposed to drought (+120%), while the other two groups showed intermediate results. Also, in the case of total phenols, the amount was higher in the apical zone (+157%) compared to the sub-apical zone (Fig. 2b and Supplementary material 2).

The amount of total flavonoids was higher in control plants (Fig. 2c and Supplementary material 2), being nearly 4-fold higher when compared to those exposed to drought. Plants exposed to heat stress showed intermediate values, while no flavonoids were detected (<LOD) in plants exposed to combined stress. Also, in this case, the amount of total flavonoid was higher in exudates collected from the apical zone than in the sub-apical one (+295%). Similarly, the amount of threonine in root exudates followed a similar trend as total flavonoids (Fig. 2d and Supplementary material 2). Indeed, control plants exuded significantly more threonine than plants exposed to drought (+105%), while both plants exposed to heat and those exposed to combined stress exhibited intermediate values. In addition, the apical zone exuded more threonine than the sub-apical one (+246%, Figure 2d and supplementary material 2). Glycine and alanine exudation were significantly influenced by the root zone, with higher amounts exuded in the apical zone (+213%) compared to the sub-apical zone (Fig. 2e and 2f and Supplementary material 2). Total flavonols were not influenced by any factor (Fig. 2g and Supplementary material 2).

### Untargeted metabolomics

The exudate profiling of the different maize root types and zones exposed to single and combined abiotic stress was investigated using UHPLC-QTOF-MS untargeted metabolomics analysis. This approach allowed the putative annotation of 337 compounds, most of which belonged to phenylpropanoids and amino acids (Supplementary material 3). We found a major effect driven by stress treatments (Fig. 3), while only minor differences between root types or root zones (Supplementary material 4). Therefore, we focused our analysis on understanding the differences among treatments. The unsupervised HCA model shows two main clusters, separating control and drought-stressed plants from plants exposed to heat and combined stress (Fig. 3), suggesting that temperature had a hierarchically stronger effect on exudate composition than drought. The supervised OPLS-DA further confirmed this separation as a function of the treatment (Fig. 4). The first latent vector allowed separating control plants and those exposed to drought from heat- and combined-stress plants. However, despite the similarity of exudate profiles between control and drought-stressed plants, the second latent vector indicated that also the soil water content influenced exudate signatures (Fig. 4). The VIP analysis highlighted the most discriminant compounds involved in OPLS-DA score plot separation. The features having a VIP score > 1 were considered as markers. Among these compounds, phenylpropanoids appeared as the major driver (Supplementary material 5). Nevertheless, several organic acids and amino acids were also highlighted as VIP markers. These results are further strengthened by the differential abundance analysis (Supplementary material 6). According to the multivariate statistics (Fig. 3 and Fig. 4), plants exposed to heat and combined stresses presented the largest differences compared to control plants, and analyses suggest that this effect was driven by 32 (heat stress) and 39 (combined stress) metabolites (Supplementary material 6).

Abiotic stressors shaped the exudation of compounds compared to the control conditions (Fig. 5). Although several compounds are down-exuded in the presence of the stresses, the stress-treated plants presented a more pronounced up-exudation of compounds, especially under combined stress. Indeed, while we found 17 down-exuded metabolites, we also found 37 compounds that were up-exuded, with 13 being specific to plants exposed to combined stress (Fig. 5). In general, all stressed plants produced less hispidulin, while 4-hydroxycoumarin, citramalic acid, and malonic acid were exuded in higher amounts (Supplementary material 6).

Regarding the metabolites specifically exuded (Tab. 1), each stress produced a unique signature on the plant exudates profile. Drought reduced the exudation of protocatechuic acid, and dihydroisocoumarin mellein, while promoted the exudation of two conjugated flavonoids and the phytoalexin resveratrol (Tab. 1). Heat stress specifically reduced the exudation of ascorbic acid bisdemethoxycurcumin, and apigenin 7-*O*-(6''-malonyl-apiosyl-glucoside), but increased the amount of 4-vinylphenol, luteolin 7-*O*-(2-apiosyl-6-malonyl)-glucoside, malate, 2,4-dihydroxybenzoic acid, and benzoic acid in root exudates (Tab. 1). Interestingly, the combined stress triggered a distinct exudation pattern, compared to the control or individual stresses. Specifically, 13 metabolites were up-exuded exclusively when plants were exposed to combined stress, most of which were identified as flavonoids, the hydroxycinnamic acid 1,2,2'-Trisinapoylgentiobiose, the alkylphenol 5-nonadecenylresorcinol, palmitic acid, L-tryptophan, sinapine, and the xanthophyll lutein (Tab. 1). A reduced exudation of alpha-aminoadipic acid, sorgolactone, and 6-geranylnaringenin was also specifically observed in combined stress (Tab. 1). Regarding the shared exudate compounds between the stress treatments, we found that the heat stress triggered changes in the exudation pattern of 12 molecules (4 and 8 down- and up-exuded, respectively), similarly to plants exposed to the combined stressors (Tab. 1.); while only the conjugated flavonoid kaempferol 3-*O*-(6''-acetyl-galactoside)7-*O*-rhamnoside was influenced by both drought and the combined stress treatment (Tab .1).

## Interactions between root exudates and rhizosphere microbiota

To test our hypothesis that stress-specific root exudates can influence the relative abundance of bacterial taxa, we built a framework that integrates both microbiota and metabolome datasets. The variance decomposition step revealed three factors explaining a high proportion of the variance, jointly for metabolome and microbiota in the three different treatments (Supplementary material 1). For each treatment, we selected the top 0.1% of bacterial taxa and the top 10% of metabolites that provided the largest explanation for the variation within each factor, and then we correlated the abundance of the bacterial taxa and metabolites.

The analysis of drought-stressed plants suggests that two compounds (p-coumaric acid ethyl ester and L-serine) negatively correlate with several bacterial OTUs. Both compounds were negatively correlated with the same pool of OTUs (classified in the genera *Mucilaginibacter*, *Sphingomonas*, *Acidibacter*, *Paenibacillus*, *Filimonas*, *Labrys*, *Mesorhizobium*, *Heliimonas*, *Rudaea*) with the only difference that L-serine negatively correlated with a single unidentified OTUs, while p-coumaric acid ethyl ester negatively correlated with 2 unidentified bacterial OTUs (Supplementary material 1).

Plants exposed to heat treatment showed five compounds (aminocyclopropane-1-carboxilic, rhamnetin, L-histidine, Quercetin 3-*O*-(6-acetyl-galactoside) 7-*O*-rhamnoside and 2-methoxy-5-prop-1-enylphenol/eugenol) correlating negatively with 23 bacterial OTUs (Supplementary material 1). Interestingly, the first four molecules negatively correlated with a common pool of 22 OTUs (Supplementary Material 1) classified in the genera *Bacillus*, *Bacteriovorax*, *Caulobacter*, *Chitinophaga*, *Dyadobacter*, *Heliimonas*, *Mesorhizobium*, *Micromonospora*, *Niastella*, *Pseudonocardia*, *Rhizobacter* together with 6 OTUs of unidentified bacteria. 2-Methoxy-5-prop-1-enylphenol/Eugenol negatively correlated with 4 OTUs: one belonging to the genus *Alsobacter* and three to *Caulobacter* (Supplementary material 1).

When plants were exposed to combined stress, the analysis suggests that two compounds (L-homocysteine and cinnamoyl glucose) negatively correlate with bacterial OTUs, while a positive correlation was observed only for carnosic acid. L-homocysteine influenced the abundance of 25 OTUs (Supplementary material 1), including *Bacillus*, *Clavibacter*, *Devosia*, *Dietzia*, *Microbacterium*, *Paenibacillus*, *Paracoccus*, *Rhizobium*, *Rhodococcus*, *Stenotrophomonas*. Cinnamoyl glucose and carnosic acid influenced, in the opposite way, a single OTU identified as *Paenibacillus* (Supplementary material 1).

## Discussion

In this study, we show that drought and heat stress differentially influence the composition of maize root exudates and, for the first time, that the combination of these two stresses produces a unique root exudation profile. We found indeed root exudates that respond in the same way regardless the stressor, but also unique signatures for each stress. Moreover, we linked the exudation of specific metabolites to changes in the relative abundance of bacterial taxa and, for each stress, we found a unique set of metabolites that the plant might exploit to modulate the root-associated microbiota. While we focused on a single timepoint for sample collection, and outcomes might be different during a longer time course (Shulaev *et al.* 2008), our results contribute to the understanding of the influence of abiotic stressors on plant-soil interaction at the rhizosphere interface.

### Root exudation patterns shared among drought, heat, and their combination

Regardless the stressor plants were exposed to, we found an increase in the exudation of citramalic acid, malonic acid, and 4-hydroxycoumarin compared to control conditions. Citramalic acid has been reported to be involved in soil phosphorus solubilization (Khorassani *et al.* 2011), suggesting that plants might increase the phosphorous uptake as a way to cope with abiotic stresses. In our study, we also found higher amounts of malonic acid in the root exudates of all stressed plants. Indeed, the exudation of malonic acid as a response to stress has been reported in wheatgrass (Henry *et al.* 2007) and chickpeas (Li and Copeland 2000). We also found that 4-hydroxycoumarin was more abundant in exudates of all our treatments. This compound belongs to the coumarins, also known as allelopathic compounds, which interact with the root microbiota improving nutrient acquisition (Harbort *et al.* 2020) and the root microbiome assembly (Stringlis *et al.* 2019).

We also found that stressed plants reduced the exudation of the amino acids L-serine, threonine, and flavones (hispidulin, in particular). In particular, L-serine was down-exuded under both heat and combined stress, while threonine was down-exuded under both drought and combined stress. While we have not found previous studies supporting this observation, our results also suggest an interaction between several amino acids and rhizosphere microorganisms. Thus, the reduced exudation of L-serine and threonine might represent a way to modulate the interaction between plants and their microbiome during stress events. Alternatively, plants might reduce the exudation of these molecules as an ‘energy saving strategy’, reducing their competition with microorganisms for resources (Doornbos *et al.* 2012). In our study, hispidulin was down-exuded as an effect of all stress treatments compared to control conditions. Hispidulin is a flavone with antioxidant activity and belongs to the flavonoids commonly present in root exudates (Lucini *et al.* 2019), which might help counteracting the oxidative imbalance generated by increased production of reactive oxygen species (Fahad *et al.* 2017).

### Specific response to drought stress

Our results from both targeted and untargeted metabolomics indicated that drought modified the maize root exudation profile, although with a lower magnitude compared to heat or combined stress. Previous studies found that drought influences root exudation rates (Canarini et al. 2016; Gargallo-Garriga et al. 2018; Williams and de Vries 2020). Interestingly, we found a reduction in the presence of mellein in root exudates. While plants might produce this molecule (Reveglia *et al.* 2020), Trotel-Aziz et al. (2019) suggest that mellein is secreted by the plant pathogen fungus *Neofusicoccum parvum* during the pathogenesis process. Thus, a reduction of mellein in response to drought might be due to a decrease in the abundance/activity of phytopathogenic fungi. Our results also suggest a decrease in the exudation of protocatechuic acid, previously observed in maize as a response to symbiosis with mycorrhizal fungi (Ma *et al.* 2022) which might be promoted under drought stress (Zhao et al., 2015). Similarly, resveratrol has been found to be produced by plants to counteract bacterial pathogens (Chen *et al.* 2016), thus the higher exudation we observed in our study might represent a stress(drought)-induced response that plants exploit to protect themselves from potential bacterial pathogens. Both Peonidin 3-

*O*-(6"-acetyl-galactoside) and Luteolin 7-*O*-(2-apiosyl-glucoside), were found to be more exuded under drought stress; given their antioxidant activity, they might contribute to help plants coping with stress.

**Specific response to heat stress**

Both targeted and untargeted metabolomics show an effect of heat stress on the root exudation profile. Phenolic compounds and derivates of benzoic acid were specifically more abundant in exudates when plants were exposed to heat stress. In line with our results, benzoic acid and hydroxybenzoic acid were previously reported to be influenced by changes in the environmental temperature (Pramanik *et al.* 2000). It is also known that benzoic acid released by root exudates plays a role in plant-microorganism interactions (Liu *et al.* 2015). Malate, another up-exuded compound observed in maize root exudates under heat stress, has been reported to play a role in recruiting beneficial microbes like *Bacillus subtilis* (Rudrappa *et al.* 2008), helping plants to cope with stresses. We also found different molecules with antioxidant capacity that are either up- (4-vinylphenol, luteolin) or down-exuded (ascorbic acid, bisdemethoxycurcumin, apigenin). While these molecules can help plants to mitigate the negative effects of reactive oxygen species generated as a response to several abiotic stresses (Venkatesh and Park 2014), it is interesting to note how they follow different patterns (up- or down- exuded), possibly indicating specific role(s) for the different classes. Future research might clarify why plants manage each of them in a different way.

**Specific response to combined heat and drought stresses**

Interestingly, the combined stress exhibited a distinct and chemically diverse exudate profile compared to the individual stresses, with 13 metabolites being specifically up-exuded by maize roots under combined stress. Apart from phenolic compounds, which appeared as a main class of compounds generally modulated by stress, the combined stress triggered the specific exudation of L-tryptophan and palmitic acid. It has been previously shown that the exudation of tryptophan-derived secondary metabolites is involved in response to biotic stress (Baetz and Martinoia 2014), and it is stimulated by the plant beneficial bacterium *Bacillus amyloliquefaciens* (Liu *et al.* 2016). The specificity of exudation response to the combined stress suggests the non-additive effects of the co-occurrence of abiotic stresses. This is in agreement with previous findings observed for other maize traits such as root morphology (Vescio, Abenavoli, *et al.* 2021), root microbiota (Vescio, Malacrinò, *et al.* 2021a), leaf proteome (Zhao *et al.* 2016), and yield and nutrient uptake (Hussain *et al.* 2019). Also in this case, we found molecules with antioxidant activity that are either up- or down-exuded, and future studies can help us to understand why plants modulate the exudation of molecules with a similar function in a different way.

## Effect of root type and zone on root exudation profile

Root exudation can change significantly along the root axis and different root types (Tiziani *et al.* 2021). In our study, only the targeted metabolomics analyses revealed differences in the metabolites exuded by different root zones. Aconitic acid, total phenols, total flavonoids, glycine, alanine, and threonine were more abundant in exudates collected from the apical root region compared to the subapical region. The root tip is considered to be very sensitive to environmental stimuli (Baluška and Mancuso 2013), so the higher exudation of these metabolites can be an effect driven by the way plants sense their surrounding environment. Some studies suggested that the root tip functions as a control center for sensing external nutrient concentrations, inducing alterations in root morphology and physiology (Canarini *et al.* 2019; R. Tiziani *et al.* 2020). Moreover, the elongation zone (located in the apical root zone) is known to be one of the physiologically most active root zones acting as a command center for root responses to the environment (Baluska *et al.* 2010; R. Tiziani *et al.* 2020). Given the different functional roles of different root types, we hypothesized that we would detect differences in the root exudation profile between primary and seminal roots. However, results suggest no differences between root types. While our plants were not root bound, the limited soil volume might have restricted the soil exploration of single root axes, contributing to reduce the differences between root types.

Furthermore, in the case of controlled experiments such as ours, resources are more evenly distributed within the pot volume, and this might have contributed to limiting the differences between root types. Indeed, in field conditions, resources in soil are not homogeneously distributed in space, and different root types might contribute differently to find and exploit them (Hodge 2004), contributing to the differentiation of root exudate profiles between root types.

### Interactions between root exudates and rhizosphere microbiota

In this study, we linked the exudation of specific metabolites to changes in the relative abundance of bacterial taxa. In drought-stressed plants, we found that p-Coumaric acid ethyl ester and L-serine negatively correlate with a pool of OTUs representing nine bacterial genera. A higher abundance of p-Coumaric acid has been observed in *Zea mays* (Alvarez *et al.* 2008), *Vitis vinifera* (Król *et al.* 2014) and *Achillea pachycephala* (Gharibi *et al.* 2019) when plants were exposed to drought stress. Muthusamy et al. (2016) observed an increase in transcripts associated with the L-serine pathway in drought-stressed banana plants. In our study, both p-Coumaric acid ethyl ester and L-serine influenced the abundance of bacteria that previous studies found to have a beneficial influence on plants. *Mucilaginibacter* has been found to alleviate salt stress in *Arabidopsis thaliana* (Fan *et al.* 2020). Luo *et al.* (2019) found that *Sphingomonas* contributed to alleviate the effects of drought stress in *A. thaliana*. Species of *Paenibacillus* are also well known to be recruited by plants during drought stress episodes (Khan *et al.* 2011; Liu *et al.* 2020). Similarly, *Mesorhizobium* has also often been linked to tolerance to drought stress (Yadav *et al.* 2021).

In plants exposed to heat stress, we identified five compounds that explain most of the variation in the structure of the root exudates for that treatment and that significantly correlate with several bacterial taxa. 1- aminocyclopropane-1-carboxylic is a precursor of ethylene, a plant hormone produced during stress episodes. Previous reports suggest that rhizobacteria are involved in the degradation of this molecule, thus reducing the physiological effects of ethylene on plants (Barnawal *et al.* 2012; Tiwari *et* *al.* 2018). L-histidine has been previously observed in higher abundance in drought-stressed (Kang *et al.* 2019) and heat-stressed plants (Thomason *et al.* 2018), while quercetin was found to alleviate the salt stress in tomato plants (Parvin *et al.* 2019). Wang *et al.* (2021) found that abiotic stressors influence the levels of Eugenol in strawberry fruits, and no previous studies linked plant abiotic stress with changes in the abundance of rhamnetin. Also in this case, we observed an interaction between these compounds and several bacterial taxa that have been previously reported to have a beneficial influence on stressed plants. Species of *Bacillus* are widely recognized to be beneficial microorganisms, helping plants to cope with a variety of stressors including heat (Khan *et al.* 2020). While *Chitinophaga, Mesorhizobium and Rhizobacter* are all well-known PGPR (Kour *et al.* 2019), no previous study reports a link between *Chitinophaga* and abiotic stressors.

When combining both drought and heat stress, we identified three metabolites that likely have a major role in the interaction between the plant and the soil microbiota. To the best of our knowledge, no previous study directly linked these root exudates to the combination of both heat and drought stress. Similarly to what we observed for the single stressors, also L-homocysteine, cinnamoyl glucose and carnosic acid correlate with taxa that have been previously reported to promote plant growth or alleviate the effect of plant stress.

Taken together, our results support the existence of a mechanism by which plants use a portion of root exudates to recruit beneficial microorganisms. Furthermore, here we show that each stress produces a specific signature in the composition of root exudates, which also vary in the way it targets bacterial taxa. Interestingly, within the same treatment, the metabolites we identified interact with the same group of bacterial taxa suggesting that, mechanistically, plants might modulate the exudation of a specific composition of molecules that act in concert to exert their influence on rhizosphere bacterial communities. This might further explain why we did not observe a clear signal for all these metabolites when comparing treatment versus control, as the influence of root exudates on bacterial taxa might need to be additive across a small group of molecules that plants produce in a stress-specific way.

## Conclusions

Considering the fundamental role of root exudates in plant-soil interactions, we investigated the impact of single abiotic stresses, drought and heat, and their combination, on the exudate profile of maize plants. Different exudate profiles were observed in the presence of drought and heat stress, involving stress-specific up- and down-exuded compounds. These metabolites likely play a role in the plant acclimation processes induced by reduced water availability or increased soil temperature. The combination of drought and heat stress produced a different root exudate composition than single stresses, thus strengthening the idea that multiple stresses can interfere with plant processes in a non-additive way.

Taken together, our results highlight that root exudation is a pivotal process through which maize plants manage their rhizosphere processes in response to abiotic stresses, with distinctive traits being observed when maize plants were exposed to the combined stress. This fine tuning is also mechanistically linked to changes in soil bacterial community composition and functioning. These new insights in rhizosphere processes could be useful to evaluate and define future mitigation strategies to limit the impact of drought and heatwaves on plant growth and productivity.

## Funding

This work was supported by the Consejería de Empleo, Universidades, Empresa y Medio Ambiente of the CARM, through the Fundación Séneca-Agencia de Ciencia y Tecnología de la Región de Murcia (Spain) (BM-M, grant number: 21252/PD/19). We thank the "Stiftung Südtiroler Sparkasse" for funding the PhD fellowship of Raphael Tiziani. This work was furthermore supported by grants from the Free University of Bolzano (grant number: NUMICS TN200E).

## Acknowledgements

We are thankful to KWS Italy for providing the maize seeds. We also acknowledge funding from the PhD course "Scienze Agrarie, Alimentari e Forestali" of the University "Mediterranea" of Reggio Calabria and the Department AGRARIA at Università Mediterranea of Reggio Calabria for supporting Rosa Vescio. The authors thank the "Romeo ed Enrica Invernizzi" foundation (Milan, Italy) for its kind support to the metabolomics facility at Università Cattolica del Sacro Cuore.

## Data Availability

Raw metabolomics data is available on Mendeley Data (DOI: 10.17632/tgcv97wwyz.1).

## Literature Cited

**Alvarez S, Marsh EL, Schroeder SG, Schachtman DP**. **2008**. Metabolomic and proteomic changes in the xylem sap of maize under drought. *Plant, cell & environment* **31**: 325–340.

**Argelaguet R, Velten B, Arnol D, *et al.* 2018**. Multi-Omics Factor Analysis—a framework for unsupervised integration of multi-omics data sets. *Molecular Systems Biology* **14**: e8124.

**Astolfi S, Pii Y, Mimmo T, *et al.* 2020**. Single and combined fe and s deficiency differentially modulate root exudate composition in tomato: A double strategy for fe acquisition? *International*

*Journal of Molecular Sciences* **21**: 1–20.

**Atanassova M, Georgieva S, Ivancheva K**. **2011**. Total Phenolic and Total Flavonoid Contents , Antioxidant Capacity and Biological Contaminants in Medicinal Herbs. *Journal of the University of Chemical Technology and Metallurgy*, **46**: 81–88.

**Baetz U, Martinoia E**. **2014**. Root exudates: the hidden part of plant defense. *Trends in Plant Science* **19**: 90–98.

**Baluška F, Mancuso S**. **2013**. Root Apex Transition Zone As Oscillatory Zone . *Frontiers in Plant Science* **4**: 354.

**Baluska F, Mancuso S, Volkmann D, Barlow PW**. **2010**. Root apex transition zone: a signalling-response nexus in the root. *Trends in plant science* **15**: 402–408.

**Barnawal D, Bharti N, Maji D, Chanotiya CS, Kalra A**. **2012**. 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase-containing rhizobacteria protect Ocimum sanctum plants during waterlogging stress via reduced ethylene generation. *Plant physiology and biochemistry : PPB* **58**: 227–235.

**Canarini A, Kaiser C, Merchant A, Richter A, Wanek W**. **2019**. Root exudation of primary metabolites: Mechanisms and their roles in plant responses to environmental stimuli. *Frontiers in Plant Science* **10**: 157.

**Canarini A, Merchant A, Dijkstra FA**. **2016**. Drought effects on Helianthus annuus and Glycine max metabolites: from phloem to root exudates. *Rhizosphere* **2**: 85–97.

**Chen J, Yu Y, Li S, Ding W**. **2016**. Resveratrol and Coumarin: Novel Agricultural Antibacterial Agent against Ralstonia solanacearum In Vitro and In Vivo. *Molecules (Basel, Switzerland)* **21**.

**Christian S-M, Joseph E, Zachary L, Bao N, Venkatesan S, M. AF**. **2021**. Drought Stress Results in a Compartment-Specific Restructuring of the Rice Root-Associated Microbiomes. *mBio* **8**: e00764-17.

**Doornbos RF, van Loon LC, Bakker PAHM**. **2012**. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. *Agronomy for Sustainable Development* **32**: 227–243.

**Fahad S, Bajwa AA, Nazir U, *et al.* 2017**. Crop production under drought and heat stress: Plant responses and management options. *Frontiers in Plant Science* **8**: 1–16.

**Fan D, Subramanian S, Smith DL**. **2020**. Plant endophytes promote growth and alleviate salt stress in Arabidopsis thaliana. *Scientific Reports* **10**: 12740.

**García-Pérez P, Miras-Moreno B, Lucini L, Gallego PP**. **2021**. The metabolomics reveals intraspecies variability of bioactive compounds in elicited suspension cell cultures of three Bryophyllum species. *Industrial Crops and Products* **163**: 113322.

**Gargallo-Garriga A, Preece C, Sardans J, Oravec M, Urban O, Peñuelas J**. **2018**. Root exudate metabolomes change under drought and show limited capacity for recovery. *Scientific Reports* **8**: 12696.

**Gelsomino A, Tortorella D, Cianci V, *et al.* 2010**. Effects of a biomimetic iron-porphyrin on soil respiration and maize root morphology as by a microcosm experiment. *Journal of Plant Nutrition and Soil Science* **173**: 399–406.

**Gharibi S, Sayed Tabatabaei BE, Saeidi G, Talebi M, Matkowski A**. **2019**. The effect of drought stress on polyphenolic compounds and expression of flavonoid biosynthesis related genes in Achillea pachycephala Rech.f. *Phytochemistry* **162**: 90–98.

**Harbort CJ, Hashimoto M, Inoue H, *et al.* 2020**. Root-Secreted Coumarins and the Microbiota Interact to Improve Iron Nutrition in Arabidopsis. *Cell Host & Microbe* **28**: 825-837.e6.

**Henry A, Doucette W, Norton J, Bugbee B**. **2007**. Changes in crested wheatgrass root exudation caused by flood, drought, and nutrient stress. *Journal of environmental quality* **36**: 904–912.

**Hodge A**. **2004**. The plastic plant: root responses to heterogeneous supplies of nutrients. *New*

*Phytologist* **162**: 9–24.

**Hund A, Fracheboud Y, Soldati A, Stamp P**. **2008**. Cold tolerance of maize seedlings as determined by root morphology and photosynthetic traits. *European Journal of Agronomy* **28**: 178–185.

**Hussain HA, Men S, Hussain S, *et al.* 2019**. Interactive effects of drought and heat stresses on morpho-physiological attributes, yield, nutrient uptake and oxidative status in maize hybrids. *Scientific Reports* **9**: 3890.

**IPCC**. **2018**. Global warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change,. *Ipcc - Sr15* **2**: 17–20.

**Jägermeyr J, Müller C, Ruane AC, *et al.* 2021**. Climate impacts on global agriculture emerge earlier in new generation of climate and crop models. *Nature Food* **2**: 873–885.

**Kang S-M, Khan AL, Waqas M, *et al.* 2019**. Integrated phytohormone production by the plant growth-promoting rhizobacterium Bacillus tequilensis SSB07 induced thermotolerance in soybean. *Journal of Plant Interactions* **14**: 416–423.

**Kawasaki A, Dennis PG, Forstner C, *et al.* 2021**. Manipulating exudate composition from root apices shapes the microbiome throughout the root system. *Plant Physiology*.

**Khan MA, Asaf S, Khan AL, *et al.* 2020**. Thermotolerance effect of plant growth-promoting Bacillus cereus SA1 on soybean during heat stress. *BMC Microbiology* **20**: 175.

**Khan N, Mishra A, Chauhan PS, Nautiyal CS**. **2011**. Induction of Paenibacillus lentimorbus biofilm by sodium alginate and CaCl2 alleviates drought stress in chickpea. *Annals of Applied Biology* **159**: 372–386.

**Khorassani R, Hettwer U, Ratzinger A, Steingrobe B, Karlovsky P, Claassen N**. **2011**. Citramalic acid and salicylic acid in sugar beet root exudates solubilize soil phosphorus. *BMC plant biology* **11**:

121.

**Kour D, Rana KL, Yadav N, Yadav AN**. **2019**. *Plant Growth Promoting Rhizobacteria for Agricultural Sustainability*.

**Król A, Amarowicz R, Weidner S**. **2014**. Changes in the composition of phenolic compounds and antioxidant properties of grapevine roots and leaves (Vitis vinifera L.) under continuous of long-term drought stress. *Acta Physiologiae Plantarum* **36**: 1491–1499.

**Li J, Copeland L**. **2000**. Role of malonate in chickpeas. *Phytochemistry* **54**: 585–589.

**Liao H, Wan H, Shaff J, Wang X, Yan X, Kochian L V. 2006**. Phosphorus and aluminum interactions in soybean in relation to aluminum tolerance. Exudation of specific organic acids from different regions of the intact root system. *Plant Physiology* **141**: 674–684.

**Liu W, Hou J, Wang Q, Yang H, Luo Y, Christie P**. **2015**. Collection and analysis of root exudates of Festuca arundinacea L. and their role in facilitating the phytoremediation of petroleum-contaminated soil. *Plant and Soil* **389**: 109–119.

**Liu W, Sikora E, Park S-W**. **2020**. Plant growth-promoting rhizobacterium, Paenibacillus polymyxa CR1, upregulates  dehydration-responsive genes, RD29A and RD29B, during priming drought tolerance in arabidopsis. *Plant physiology and biochemistry : PPB* **156**: 146–154.

**Liu L, Yang DF, Liang TY, Zhang HH, He ZG, Liang ZS**. **2016**. Phosphate starvation promoted the accumulation of phenolic acids by inducing the key enzyme genes in *Salvia miltiorrhiza* hairy roots. *Plant Cell Reports* **35**: 1933–1942.

**Love MI, Huber W, Anders S**. **2014**. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**: 550.

**Lucini L, Colla G, Miras Moreno MB, *et al.* 2019**. Inoculation of Rhizoglomus irregulare or Trichoderma atroviride differentially modulates metabolite profiling of wheat root exudates.

*Phytochemistry* **157**: 158–167.

**Luo Y, Wang F, Huang Y, *et al.* 2019**. Sphingomonas sp. Cra20 Increases Plant Growth Rate and Alters Rhizosphere Microbial Community Structure of Arabidopsis thaliana Under Drought Stress . *Frontiers in Microbiology* **10**: 1221.

**Lupini A, Sorgonà A, Princi MP, Sunseri F, Abenavoli MR**. **2016**. Morphological and physiological effects of trans-cinnamic acid and its hydroxylated derivatives on maize root types. *Plant Growth Regulation* **78**: 263–273.

**Lynch JP**. **2013**. Steep, cheap and deep: an ideotype to optimize water and N acquisition by maize root systems. *Annals of Botany* **112**: 347–357.

**Ma J, Wang W, Yang J, *et al.* 2022**. Mycorrhizal symbiosis promotes the nutrient content accumulation and affects the root exudates in maize. *BMC Plant Biology* **22**: 64.

**Mariano ED, Keltjen WG**. **2003**. Evaluating the role of root citrate exudation as a mechanism of aluminium resistance in maize genotypes. *Plant and Soil* **256**: 469–479.

**McPherson MR, Wang P, Marsh EL, Mitchell RB, Schachtman DP**. **2018**. Isolation and Analysis of Microbial Communities in Soil, Rhizosphere, and Roots in Perennial Grass Experiments. *JoVE*: e57932.

**Mickelsen O, Yamamoto RS**. **1958**. Methods for the Determination of Thiamine. *Methods of Biochemical Analysis*: 191–257.

**Miliauskas G, Venskutonis PR, van Beek TA**. **2004**. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry* **85**: 231–237.

**Muthusamy M, Uma S, Backiyarani S, Saraswathi MS, Chandrasekar A**. **2016**. Transcriptomic Changes of Drought-Tolerant and Sensitive Banana Cultivars Exposed to Drought Stress . *Frontiers in Plant Science* **7**: 1609.

**Oburger E, Jones DL**. **2018**. Sampling root exudates – Mission impossible? *Rhizosphere* **6**: 116–133.

**Pandey P, Ramegowda V, Senthil-Kumar M**. **2015**. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms . *Frontiers in Plant Science* **6**: 723.

**Parvin K, Hasanuzzaman M, Bhuyan MHMB, Mohsin SM, Fujita M**. **2019**. Quercetin Mediated Salt Tolerance in Tomato through the Enhancement of Plant Antioxidant Defense and Glyoxalase Systems. *Plants* **8**.

**Pramanik MHR, Nagai M, Asao T, Matsui Y**. **2000**. Effects of Temperature and Photoperiod on Phytotoxic Root Exudates of Cucumber (Cucumis sativus) in Hydroponic Culture. *Journal of Chemical Ecology* **26**: 1953–1967.

**Quast C, Pruesse E, Yilmaz P,** ***et al.*** **2013**. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* **41**: D590-6.

**Reveglia P, Masi M, Evidente A**. **2020**. Melleins-Intriguing Natural Compounds. *Biomolecules* **10**.

**Rivero RM, Mestre TC, Mittler R, Rubio F, Garcia-Sanchez F, Martinez V**. **2014**. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. *Plant, cell & environment* **37**: 1059–1073.

**Rognes T, Flouri T, Nichols B, Quince C, Mahé F**. **2016**. VSEARCH: a versatile open source tool for metagenomics (T Hrbek, Ed.). *PeerJ* **4**: e2584.

**Rudrappa T, Czymmek KJ, Paré PW, Bais HP**. **2008**. Root-Secreted Malic Acid Recruits Beneficial Soil Bacteria. *Plant Physiology* **148**: 1547 LP – 1556.

**Sasse J, Martinoia E, Northen T**. **2018**. Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? *Trends in Plant Science* **23**: 25–41.

**Schneider HM, Wojciechowski T, Postma JA,** ***et al.*** **2017a**. Root cortical senescence decreases root respiration, nutrient content and radial water and nutrient transport in barley. *Plant, Cell & Environment* **40**: 1392–1408.

**Schneider HM, Wojciechowski T, Postma JA, *et al.* 2017b**. Root cortical senescence decreases root respiration, nutrient content and radial water and nutrient transport in barley. *Plant, Cell & Environment* **40**: 1392–1408.

**Shulaev V, Cortes D, Miller G, Mittler R**. **2008**. Metabolomics for plant stress response. *Physiologia Plantarum* **132**: 199–208.

**Sorgonà A, Lupini A, Mercati F, Di Dio L, Sunseri F, Abenavoli MR**. **2011**. Nitrate uptake along the maize primary root: an integrated physiological and  molecular approach. *Plant, cell & environment* **34**: 1127–1140.

**Stringlis IA, de Jonge R, Pieterse CMJ**. **2019**. The Age of Coumarins in Plant-Microbe Interactions. *Plant & cell physiology* **60**: 1405–1419.

**Sun H, Jiang S, Jiang C, Wu C, Gao M, Wang Q**. **2021**. A review of root exudates and rhizosphere microbiome for crop production. *Environmental Science and Pollution Research*.

**Thomason K, Babar MA, Erickson JE, Mulvaney M, Beecher C, MacDonald G**. **2018**. Comparative physiological and metabolomics analysis of wheat (Triticum aestivum L.) following post-anthesis heat stress. *PLOS ONE* **13**: e0197919.

**Tiwari G, Duraivadivel P, Sharma S, P. H**. **2018**. 1-Aminocyclopropane-1-carboxylic acid deaminase producing beneficial rhizobacteria ameliorate the biomass characters of Panicum maximum Jacq. by mitigating drought and salt stress. *Scientific Reports* **8**: 17513.

**Tiziani R., Mimmo T, Valentinuzzi F, Pii Y, Celletti S, Cesco S**. **2020**. Root Handling Affects Carboxylates Exudation and Phosphate Uptake of White Lupin Roots. *Frontiers in Plant Science* **11**: 1403.

**Tiziani R, Pii Y, Celletti S, Cesco S, Mimmo T**. **2020**. Phosphorus deficiency changes carbon isotope fractionation and triggers exudate reacquisition in tomato plants. *Scientific Reports* **10**.

**Tiziani R, Puschenreiter M, Smolders E, *et al.* 2021**. Millimetre-resolution mapping of citrate exuded from soil-grown roots using a novel, low-invasive sampling technique. *Journal of Experimental Botany* **72**: 3513–3525.

**Tsugawa H, Cajka T, Kind T, *et al.* 2015**. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nature Methods* **12**: 523–526.

**Venkatesh J, Park SW**. **2014**. Role of L-ascorbate in alleviating abiotic stresses in crop plants. *Botanical Studies* **55**: 38.

**Vescio R, Abenavoli MR, Sorgonà A**. **2021**. Single and Combined Abiotic Stress in Maize Root Morphology. *Plants* **10**.

**Vescio R, Malacrinò A, Bennett AE, Sorgonà A**. **2021a**. Single and combined abiotic stressors affect maize rhizosphere bacterial microbiota. *Rhizosphere* **17**: 100318.

**Vescio R, Malacrinò A, Bennett AE, Sorgonà A**. **2021b**. Single and combined abiotic stressors affect maize rhizosphere bacterial microbiota. *Rhizosphere* **17**: 100318.

**Vives-Peris V, Gómez-Cadenas A, Pérez-Clemente RM**. **2017**. Citrus plants exude proline and phytohormones under abiotic stress conditions. *Plant Cell Reports* **36**: 1971–1984.

**Vives-Peris V, de Ollas C, Gómez-Cadenas A, Pérez-Clemente RM**. **2019**. Root exudates: from plant to rhizosphere and beyond. *Plant Cell Reports*.

**Waisel, Y.; Eshel, A. and Kafkafi U**. **2003**. Plant Roots: The Hidden Half. 3rd Edition. *Experimental Agriculture* **39**: 219–222.

**Wang S, Zhou D, Shi M, *et al.* 2021**. Expression patterns of four key genes involved in strawberry eugenol synthesis under abiotic stresses. *Acta Ecologica Sinica*.

**Williams A, de Vries FT**. **2020**. Plant root exudation under drought: implications for ecosystem functioning. *New Phytologist* **225**: 1899–1905.

**Wu A-L, Jiao X-Y, Wang J-S, *et al.* 2021**. Sorghum rhizosphere effects reduced soil bacterial diversity by recruiting specific bacterial species under low nitrogen stress. *Science of The Total Environment* **770**: 144742.

**Yadav A, Yadav A, Singh RP, Singh AL, Singh M**. **2021**. Identification of genes involved in phosphate solubilization and drought stress tolerance in chickpea symbiont Mesorhizobium ciceri Ca181. *Archives of microbiology* **203**: 1167–1174.

**Yuan Y, Brunel C, van Kleunen M, Li J, Jin Z**. **2019**. Salinity-induced changes in the rhizosphere microbiome improve salt tolerance of Hibiscus hamabo. *Plant and Soil* **443**: 525–537.

**Zhan A, Schneider H, Lynch JP**. **2015**. Reduced Lateral Root Branching Density Improves Drought Tolerance in Maize. *Plant physiology* **168**: 1603–1615.

**Zhang J, Kobert K, Flouri T, Stamatakis A**. **2014**. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics (Oxford, England)* **30**: 614–620.

**Zhao F, Zhang D, Zhao Y, *et al.* 2016**. The Difference of Physiological and Proteomic Changes in Maize Leaves Adaptation to Drought, Heat, and Combined Both Stresses. *Frontiers in Plant Science* **7**.

# Figures

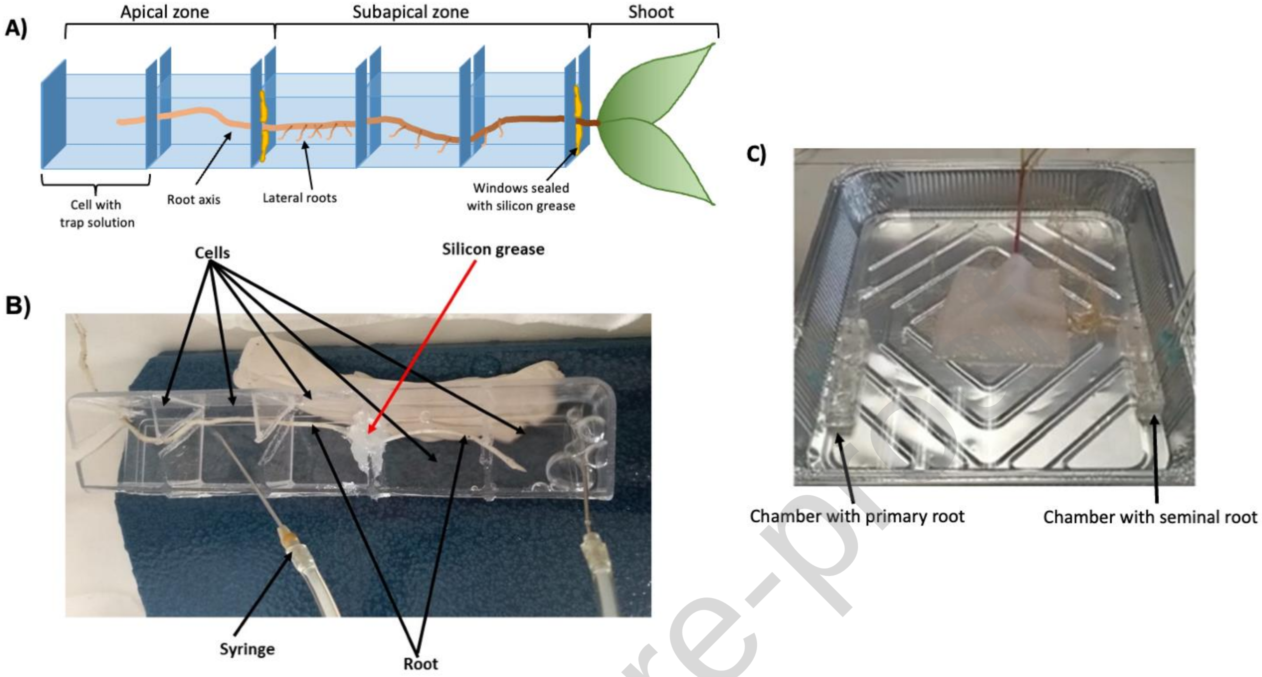


**Figure 1.** Scheme (A) and picture (B) of the compartmented chambers, and (C) the sampling method used for the collection of the maize root exudates.

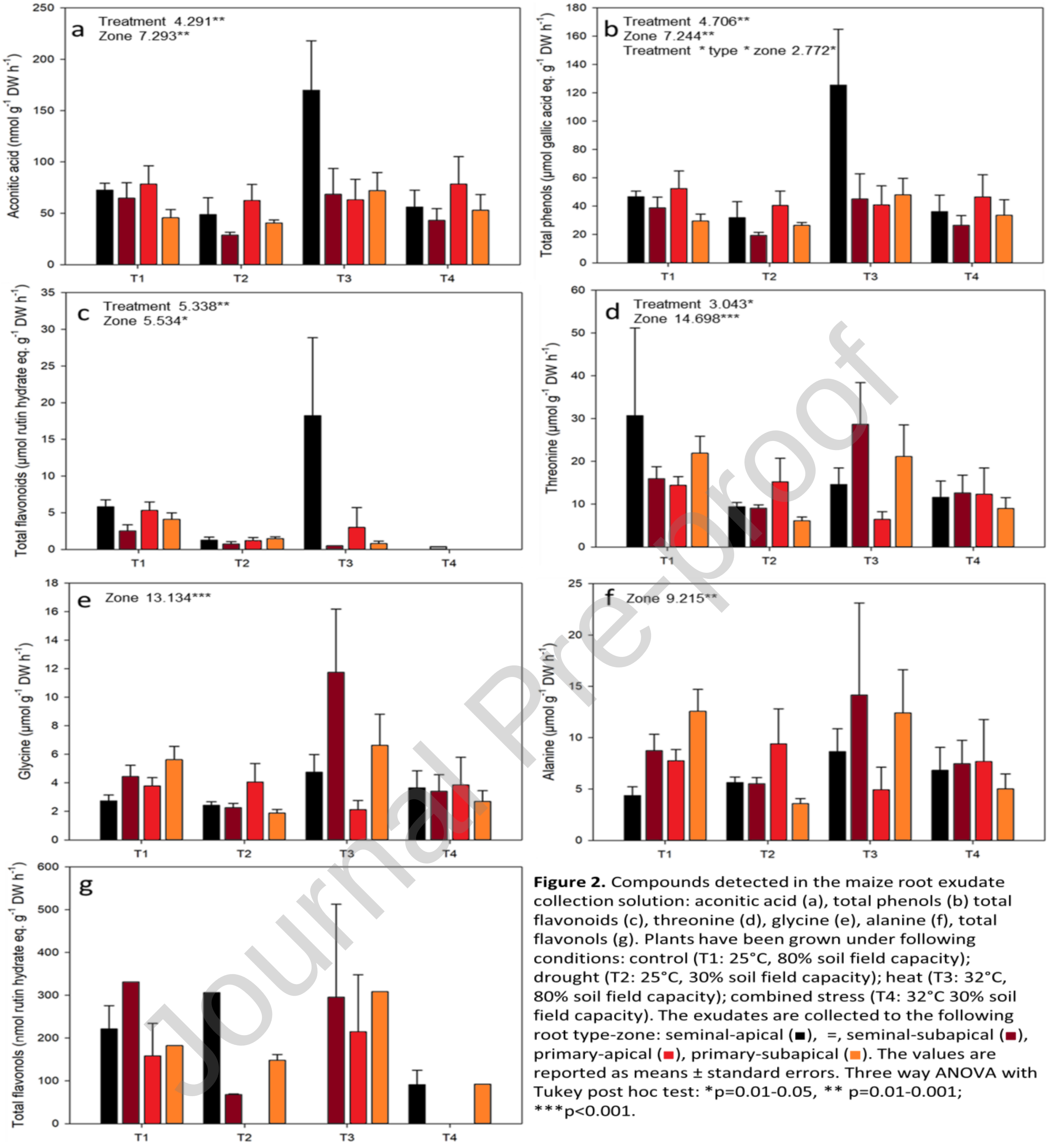


**Figure 2.** Compounds detected in the maize root exudate collection solution: aconitic acid (a), total phenols (b) total flavonoids (c), threonine (d), glycine (e), alanine (f), total flavonols (g). Plants have been grown under following conditions: control (T1: 25°C, 80% soil field capacity); drought (T2: 25°C, 30% soil field capacity); heat (T3: 32°C, 80% soil field capacity); combined stress (T4: 32°C 30% soil field capacity). The exudates are collected to the following root type-zone: seminal-apical (■), =, seminal-subapical (■), primary-apical (■), primary-subapical (■). The values are reported as means ± standard errors. Three way ANOVA with Tukey post hoc test: *p=0.01-0.05, ** p=0.01-0.001; ***p<0.001.

**Figure 2**. Compounds detected in the maize root exudate collection solution: aconitic acid (A), total phenols (B) total flavonoids (C), threonine (D), glycine (E), alanine (F), total flavonols (G). Plants have been grown under following conditions: control (T1: 25°C, 80% soil water holding capacity); drought (T2: 25°C, 30% soil water holding capacity); heat (T3: 32°C, 80% soil water holding capacity); combined stress (T4: 32°C 30% soil water holding capacity). The exudates are collected to the

following root type-zone: seminal-apical (■), seminal-subapical (■), primary-apical (■), primary-subapical (■). The values of the bars are reported as means ± standard errors. Three way ANOVA results are only reported when $p<0.05$ and are indicated as F values and *$p<0.05$, ** $p<0.01$; ***$p<0.001$. The Tukey post-hoc test results are reported in the Supplemental material 2.

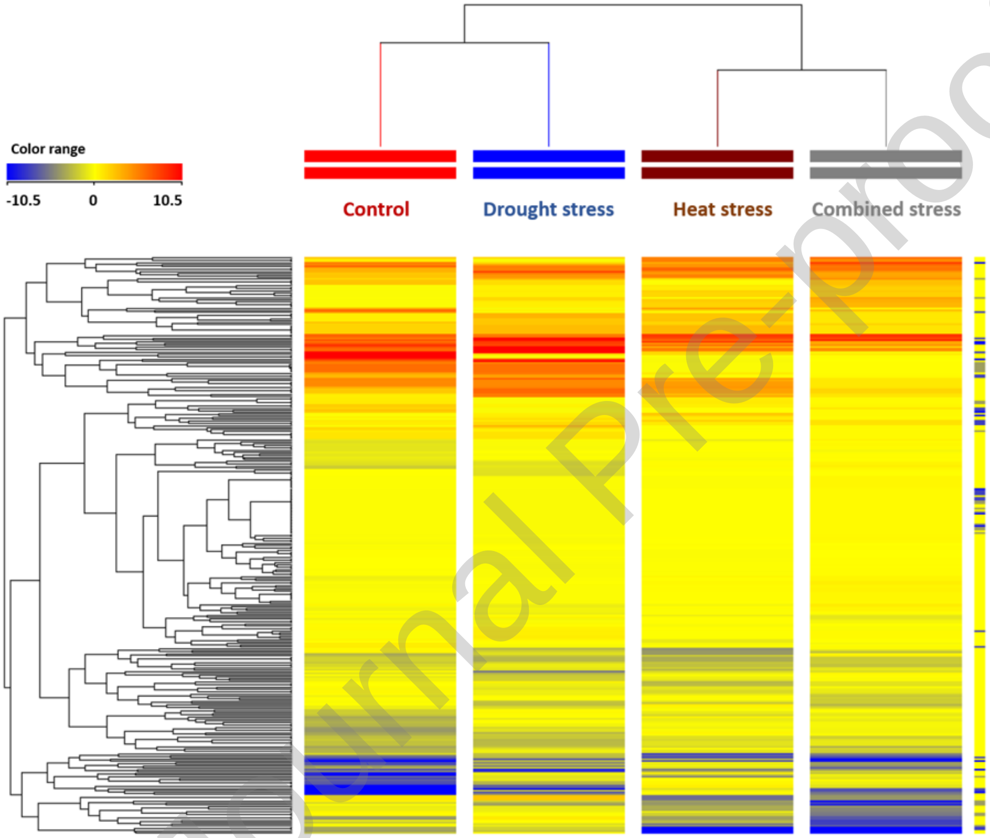


**Figure 3.** Unsupervised hierarchical cluster analysis (Euclidean distance; linkage rule: Ward). Metabolites were identified by UHPLC-ESI/QTOF-MS untargeted analysis, and their intensities used to build the fold-change heatmap.

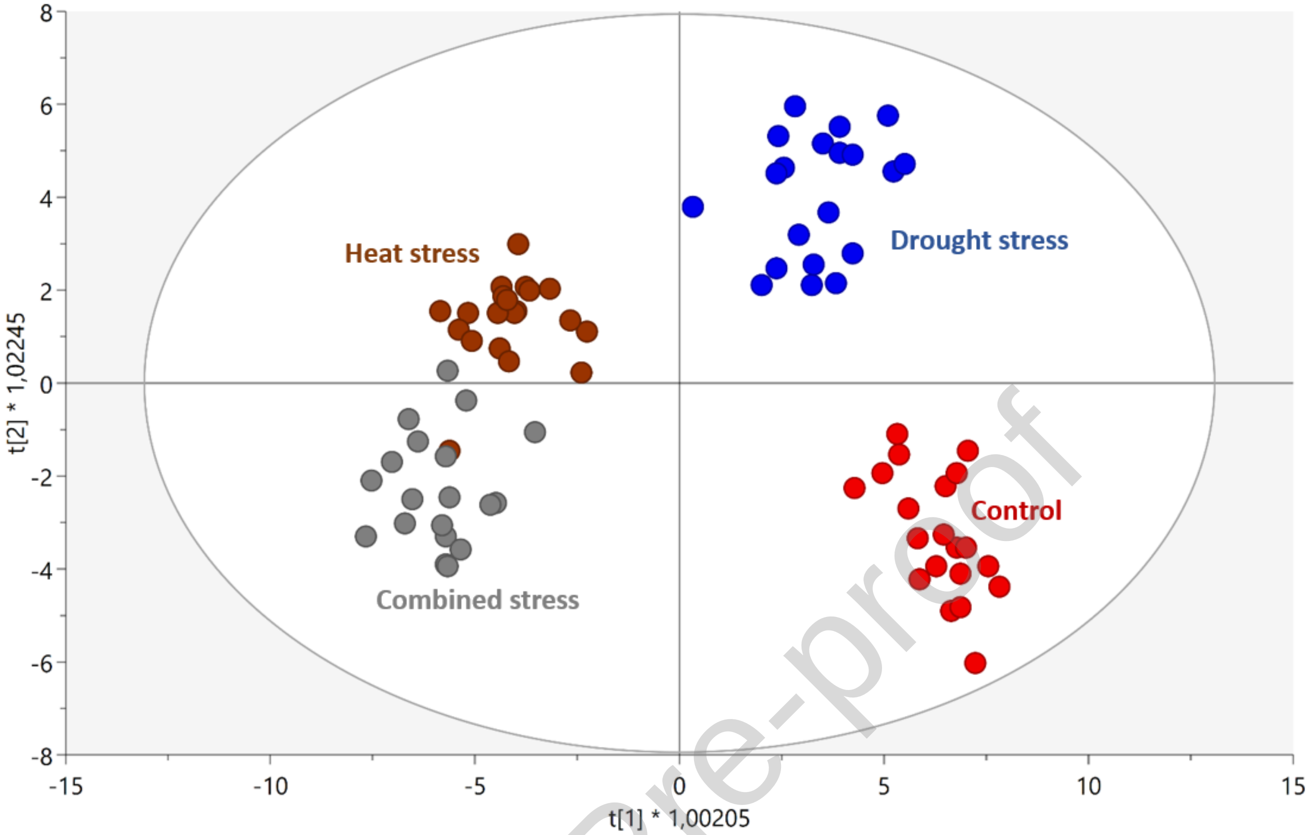


**Figure 4.** Score plot of orthogonal projection to latent structures discriminant analysis (OPLS-DA) supervised modelling carried out from untargeted metabolomics profiles of root exudates (correlation $R^2Y = 0.83$, prediction ability $Q^2Y = 0.48$).

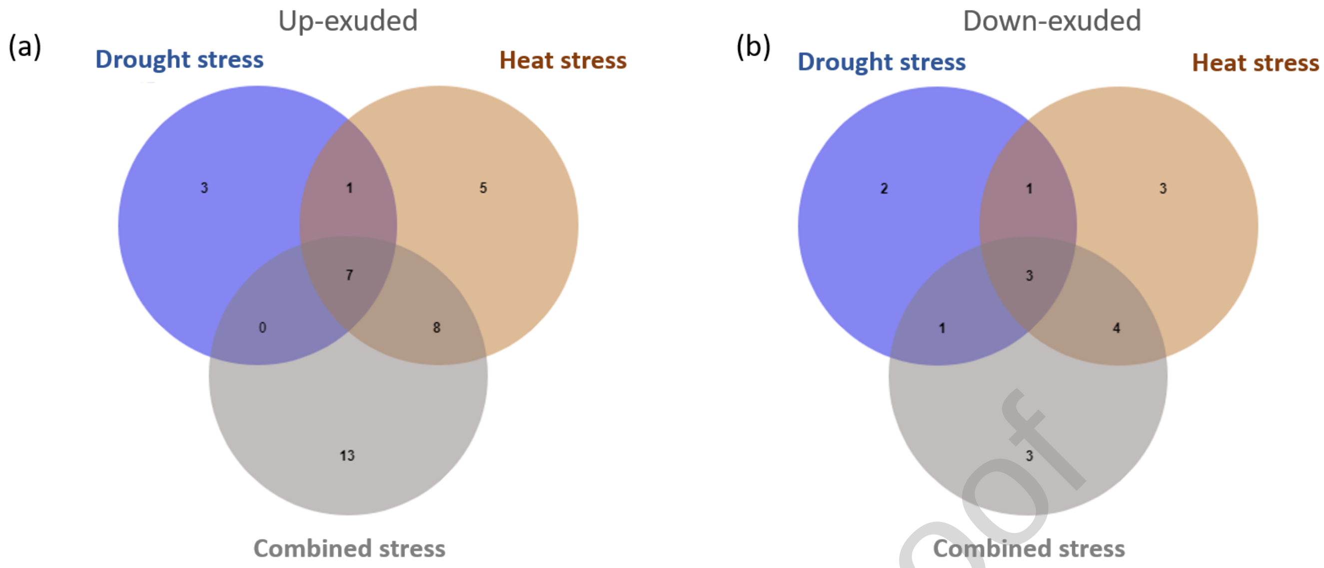


**Figure 5**. Venn diagram summarizing the metabolites differentially up-exuded (A) and down-exuded (B) that resulted from the volcano plot.

**Table 1.** Metabolites specifically exuded in the presence of drought, heat and/or combined stress (metabolites have been detected through untargeted metabolomics).

| | **DROUGHT AND DROUGHT+COMBINED STRESS** | **HEAT AND HEAT+COMBINED STRESS** | **COMBINED STRESS** |
|---|---|---|---|
| **DOWN-EXUDED** | **Mellein** | **Ascorbic acid** | **alpha-aminoadipic acid** |
| | **protocatechuic acid** | **Bisdemethoxycurcumin** | **sorgolactone** |
| | Kaempferol 3-O-(6"-acetyl-galactoside) 7-O-rhamnoside | **Apigenin 7-O-(6''-malonyl-apiosyl-glucoside)** | **6-Geranylnaringenin** |
| | | L-serine | |

| | | | |
|---|---|---|---|
| | | 3-Methylcatechol | |
| | | 3,5-Dihydroxybenzoic acid | |
| | | 2-S-Glutathionyl caftaric acid | |
| **UP-EXUDED** | **Resveratrol** | **4-Vinylphenol** | **Lutein** |
| | **Peonidin 3-O-(6''-acetyl-galactoside)** | **Luteolin 7-O-(2-apiosyl-6-malonyl)-glucoside** | **Palmitic acid** |
| | **Luteolin 7-O-(2-apiosyl-glucoside)** | **malate** | **Luteolin 7-O-rutinoside** |
| | | **2,4-Dihydroxybenzoic acid** | **Hesperidin** |
| | | **Benzoic acid** | **Cyanidin 3-O-(6''-p-coumaroyl-glucoside)** |
| | | 6"-O-Malonylgenistin | **Naringin 6'-malonate** |
| | | Pelargonidin 3-O-(6"-malonyl-glucoside) | **5-Nonadecenylresorcinol** |
| | | Quercetin 3-O-xylosyl-rutinoside | **Jaceidin 4'-O-glucuronide** |
| | | Cyanidin 3-O-sambubiosyl 5-O-glucoside | **Sinapine** |
| | | Petunidin 3-O-(6"-acetyl-galactoside) | **Luteolin 7-O-malonyl-glucoside** |
| | | 4-Ethylphenol | **L-tryptophan** |

| | | |
|---|---|---|
| | 3,4-Dihydroxyphenylglycol | **1,2,2'-Trisinapoylgentiobiose** |
| | 2,6-Dihydroxybenzoic acid | **Patuletin 3-O-(2''-feruloylglucosyl)(1-6)-[apiosyl(1-2)]-glucoside** |

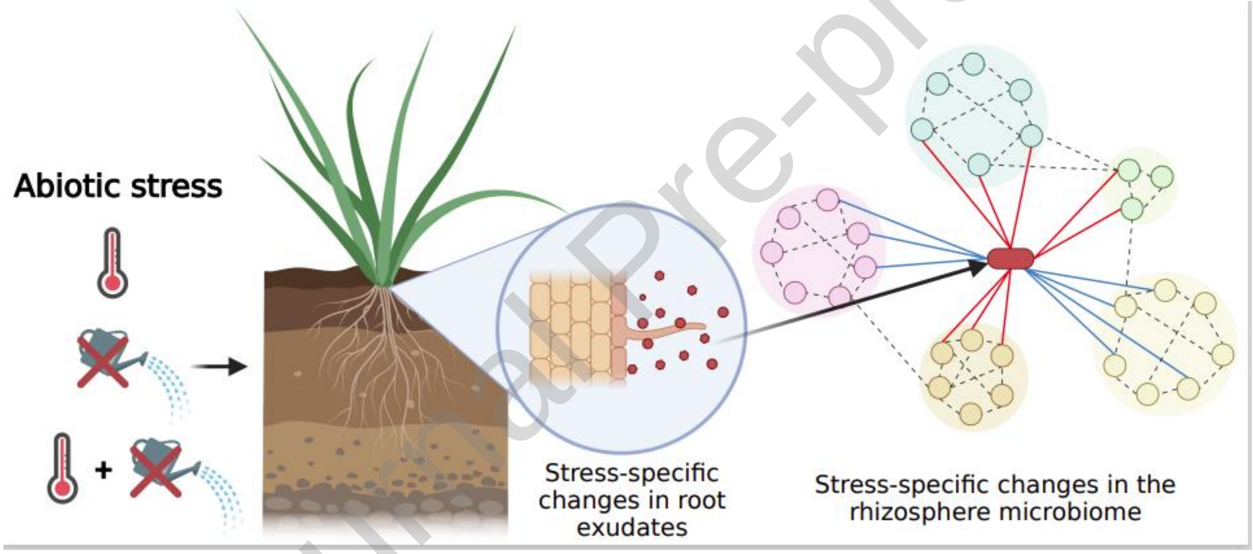
*Metabolites in bold indicate their modulation exclusively in the presence of the single stress or the combined stress, while the others were shared between the single (drought and heat) and combined stress*

## CRediT authorship contribution statement

AS: Conceptualization; AS, TM, LL, SC: Methodology and Resources; RV, RT, BM-M: Investigation; AM, RT, BM-M: Formal analysis; RT, BM-M, AM: Writing - Original Draft; all coauthors: Writing - Review & Editing.

### Declaration of Competing Interest

### Graphical abstract



## Highlights

- Drought, heat, and their combination have a specific influence on root exudation
- The combination of stresses influences exudates differently from the single stresses
- Changes in root exudation are associated with changes of specific microbial taxa