

Impact of cyclic-mild-drought stress on the metabolism of *Mentha spicata* L.: A strategy to improve quality traits

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ABSTRACT

Studying the impact of mild drought stress on plant productivity and product quality is essential in the context of climate change and dwindling water resources. Moreover, understanding how mild drought stress affects plants is crucial for sustainable agriculture, since it can potentially lead to the development of more efficient irrigation techniques, which, in turn, could enhance the resilience of agricultural systems, improve crop quality and reduce environmental impacts. In this contest, the study reports on the physiological and metabolic responses of spearmint (*Mentha spicata* L.) plants to cyclic-mild-drought stress. The research aimed to understand how plants adapt to water stress conditions and whether the nutraceutical and sensory characteristics of the plant can be enhanced by managing the water supply. Cyclic-mild-drought stress affected various physiological parameters of the plants [i.e. stomatal conductance, net photosynthesis, leaf temperature and leaf osmolality] but did not impact plant biomass and the photosynthetic machinery. Changes in leaf cation and anion concentration were not related to the observed changes in leaf osmolality, suggesting that the accumulation of organic compounds such as amino acids, sugars, organic acids, and phenolic acids mainly drove the osmotic adjustment. Finally, the comparison between the leaves' volatile profile of stressed and unstressed plants revealed that the cyclic-mild-drought stress significantly increased the concentration of carvone, the most representative and industrially important molecule produced by spearmint. Overall, the study's novelty is to provide insights into spearmint plants' physiological and metabolic responses to cyclic-mild-drought stress, highlighting potential strategies, after verification in open fields, to enhance plant productivity and quality by modulating water supply.

1. Introduction

Drought is one of the primary factors affecting crop productivity in the Mediterranean area as, among other effects, it exposes plants to a high reactive oxygen species (ROS) generation, which compromises plant growth and development (Hasanuzzaman et al., 2013). Plants evolved a plethora of systems and adaptive features that enable them to escape, avoid, or tolerate drought (Basu et al., 2016). Certain species employ drought escape to navigate the challenges of limited water availability by completing their life cycle before the onset of a drought (Jones et al., 1981). Drought avoidance is the ability to maintain a relatively higher tissue water content even when the soil water content is reduced (Levitt, 1980). In contrast, drought tolerance is the capacity of plants to endure low tissue water content through specific adaptive traits. This strategy involves maintaining cell turgor through mechanisms like osmotic adjustment and cellular plasticity (Morgan, 1984).

Concerning the latter strategy, drought-tolerant species can effectively maintain cell structure, resist wilting, and continue critical metabolic processes, even in the face of limited water availability, through the production of osmoprotectant/antioxidant molecules and the activity of enzymes that reduce the level of reactive oxygen species (ROS), preventing their genesis or tissue accumulation (Considine and Foyer, 2021; del Río, 2015).

Since drought stress directly affects plant metabolism, using novel metabolomics approaches is an effective tool for garnering comprehensive information on metabolite profiling and identifying the metabolic pathways affected by the stress (Ghatak et al., 2018). It was reported that drought stress significantly impacts the carbohydrate and organic acid metabolism of plants. For example, a study carried out by Yang et al. (2018), on drought-tolerant and drought-sensitive lines of *Z. mays*, reported that carbohydrates such as sucrose, fructose, galactinol, raffinose, and ketose tend to increase in the drought-sensitive line

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and decrease in the drought-tolerant line under drought stress. Similarly, the tricarboxylic acid (TCA) cycle intermediates, such as citrate, succinate, α -ketoglutarate, and fumarate, decrease in the drought-tolerant lines but increase in the drought-sensitive. This variation in metabolic responses plays a pivotal role in osmoprotection, membrane maintenance, and ROS defence during drought stress (Yang et al., 2018). Concerning amino acids, there is a noteworthy variation in their accumulation in different plant genotypes. For instance, some chickpea genotypes accumulate proline, arginine, histidine, isoleucine, and tryptophan in the leaves, especially in the tolerant varieties. Conversely, alanine, α -ketoglutaric acid, GABA, choline, tyrosine, glucosamine, adenosine, guanine, and aspartic acid decrease in tolerant and sensitive genotypes under drought conditions (Khan et al., 2019). Aromatic amino acids, like phenylalanine and tyrosine, also function as secondary energy sources and play a role in stress tolerance (Maeda and Dudareva, 2012; Suguiyama et al., 2014). Also, drought stress significantly alters lipid metabolism (Gigon et al., 2004; Toumi et al., 2008). Studies reveal that the profiles of different lipid classes can vary in drought-tolerant and -sensitive cultivars. For instance, drought-sensitive cultivars show increased levels of saturated fatty acids, particularly palmitic acid. In contrast, drought-tolerant varieties exhibit the early induction of signaling-related fatty acids and lipids, such as linolenic acid and diacylglycerols, as part of their response to drought stress. Drought stress also induces changes in lipid composition, resulting in membrane lipid remodelling and activation of defence mechanisms against biotic and abiotic stressors, including drought (Moradi et al., 2017; Sharma et al., 2023).

Besides the effects of drought on the primary metabolism, it should be highlighted that stress also triggers changes in the biosynthesis of specialized metabolites which, especially those from the phenylpropanoid pathway (Xu et al., 2021), play a crucial role in scavenging ROS to protect plant cells from lipid peroxidation and perform other defence-related functions (Sharma et al., 2019). Increased levels of specialized metabolites could enhance stress tolerance by influencing various physiological and biochemical parameters in plants. These adaptive responses are particularly evident in the case of phenolic compounds like flavonoids, tannins, and lignins (Sharma et al., 2019). Flavonoids, for example, increase in response to drought stress, helping the defence mechanisms. Similarly, lignin is essential for secondary cell wall formation and contributes to plant biotic and abiotic stress responses (Bhardwaj et al., 2014). Drought stress also influences terpenes and polyamines metabolism. The biosynthesis of terpenes, including monoterpenes, diterpenes, and sesquiterpenes, is upregulated under drought conditions (Turtola et al., 2003). These molecules could enhance drought tolerance and induce the ROS defence system. Polyamines, such as spermine and spermidine, increase significantly in response to drought stress and play a role in plant adaptation (Vaughan et al., 2015; Yadav et al., 2021).

Despite the abundance of information concerning the molecular, biochemical and physiological response of plants to drought stress, relatively little information is reported concerning the response of plants to mild drought stress. Recent research highlighted that, unlike what happens in case of high-intensity stress where plant growth and development are strongly impacted, plants exposition to moderate stress (mild stress) also triggers the activation of primary and specialized metabolism, which, by increasing the production of metabolites with osmoprotectants and antioxidant activity, supports the steady growth and development of the plant (Al-Gabbiesh et al., 2015; Men et al., 2018; Wu et al., 2016). The studies carried out on mildly stressed plants reported that these specialized metabolites, mainly belong to the classes of terpenoids and phenolic compounds which, besides their ecological role, have high commercial importance as their levels in plant tissues are considered as quality index, especially for the medicinal and aromatic plants market (Gutiérrez-del-Río et al., 2018). In fact, they possess nutraceutical and pharmacological properties, thereby adding to their significance in scientific research (Selmar and Kleinwächter, 2013).

According to ISMEA (Institute of Services for the Agricultural and Food Market), the aromatic and medicinal plant sector has grown by 110% in the last years, developing a market of great interest for Italy (Fichera et al., 2013). This market produces 25.000 tons of products of various kinds (drugs, supplements, aromas, cosmetics, liqueurs, etc.) in over 6 K companies involved (about 24 K cultivated hectares), covering only the 70% of the entire national requirement (Fichera et al., 2013). In Italy, according to sources from the National Federation of Medicinal Plant Producers, both peppermint (*Mentha × piperita*) and spearmint (*Mentha spicata* L.) are the crops among the aromatics, with the highest planted area (Fichera et al., 2013). In particular, *Mentha spicata* (from now on, spearmint) is a perennial herb that is widely cultivated for fresh consumption and for its essential oils, used in a variety of foods, beverages, and personal care products (Mahendran et al., 2021; Prakash et al., 2016). This species is shallow-rooted, making it a high-water-demanding crop. Several studies previously demonstrated its dependence on irrigation, highlighting that inadequate water management alters plant water status and metabolism, significantly affecting yield (Juárez-Rosete et al., 2014; Lakara and Verma, 2021).

Considering the high spearmint's water demand and the main problems related to climate change, finding new approaches that could help achieve an optimal yield while reducing water application could be agronomically important. In this context, recent studies investigated the effect of cyclic-mild-drought stress on several species, aiming to reduce the amount of water for their cultivation without affecting the yield and improving at the same time qualitative traits (Selmar and Kleinwächter, 2013). Numerous studies documented that plants subjected to drought stress exhibit elevated levels of specialized metabolites. These heightened concentrations have been observed in various classes of compounds, including simple and complex phenols and numerous terpenes. For instance, plants of *Hypericum brasiliense* exposed to non-cyclic drought stress were characterized by reduced growth and a drastically increased concentration of phenolics (Nacif de Abreu and Mazzafera, 2005). Similarly, in experiments on *Salvia officinalis*, a species cultivated mainly for its terpenic profile, drought elicited a massive production of monoterpenes (Nowak et al., 2010). At the same time, it has been reported that mild drought stress applied to lettuce significantly increased biomass production, sensory traits, and product shelf life (Paim et al., 2020). Little information is available regarding plants' physiological and metabolic responses to cyclic mild drought stress, and no information, evaluated through an integrated physiological and metabolomic approach, has been published concerning the changes induced by this stress typology on the nutraceutical properties of *M. spicata*.

In light of climate change and the decreasing availability of water for agricultural practices, identifying novel strategies to sustain or enhance crop production in response to dwindling water resources is of paramount significance. Equally important is the understanding of the metabolic and molecular processes underpinning such adaptation. Therefore, this study has a double purpose. On the one hand, it aims to study the physiological responses and the metabolic adjustments that plants activate to counteract a cyclic-mild-drought stress condition. On the other, it aims to evaluate if this water management could also be adopted to improve the nutraceutical and sensory characteristics of the product.

2. Materials and methods

Preliminary experiments, which aimed to identify the stress thresholds, were carried out on rooted mature cuts, as described below in detail. In particular, plants were subjected to water deficit, and both soil tension and stomatal conductance were measured daily to individuate the minimal soil tension value at which the stomatal conductance reached 50% with respect to the control. This value, corresponding to -300 mbar, was considered a mild-drought stress and applied for subsequent experiments.

2.1. Plant species and growth condition

For all the experiments was employed a mixture of silver sand (0–5 mm) and a professional peat-based potting mix [COMPLETO, produced by Vigor Plant Italia S.R.L., Fombio (LO), Italy] with the following characteristics: 23% coconut fibre (0–5 mm), 27% Irish peat (0–5 mm), 14% volcanic pumice (3–8 mm), 36% ultrathin peat (0–10 mm), pH (in H₂O) 6.5, electrical conductivity (0.35 dS m⁻¹), total porosity (92%, v/v), low release NPK]. Professional soil and sand were mixed with the ratio soil:sand (2:1, v:v). Plants of spearmint were bought from a commercial nursery (Ingegnoli nursery, Milan, Italy) and grown in a greenhouse with a supplementary cooling and light system. The photoperiod was characterized by 16-h light (PPFD of ~300 μmol of photons m⁻² s⁻¹) and 8-h darkness. The daily average temperature was settled to 26.4 ± 0.5 °C during the light period and 24.5 ± 0.5 °C during darkness. The mean daily vapor pressure deficit (VPD) was 2.3 ± 0.3 kPa.

Before starting the experiment, plant variability was reduced by producing uniform apical cuttings from mother plants. spearmint cuttings were rooted using a substrate composed of the previously described professional peat-based substrate soil mixed with silver sand (2:1 v:v) (Supplementary Fig. S1). After rooting and establishment, six rooted cuttings, selected for growth uniformity, were transplanted in 9 L pots (35 l x 25 d x 14 h cm) filled with the substrate previously described, where plants were left to grow for one month till vegetative maturity, which coincided with the rhizome formation. During this period, plants were fertilized every week using 11 g of slow-release fertilizer (15% total N, 15% K₂O, 9% P₂O₅, 2% MgO, 22.5% SO₃, 0.01% B, 0.002% Cu, 0.3% Fe, 0.1% Mn and 0.002% Zn) and watered every other day, maintaining a constant pot soil water capacity (90%).

2.2. Stress application

The experiment was done using a randomized design with four replications, each consisting of 1 pot with six plants, two treatments [watered (C) and drought-stressed (T)] and two-time cycles (T1 and T2). One tensiometer for each group of replicates was used. To apply middle cyclic stress, the pots were irrigated to achieve a pot soil water capacity of 90%, and then the watering was stopped only in T plants, whereas C plants were continuously maintained at the same pot soil water capacity. When the soil tension in T pots was equal to -300 mbar, the plants were considered mild stressed and an entire pot, consisting of six plants, was sampled (four replications), while the remaining not harvested T pots were restored to a pot water capacity of 90%. Plants were kept under this condition (90% of the water capacity) for 6 days to allow plant recovery from stress, and then the water stress was repeated once again (second mild stress cycles), following the criteria previously described.

Concerning the sampling timeline T0 was considered as day 1 of the experiment, and the first sampling was carried out on day 9 (T1). Immediately after sampling, all plants (treated and untreated) were watered and maintained at field capacity to allow plant recovery (from day 9 to day 15, when the irrigation was newly stopped in treated plants). Finally, on day 23, plants were newly sampled (T2) (Supplementary Fig. S1). Before each sample collection, all the in-vivo measurements were carried out (see § 2.3).

2.3. In-vivo analysis

All the in-vivo analyses were measured at the same time of the day (9:30 am) to avoid biased results due to circadian rhythms.

2.3.1. Chlorophyll a fluorescence, net photosynthesis, stomatal conductance and leaf temperature

Chlorophyll a fluorescence transients were determined on 30 min dark-adapted leaves following the protocol previously reported by Bulgari et al. (2020), whereas the portable photosynthesis system CIRAS-3

(PP Systems International, Inc., Amesbury, USA) was used to monitor the stomatal conductance (gs) and the net photosynthesis (An). During the CIRAS-3 analyses, the air temperature was 25 °C ± 1.5, the Vapor Pressure Deficit was 2.3 ± 0.3, and the analysis was carried out on a leaf surface of 1.75 cm².

Finally, the temperature of the leaves was monitored using an infrared thermometer (Kizen LaserPro DT-8380 Infrared Thermometer).

2.4. Post-sampling analysis

For post-sampling analysis, all plants were sampled at the same hour (01:00 pm), weighed, and stored as required by the different analytical protocols. In particular, part of them was immediately snap-frozen in liquid nitrogen (i.e., for metabolomic analysis), a part was used for relative water content calculation, and another part was collected and dried at room temperature in dark conditions (i.e., for volatilomic and IRMS analysis). The remaining part was weighed and oven-dried at 80 °C for 72 h for dry biomass calculation.

2.4.1. Fresh weight (FW), dry weight (DW), water content (WC), leaf Relative Water Content (RWC) and leaf osmolality

The plant material from four pots (six plants per pot) was collected at the end of the experiment, and the FW of the aerial parts was evaluated. Successively, plants were oven dried at 80 °C for 72 h to get DW. Water content (WC) was determined as follows: WC = [(FW - DW) / FW] * 100, and expressed as % H₂O. Leaf osmolality (mOsm) was determined using a semi-micro osmometer (model K-7400, Knauer GmbH, Berlin, Germany) as previously described by Meggio et al. (2014).

Leaf relative water content (RWC) was determined following the methodology outlined by Mullan and Pietragalla (2012), with certain adaptations. Each replicate involved weighing a single leaf (FW) before placing it in a 50 mL Falcon tube filled with 15 mL of ultrapure water, where it was incubated for 24 h at 4 °C. Subsequently, the turgid weight (TW) was recorded, and the leaf samples were transferred to an oven, where they were dried at 60 °C for 48 h and then weighed (DW). The RWC parameter was calculated using the following equation: RWC = [(FW - DW)/(TW - DW)] * 100.

2.4.2. Ion content: ICP-MS and HPLC analysis

The ionic analysis, in control and mildly cyclic-stressed plants, was carried out on 0.3 g of dried plant material using an ICP-MS (Varian, Inc., Palo Alto, CA, USA) apparatus and following the protocol previously described by Álvarez-Rodríguez et al. (2023).

To determine the concentrations of anions, the following procedure was conducted. Liquid nitrogen freeze-dried samples weighing 100 mg were processed as described by Sorin et al. (2015). The supernatant, obtained during the extraction, was filtered using 20 μm filters and evaporated under vacuum at 50 °C using a CHRIST Rotary vacuum concentrator 2-18 CDplus, and suspended in 0.2 mL of ultra-pure water. Anion contents were measured using a HPLC coupled to a UV detector (Agilent Technologies - 1260 Infinity). Chromatographic separation was performed using a PRP-X100 Anion Exchange column (dimensions: 100 × 4.1 mm; particle size: 5 μm). The mobile phase, consisting of a 4 mM Na-benzoate buffer at pH 5, was pumped isocratically. The flow rate was set to 1.4 mL min⁻¹, and an injection volume of 5 μL was used. The analysis was conducted at 25 °C. Anion levels were determined by referring to the absorbances obtained from the standard curves.

2.4.3. δ¹³C determination

To prepare the samples, dry powdered leaf tissues weighing 1 mg were placed into 5 × 9 mm tin capsules. The capsules were carefully sealed by folding them with clean tweezers and then transferred to an auto-sampler. The δ¹³C values of the samples were determined using a Flash 2000 HT elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer (IRMS) via a ConFlo IV Interface, following the protocol previously described by Bononi et al. (2022).

The isotope ratio $^{13}\text{C}/^{12}\text{C}$ was expressed using the standard $\delta^{13}\text{C}$ notation: $\delta^{13}\text{C} = [(^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{VPDB}} - 1] \times 1000$.

This equation represents the part per thousand deviations of the sample's isotope ratio $^{13}\text{C}/^{12}\text{C}$ relative to the Vienna Pee Dee Belemnite international standard, as described by Brand et al. (2014).

2.4.4. Head space-solid phase micro extraction-GC-MS analysis of plant volatiles

Volatile organic compounds' analysis (VOCs) in 1 g of dried leaf samples was performed using the HS-SPME-GC-MS method following the protocol previously described by Landi et al. (2022). The obtained chromatograms of the samples were previously aligned and then deconvoluted using the open-source software MS-DIAL 4.9. Data were subsequently normalized using the mTIC (total ion current) method in MS-DIAL, and the ions intensity was extracted and averaged. The annotation of the peaks was achieved using the retention index (RI) and spectral similarity matching with an in-house EI spectral library (Misra, 2019). The annotations were categorized as level 2 and/or level 3, following the criteria proposed by Sumner et al. (2007).

2.4.5. Untargeted-metabolomic analysis

The untargeted metabolomics analysis using GC-MS was conducted on spearmint aerial tissues, following the extraction, derivatization, and analysis methods outlined in the study by Lisec et al. (2006). Derivatized samples, containing ribitol as an internal standard (0.2 mg/mL), were injected into the GC-MS apparatus equipped with a 5MS column (30 m \times 0.25 mm \times 0.25 μm + 10 m of precolumn). The instrument's temperatures, analytical settings, and the MS-DIAL analysis for sample alignment, deconvolution, peak extraction, and annotation were configured according to the methods previously established by Misra et al. (2020). To ensure quality control and monitor instrumental performance and RI shifts, blank solvents, qualitative controls (QC), and n-Alkane standards (ranging from C10 to C40, all even-numbered) were injected at regular intervals for potential identification. The annotation of peaks followed the approach described in the previous paragraph.

2.5. Statistical analysis

The experiments used a completely randomized design with different replications depending on the experiment (3 for physiological data and 4 for metabolomic data), treatment, and stress cycle. Except for the metabolomic experiments, all the data were analyzed through two-way ANOVA using Tukey's test as post-hoc ($P \leq 0.05$). Metabolomic statistical analyses (VOCs analysis and untargeted-metabolomic) were carried out using the open-source software Metaboanalyst 5.0 using the one-factor and time-series plus one-factor statistical tools. The integrity of metabolite concentrations was assessed, and in cases where missing values were detected, they were substituted with a small positive value. This replacement value was determined as half of the minimum positive number observed in the dataset (Xia and Wishart, 2011). Before performing univariate (t-tests - $P \leq 0.05$) and multivariate analysis VOCs data were normalised using the MS-DIAL mTIC (Total Ion Current) method (TIC of the identified metabolites). In contrast, normalisation was based on ribitol (internal standard) for untargeted metabolomics. Successively, all data were Log_{10} transformed and scaled through the "Pareto-Scaling" method. Next, the data underwent unsupervised Principal Component Analysis (PCA) to generate a score plot for visualizing group differentiation and a loadings plot to identify metabolites contributing to group separation. Furthermore, cluster analysis was performed using the Euclidean distance measure and the Ward algorithm for cluster formation to examine the classification further. Data were further analysed through the multivariate ANOVA Simultaneous Component Analysis (ASCA), which is an approach designed to identify major patterns about the two given factors (treatment and time of treatment) and their interaction (Smilde et al., 2005). The leverage and alpha thresholds were set to 0.7 and 0.05 for feature selection and model

validation, respectively. Since the ASCA analysis did not highlight any significant model for interaction (time \times treatment), data were analysed through the limma-based metaboanalyst tool "linear models with covariate adjustments", which uses linear models (limma or lm) to perform significance testing with covariate adjustments and is widely used for statistical analysis involving complex metadata without considering interactions (Pang et al., 2022).

3. Results

3.1. Effects of cyclic-mild-drought stress on physiological parameters

The data are presented as a time series (T0-T2) of two different treatments (watered and drought-stressed) and a control watered group (T0). At the first time point of treatment (T1), there were no significant differences in the aerial parts FW of watered and stressed plants (Fig. 1a). Moreover, no significant differences were observable between T0 and T1 (Fig. 1a). However, at the second time point (T2), the watered plants showed a 54% increase in FW, whereas stressed plants FW increased by a 28% (Fig. 1a). Anyway, even if at T2 the FW of drought-stressed plants was lower than the control plants by 26%, the parameter was not significantly different between the two conditions (Fig. 1a). Similarly, excluding the watered plants at T2, where an increment in DW was observed (95% compared to T0), in all the other treatments no significant differences in this parameter were detected (Fig. 1b). On the contrary, water content, estimated as the percentage of water in the fresh biomass, was weakly reduced in T1 stressed plants compared to T0 (7% lower) (Fig. 1c). However, the same parameter was not significantly different in stressed plants at T1 and T2 compared to their respective controls (Fig. 1c). Similarly, no difference in relative water content were observed among treatments and stress cycles (Fig. 1d).

In plants, the stomatal conductance (gs) (Fig. 2a), leaf transpiration rate (E) (Fig. 2d) and net photosynthesis (An) (Fig. 2b) were strongly affected by water stress at both exposure times (T1 and T2). Specifically, there was a 72% reduction in gs and a 67% reduction in E at T1, and a 67% reduction in gs and a 69% reduction in E at T2. (Figs. 2a and 2d). Similarly, at T1 the An parameter in stressed plants was 82% lower than control and 90% at T2 (Fig. 2b). In addition, water-stressed plants were characterised by a sensitive increment in leaf temperature (c.a. 7% higher compared to the respective controls) at both T1 and T2 (Fig. 2c). In contrast, no significant differences in the parameter Fv/Fm (maximum quantum efficiency of PSII in dark-adapted leaves) were observed (Fig. 2e).

Finally, the carbon stable isotope analysis, according to gs and E, revealed that plants treated with the cyclic-mild-drought stress were characterised by a $\delta^{13}\text{C}$ less negative than in well-hydrated plants (Fig. 2f). This increment in $\delta^{13}\text{C}$ value was affected by the time, treatment, and their interaction, as indicated by the two-way ANOVA analysis (Supplementary Table S1).

3.2. Effects of cyclic-mild-drought stress on the plant ionome and osmolality

The effects of the cyclic-mild-drought stress on osmolality and ionome (cations and anions) were analysed through two-way ANOVA to evaluate if the changes observed were influenced by the time, the treatment or their interactions. The analysis highlighted that the leaf osmolality was significantly affected by the time and the treatment, whereas the factors interaction was not significant (Supplementary Table S1). In particular, at T1 and T2, the osmolality of treated plants was characterised by a 17% and 26% increase, respectively (Fig. 3a).

Concerning the cation content, the ICP-MS analysis highlighted that Na was affected by time and stress treatment, Mo by the treatment, whereas Mg, Se, Mn and Ca were affected only by time (Fig. 3b-g and Supplementary Table S1). On the contrary, ions such as K, Fe, Cu, Zn, Ni, and Co were unaffected by the time or the treatment. The interaction

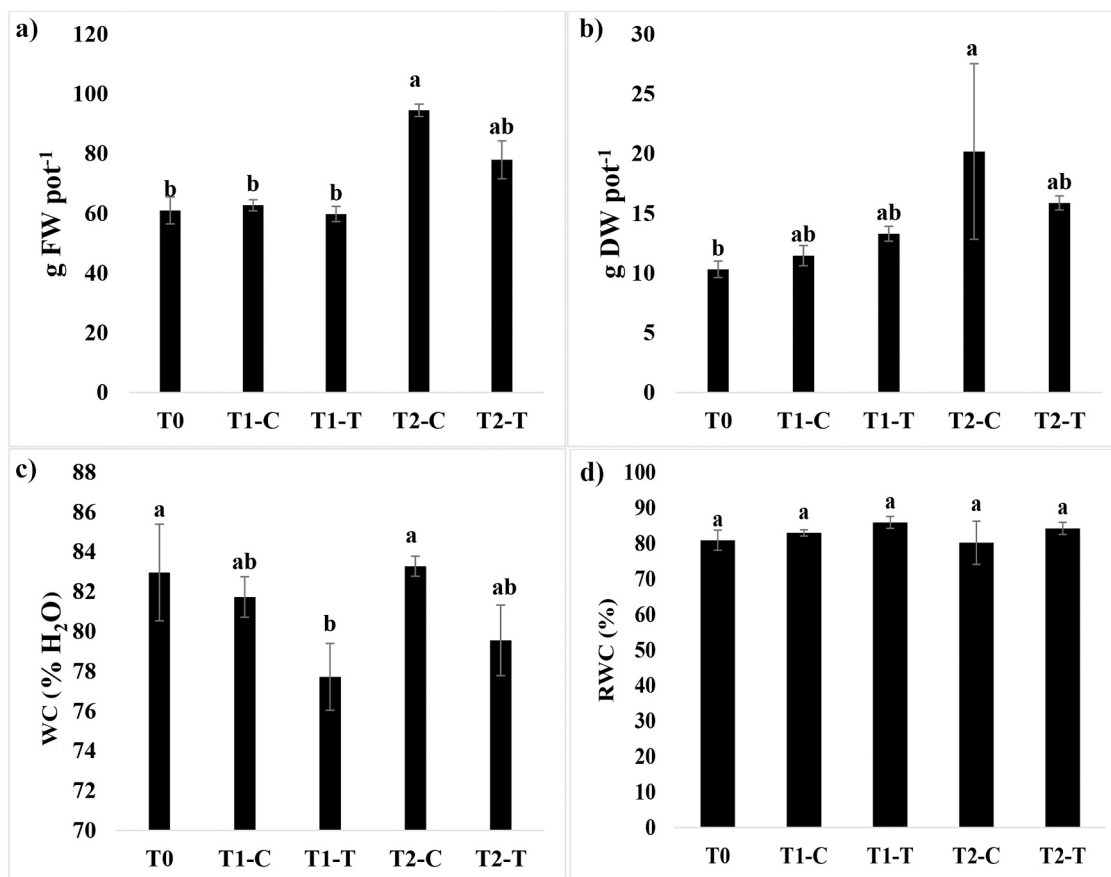


Fig. 1. : Effects of cyclic-mild-drought stress on different parameters related to the plant aerial parts in *Mentha spicata*: a) fresh weight (FW), b) dry weight (DW), c) water amount expressed in percentage *per FW* (% H₂O), and d) leaf relative water content (RWC). T0 – plant material collected at the beginning of the experiment; T1-C – watered plants collected at the end of the first cycle (T1); T1-T – drought-stressed plants collected at the end of the first cycle (T1); T2-C – watered plants collected at the end of the second cycle (T2); T2-T – drought-stressed plants collected at the end of the second cycle (T2). Data were analysed through two-way ANOVA using Tukey's test as post-hoc ($P < 0.05$). Data are expressed as mean \pm standard deviation. $n = 3$.

Time \times Treatment affected none of the ions analysed (Supplementary Table S1).

Sodium content decreased over time. However, no differences were observed comparing Na content in C and T at T1 and T2 (Fig. 3b). Molybdenum ion was only significantly reduced by the treatment at T1 (20% lower than its control) (Fig. 3c). Mg, Mn and Ca, generally increased over time, even if no differences were observed between the treatments at the same time (Fig. 3d, f-g). On the contrary, Se increased at T1 and remained constant (Fig. e).

Concerning the anions, NO₃ and P_i were affected only by time, whereas Cl was affected only by the treatment (Fig. 3h-l).

3.3. Metabolomic analysis driven by GC-MS

The untargeted metabolomic analysis on spearmint plant material identified 132 compounds. The annotated metabolites belonged to different classes of chemicals, such as organic acids, amino acids, fatty acids and sugars (see supplementary Table S2). The data underwent analysis using a multivariate unsupervised principal component analysis (PCA) technique, which explained 58% of the total variance (PC1 – 38.5% and PC2 – 19.5%). The data highlighted an overlap between T0 and the control at T1 (T1-C), whereas T2-C was slightly separated. On the contrary, the treated samples, T1-T and T2-T, were completely separated from the other groups (Fig. 4a). As revealed by the loading plots, PC1 was mainly influenced by palmitic acid, lactic acid, asparagine, heptadecanoic acid, 4-hydroxybutyric acid, stearic acid, citraconic acid, icosanoic acid, glutamine, whereas PC2 by salicyl alcohol-b-

glucoside, serine, protein serine, mannose, 4-hydroxybutyric acid, among others (Supplementary Table S2). The cluster analysis confirmed the group separation trend in the PCA, highlighting the formation of two macro groups: the first including T0 and control plants and the second mildly stressed plants (Fig. 4b).

The multivariate ASCA model successfully analyzed the data, highlighting that the treatment and time influenced the metabolome. In contrast, the factors' interaction was not significant (data not shown). Therefore, we decided to analyze the data through the univariate limma-based linear models with covariate adjustments (Fig. 4c,d), highlighting that 51 metabolites were significantly affected by the treatment and 93 by the time (Fig. 4c,d and Supplementary Table S2).

Considering the metabolites altered by the drought, most belonged to the class of organic acids, sugars, amino acids, and phenolics, among others (Figs. 5–8 and Supplementary Table S2). Concerning the amino acids, the mild cyclic stress induced a significant accumulation at T1 and T2 of the metabolites GABA, leucine, valine, *n*-acetylserine, proline, and its derivative pyroglutamic acid (Fig. 5), whereas glycine was accumulating only at T2. On the contrary, glutamine and asparagine were significantly reduced over time, but the treatment exacerbated this effect. The treatment strongly reduced these two molecules at both exposure times (T1 and T2). Finally, β -alanine significantly reduced over time only in control plants, whereas in drought-stressed plants maintained higher levels both at T1 and T2 (Fig. 5).

Similarly, sugars such as glucose, maltose, galactose, rhamnose, and arabinose significantly accumulated in mildly stressed plants at T1 and T2, ribose only at T2, whereas fructose in control plants dropped down

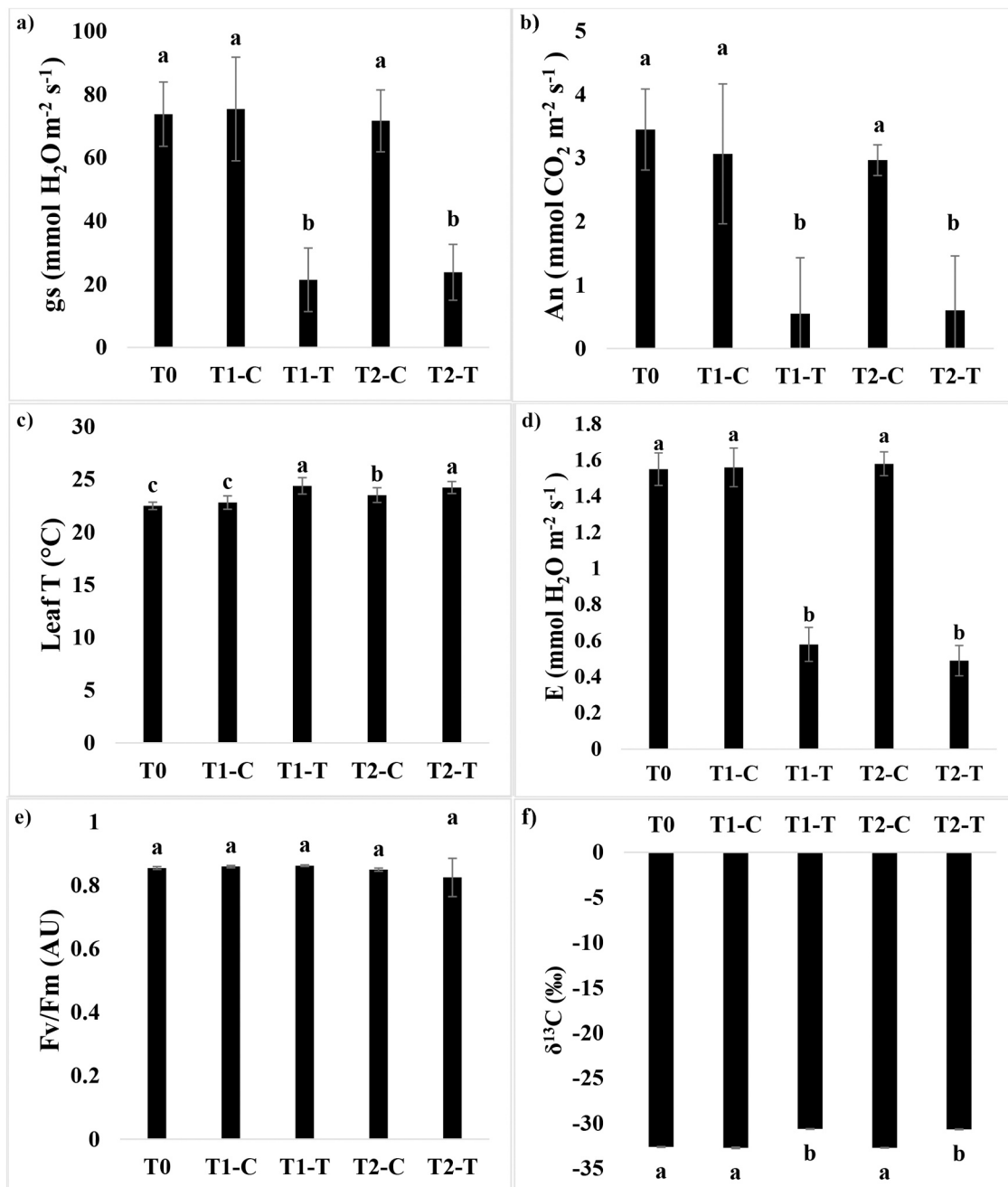


Fig. 2. : Effects of cyclic-mild-drought stress on different *Mentha spicata* parameters: a) stomatal conductance (gs); b) net photosynthesis (An); c) leaf temperature; d) leaf transpiration rate (E); e) the total efficiency of the dark-adapted PSII (Fv/Fm); and f) leaves stable carbon isotopic composition ($\delta^{13}C$). T0 – plant material collected at the beginning of the experiment; T1-C – watered plants collected at the end of the first cycle (T1); T1-T – drought-stressed plants collected at the end of the first cycle (T1); T2-C – watered plants collected at the end of the second cycle (T2); T2-T – drought-stressed plants collected at the end of the second cycle (T2). Data were analysed through two-way ANOVA using Tukey's test as post-hoc ($P \leq 0.05$). Data are expressed as mean \pm standard deviation. n = 3.

at T1, while in treated plants was only slightly reduced compared to control (Fig. 6). Finally, sugars phosphates such as fructose-6-phosphate and glucose-6-phosphate at T1 were characterized by an inverted trend compared to control. In fact, in treated plants, both sugars strongly accumulated, whereas in control, they were significantly reduced. At T2, both sugars restored their concentrations (Fig. 6).

In treated plants, organic acids such as malic, succinic, citramalic, and glyceric acid were more abundant than in the control at T1 and T2. On the contrary, citraconic acid content was lower (Fig. 7). Finally, all the phenolic acids, nicotinic acid and phenylalanine were in higher concentrations in treated plants if compared to control at the respective time of treatment (T1 and T2) (Fig. 8). A similar trend was also observed

in ascorbic and pantothenic acid content. In contrast, an inverted trend characterised gulonic acid at T1 compared to the control. In fact, in treated plants at T1 it accumulated, whereas, in control, it was significantly reduced. At T2, gulonic acid was restored to T0 levels, but its concentration was still higher than in control at the respective time (Fig. 8).

Data were further analysed through the pathway analysis, which combines the topology and enrichment analysis. The results highlighted that the treatment significantly affected several pathways, and only eleven at T1 and thirteen at T2 were characterized by an impact higher than 0.2 (Table 1 and Supplementary Table S2). In particular, the most affected pathways were related to the biosynthesis of secondary

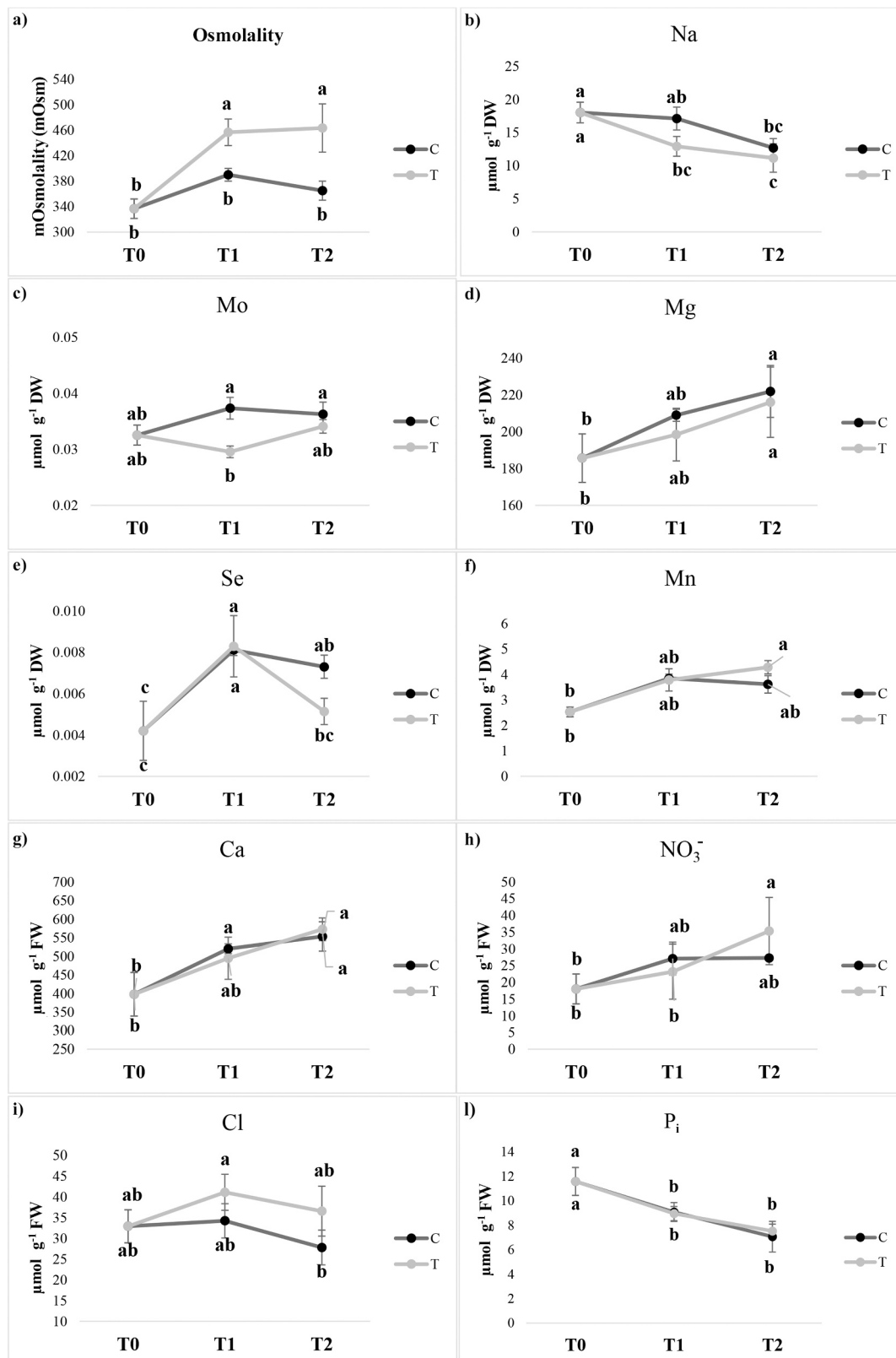


Fig. 3. : Effects of the cyclic-mild-drought stress on osmolality and ion levels in the aerial parts of plants of *Mentha spicata*. Data were analysed through two-way ANOVA using Tukey's test as post hoc ($P \leq 0.05$). Only the ions significantly affected by treatments, time of exposure and/or Time×Treatment, are reported in the graphs. a) osmolality; b) Na, sodium; c) Mo, molybdenum; d) Mg, magnesium; e) Se, selenium; f) Mn, manganese; g) Ca, calcium; h) NO₃⁻, nitrate; i) Cl, chloride; j) P_i, inorganic phosphate. C – watered plants; T – drought stressed plants; T0 – beginning of the experiment; T1 – end of the first cycle of the experiment; T2 – end of the second cycle of the experiment. Data are expressed as mean ± standard deviation. n = 3.

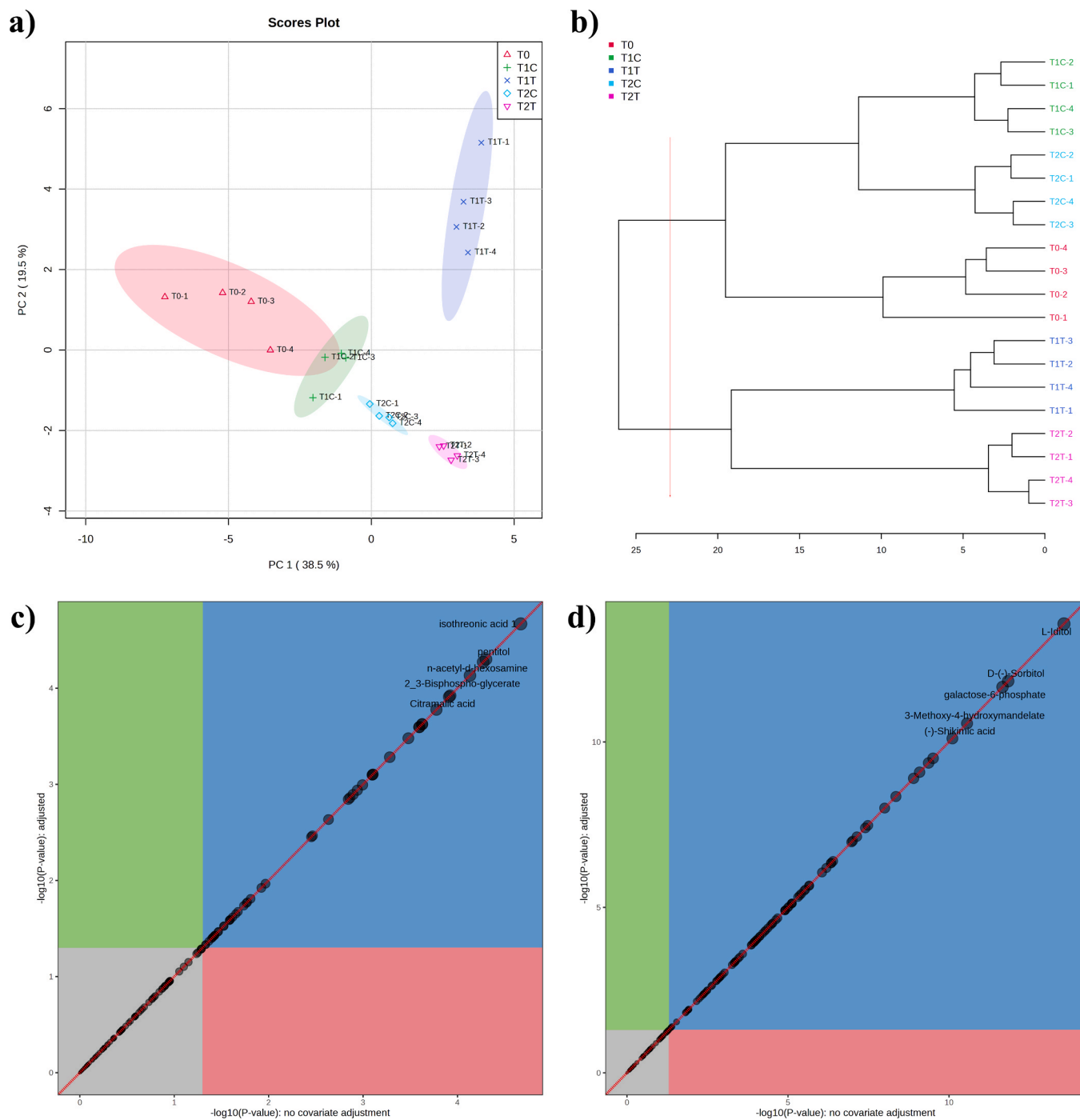


Fig. 4. : Effects of cyclic-mild-drought stress over time on plant metabolome. (a) Unsupervised PCA Scores plot between the two selected PCs (the explained variances are shown in brackets); (b) Clustering result shown as a dendrogram (distance measure using Euclidean, and clustering algorithm using ward); (c) Significant metabolites resulting from the linear models with covariate adjustments considering the treatment [control (C) and drought stress (T)] d) and considering the time (T0-T2). Only the metabolites in the light blue quadrant were characterised by a P value ≤ 0.05 . $n = 4$.

metabolites, amino acids, sugars and organic acids metabolisms, among others (Table 1).

3.4. Head space-solid phase micro extraction-GC-MS analysis of VOCs

To identify the impact of cyclic-mild-drought stress on spearmint's VOCs profile, dry leaves were analyzed through the HS-SPME-GC-MS technique. The analysis allowed the isolation of 196 compounds, but only 55 were correctly annotated (Supplementary Table S3). To assess the influence of the treatments on overall metabolites, annotated raw

data were analyzed through PCA built by virtue of the first two PCs components (PC1 vs PC2), which described 54.6% of the total variability (Fig. 9a). The data highlighted an overlap among the control (T0, T1C, and T2C) and the first drought stress-treated group (T1-T) and a clear separation from T2-T (Fig. 9a). The trend observed during the unsupervised PCA was further confirmed by the cluster analysis, which at a higher level, highlighted the presence of two separate clusters, one formed by control treatments (T0 - T1-C - T2-C) and the other by drought-stressed plants (T1-T - T2-T) (Fig. 9b).

Also, the volatilome was analyzed through the multivariate ASCA

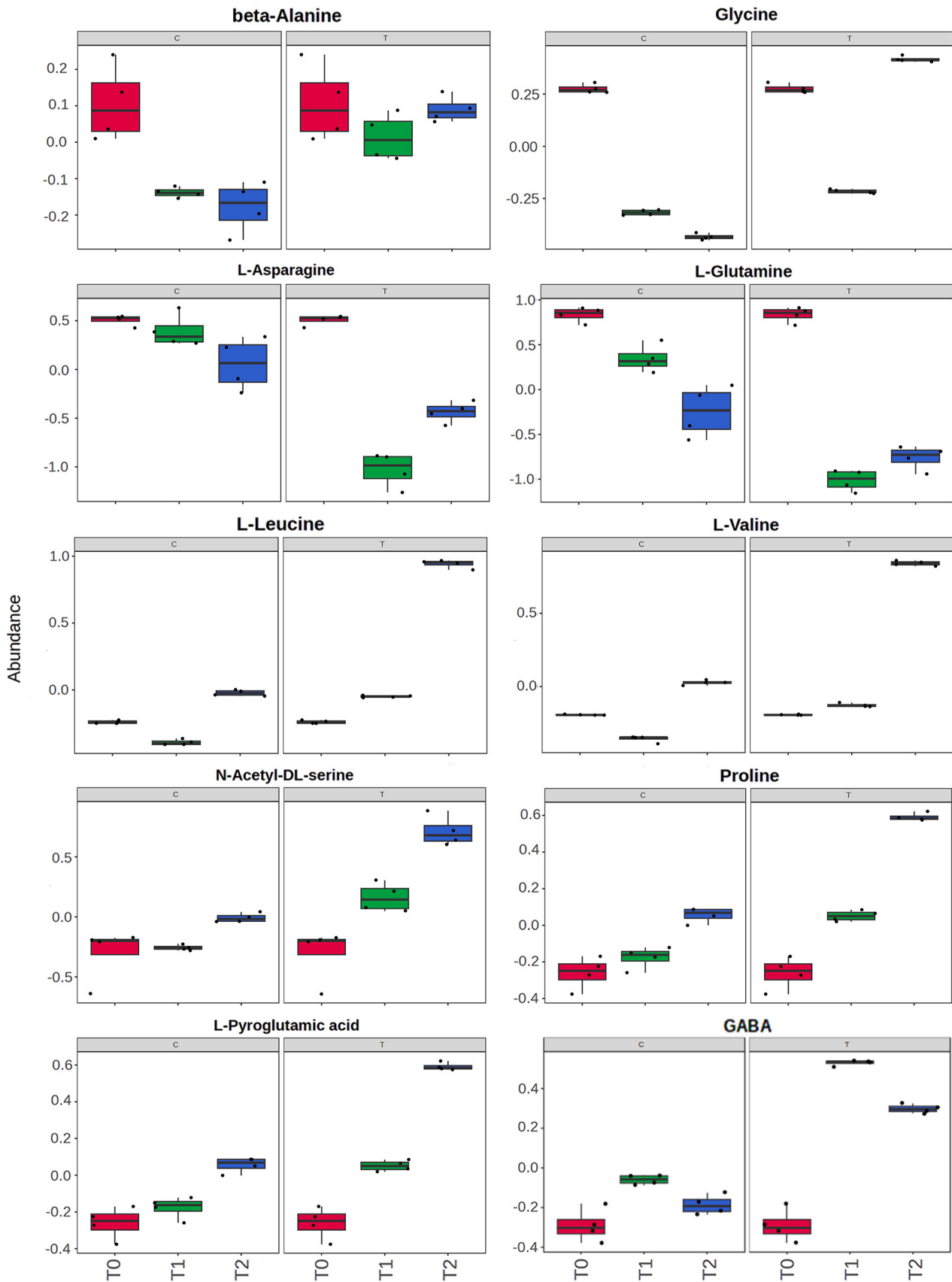


Fig. 5. : Box plots reporting the effects of cyclic-mild-drought stress on the relative abundance of the significantly affected amino acids identified in *Mentha spicata* plants. The identified metabolites were generated by linear models with covariate adjustments considering the treatment, reporting the trend of the metabolites along the time in control (CT) and drought-stressed samples (TR). T0 – beginning of the experiment; T1 – end of the first cycle of the experiment; T2 – end of the second cycle of the experiment. n = 4.

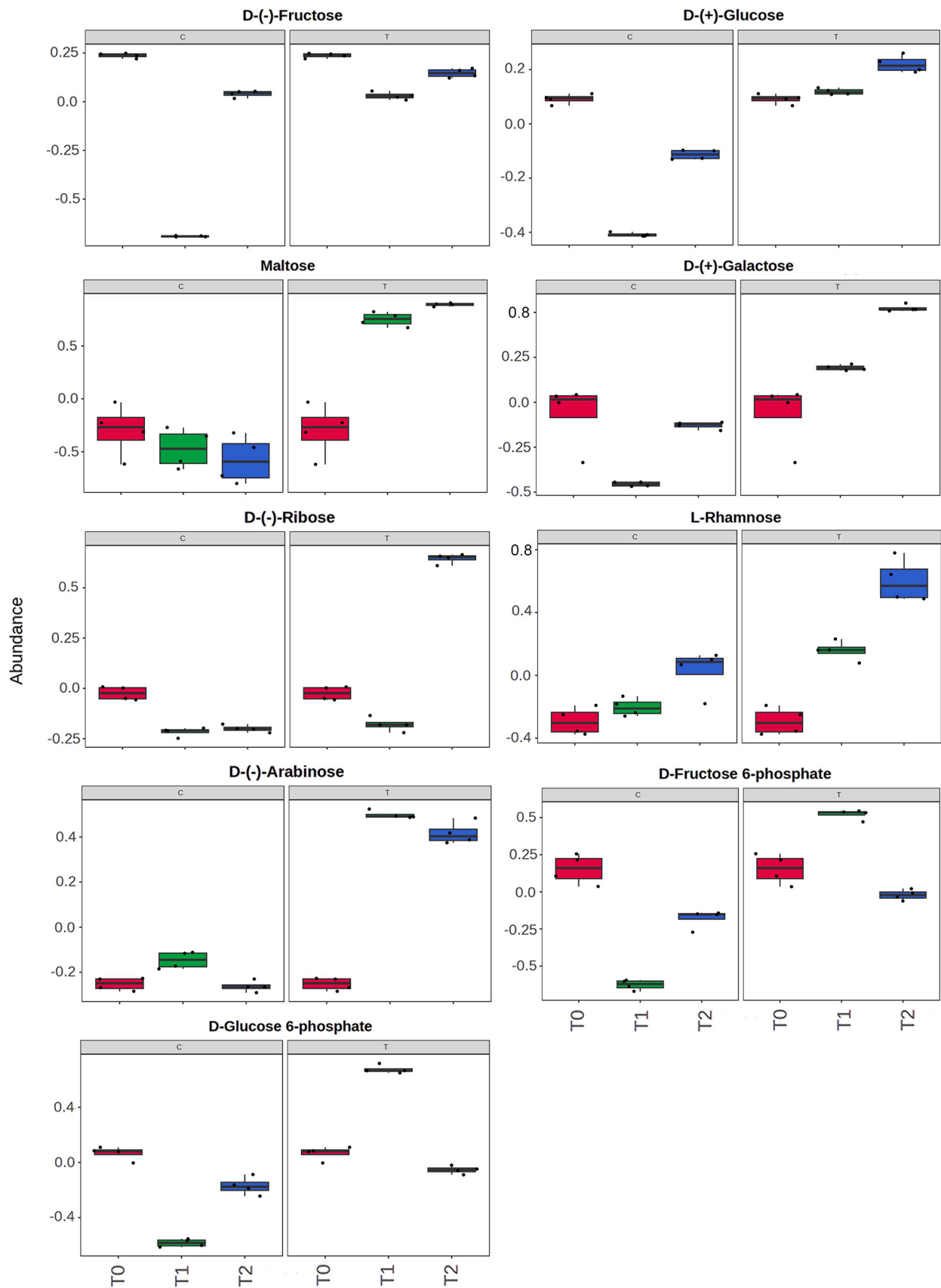


Fig. 6. : Box plots reporting the effects of cyclic-mild-drought stress on the relative abundance of the main significantly affected sugars identified in *Mentha spicata* plants. The identified metabolites were generated by linear models with covariate adjustments considering the treatment, reporting the trend of the metabolites along the time in control (CT) and drought stressed samples (TR). T0 – beginning of the experiment; T1 – end of the first cycle of the experiment; T2 – end of the second cycle of the experiment. n = 4.

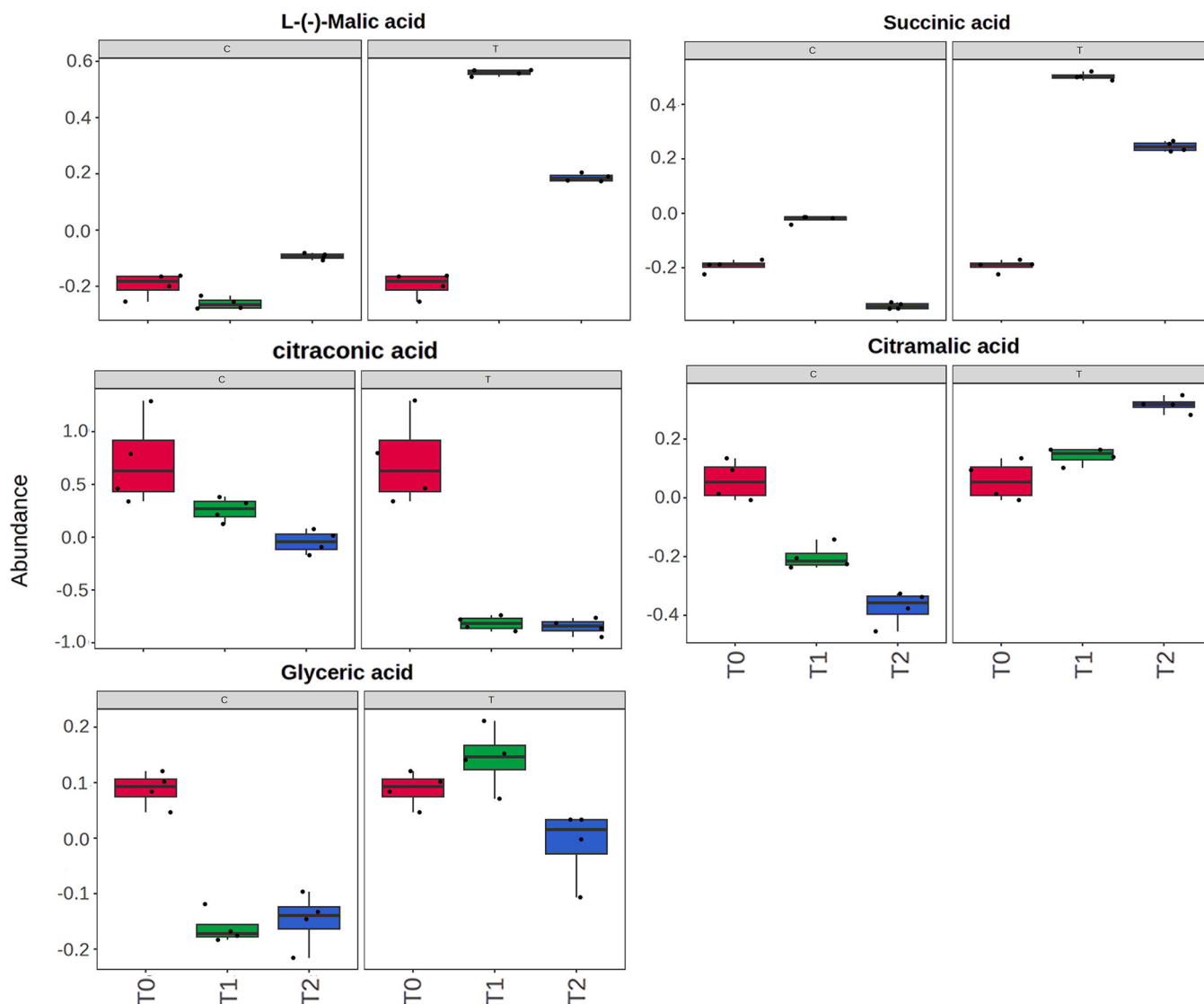


Fig. 7. : Box plots reporting the effects of cyclic mild-drought stress on the relative abundance of the main organic acids identified in *Mentha spicata* plants. The identified metabolites were generated by linear models with covariate adjustments considering the treatment, reporting the trend of the metabolites along the time in control (CT) and drought stressed samples (TR). T0 – beginning of the experiment; T1 – end of the first cycle of the experiment; T2 – end of the second cycle of the experiment. n = 4.

model, which confirmed that VOCs were influenced by the treatment and time but not by their interaction. Therefore, as previously described, we adopted the univariate limma-based linear models with covariate adjustments to analyze the data (Fig. 9c and d). This model highlighted that the treatment significantly affected eight compounds, whereas 32 compounds were affected by the time (Fig. 9c and d). The trend of the metabolites involved in the treatment is reported in Fig. 10, whereas those affected by the time can be found in Supplementary Table S3. Excluding 3-octanol, all the significantly affected metabolites were up-accumulating in response to the treatment (Fig. 10).

4. Discussions

4.1. Plant response to the cyclic-mild-drought stress

Numerous studies have assessed the effects of abiotic elicitors, such as moderate thermal and/or drought stress, on enhancing crop production and quality (Paim et al., 2020; Thakur et al., 2019). This strategy is based on the principle that plants have evolved biochemical and physiological mechanisms to adapt and tolerate short stress periods

(Thakur et al., 2019), including the production of specialized metabolites, which are often important determinants of quality traits, especially in aromatic plants (Mahajan et al., 2020). Evidence suggests that plants cyclically exposed to mild drought stress can induce these mechanisms without suffering significant growth alterations (Paim et al., 2020). The presence of cyclic-mild-drought stress allows the plant to enable osmotic adjustment if, in the species/genotype considered, this adjustment ability exists (Tschaplinski et al., 2019). In this study, two cycles of mild drought stress were applied throughout the growth and development of *M. spicata*. The first and the second cycle, each of which concluded when soil tension was around – 300 mbar, were separated by a recovery stage during which water in the substrate was restored to the field capacity. As a result, crop biomass and other physiological parameters monitored were affected neither by the first stress cycle or the second, confirming that the conditions chosen for the experiment could not be considered severe stresses. These results also suggest that spearmint can effectively tolerate the stress levels applied. In particular, in this experiment, the physiological data highlighted that the main factor affecting plant biomass was time rather than treatment.

Usually, during drought stress, the cell water potential drops down

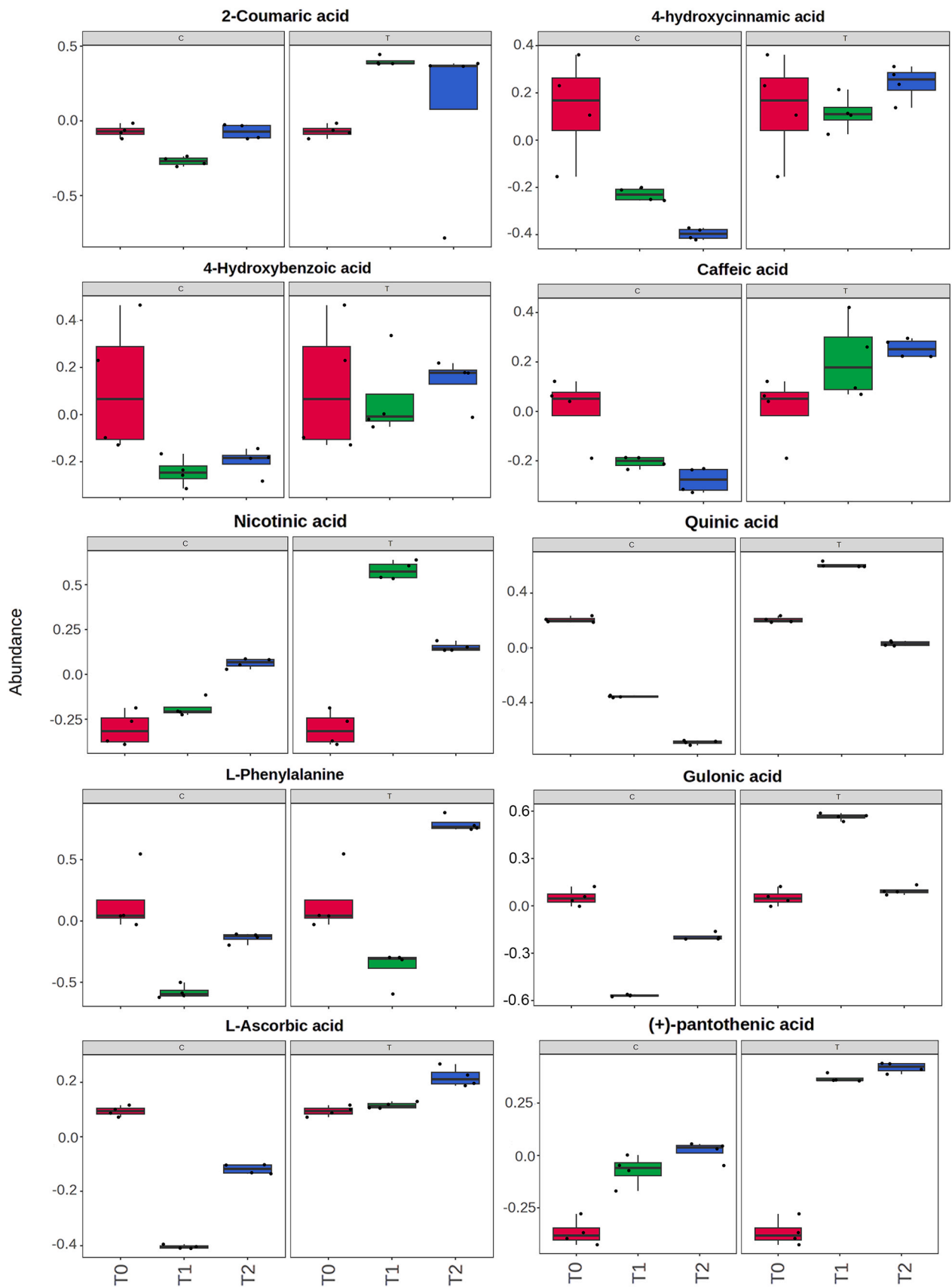


Fig. 8. Box plots reporting the effects of cyclic mild-drought stress on the relative abundance of the main phenolic acids and miscellaneous identified in *Mentha spicata* plants. The identified metabolites were generated by linear models with covariate adjustments considering the treatment, reporting the trend of the metabolites along the time in control (CT) and drought stressed samples (TR). T0 – beginning of the experiment; T1 – end of the first cycle of the experiment; T2 – end of the second cycle of the experiment. n = 4.

Table 1

Result from "Pathway Analysis" (topology + enrichment analysis) reporting the significant pathways affected in *Mentha spicata* after the first (T1) and the second (T2) cycle of mild drought stress.

Pathways	Total Cmpd	Hits	Raw P		Impact
			T1	T2	
Biosynthesis of secondary metabolites - unclassified	5	1	0.00016	3.67E-06	1
Alanine aspartate and glutamate metabolism	22	7	6.54E-06	0.00020667	0.72
Glycine serine and threonine metabolism	33	5	1.46E-08	6.33E-08	0.54
C5-Branched dibasic acid metabolism	6	1	3.84E-06	2.09E-05	0.5
Phenylalanine metabolism	11	1	0.04041	2.77E-07	0.47
Starch and sucrose metabolism	22	6	2.77E-09	0.04337	0.42
Glyoxylate and dicarboxylate metabolism	29	10	0.0002	1.14E-05	0.33
β-Alanine metabolism	18	4	//	0.039361	0.32
Citrate cycle (TCA cycle)	20	4	2.14E-09	6.25E-10	0.25
Pantothenate and CoA biosynthesis	23	5	//	0.0072916	0.24
Arginine and proline metabolism	34	4	5.35E-05	3.51E-07	0.22
Pentose and glucuronate interconversions	16	2	0.00046	1.22E-07	0.22
Carbon fixation in photosynthetic organisms	21	4	1.20E-11	1.33E-06	0.21

Total Cmpd: the total number of compounds in the pathway; Hits: the matched number from the uploaded data; P value: the original P value calculated from the enrichment analysis; Impact: the pathway impact value calculated from pathway topology analysis; //: not significantly impacted pathways. n = 4.

and, to reduce transpiration, the leaf stomata close, preventing water loss but reducing CO₂ absorption, which negatively affects the photosynthetic rate (Cornic, 2000). Moreover, it has been reported that prolonged drought stress severely affected the photosynthetic machinery of *M. spicata*, causing physical damage to the PSII (highlighted by a reduction in Fv/Fm parameter) and reducing plant growth and development (Matraka et al., 2010). Our experiments highlighted that the stressed plants were characterised by a higher δ¹³C (i.e., less C stable isotope discrimination), typical of plants that reduce transpiration by decreasing stomatal conductance (Farzadfar et al., 2017). Moreover, at T1 and T2 the applied stress induced a drop-down of stomatal conductance (gs) and net photosynthesis (An). An increase in leaf blade temperature also characterised plants, according to the behaviour generally observed during reduced transpiration (Matraka et al., 2010); however, no negative effects were observed for the Fv/Fm parameter. Finally, during the recovery step, when water in the growing medium of treated plants was restored to the field capacity, the gs and An parameters were restored to control conditions (data not shown). These results further confirm the mildness of the stress since plants were not experiencing physical damage to the photosynthetic machinery, and only those physiological and biochemical processes involved in early response to drought stress were activated.

One of the most important mechanisms adopted by plants to resist drought stress is reducing the osmotic potential, which could be achieved through three main ways: *i*) decreasing the intracellular water, *ii*) decreasing the cell volume, and *iii*) increasing osmolyte concentration (Yang et al., 2021). The latter could be achieved by increasing the cellular concentrations of soluble proteins, inorganic ions and/or organic compounds (Yang et al., 2021). Among the organic compounds, the main metabolites involved in osmoprotection belong to the classes of *i*) sugars and sugar alcohols (i.e., glucose, sucrose, raffinose, trehalose,

inositol, mannitol, sorbitol, galactinol, among others), which act in membrane protection, scavenging ROS (Ozturk et al., 2021; Traversari et al., 2018; Koyro et al., 2012); *ii*) quaternary ammonium compounds such as choline betaine, polyamines, proline, and glycine betaine, which facilitate plant adaptation under stress, protecting enzymes and membranes and stabilising the PSII protein pigment complex (Ozturk et al., 2021; Ramanjulu and Sudhakar, 2000; Papageorgiou and Murata, 1995); *iii*) free amino acids (i.e., arginine, asparagine, glutamine, proline, and gamma-aminobutyric acid), which strongly accumulate in a wide variety of species subjected to abiotic stress (Ozturk et al., 2021; Sankar et al., 2007; Nath et al., 2005; Yadav et al., 2005).

In the experiment, we observed an increase in leaf osmolality; however, changes in cations and anions concentrations do not seem related to the observed changes in this parameter. Moreover, the RWC was not affected by the treatments, suggesting that the observed increase in osmolality was not related to a loss of water in leaf tissues. Such a finding allowed us to speculate that changes in leaf osmolality could be mainly due to changes in organic compounds accumulation. In fact, according to our data, during drought stress, the metabolism of alanine, glutamate and aspartate, the glycine, serine and threonine metabolism, the arginine and proline metabolism, and the starch and sucrose metabolism, among others, are the most affected pathways belonging to the primary metabolism (Neto et al., 2021). Also, the pathways related to the biosynthesis of secondary metabolites, like the phenylalanine metabolism, on which the production of phenolic compounds depends, and vitamin B-related metabolism (pantothenate and CoA biosynthesis) were similarly affected (Subramani et al., 2022; Zhao et al., 2021). Indeed, treated plants pointed out an accumulation of sugars (i.e. maltose, fructose, glucose), amino acids (i.e. GABA, proline, 5-oxoproline), organic acids (i.e. malic acid, succinic acid) and other metabolites (caffeic acid, nicotinic acid, ascorbic acid among others) involved in plant protection during abiotic stress (Singh et al., 2015; Zulfiqar et al., 2020). An increase in osmotic potential accompanied by the accumulation of organic compounds, such as sugars and sugar alcohol, was also observed by Aranda et al. (2020) in seedlings of *Quercus pyrenaica* exposed to moderate drought stress. The same authors reported that the pool of carbohydrates, cyclitols and some organic acids were the main osmolytes explaining osmotic potential across four *Quercus* species exposed to drought stress (Aranda et al., 2021).

The accumulation of organic compounds derived from the primary carbon metabolism, such as sugars, tertiary sulphonium compounds and tertiary and quaternary ammonium compounds (Sairam et al., 2002), is generally used by plants in osmoregulation adaptive mechanisms to cope with salinity, drought, heat or cold stress (Ozturk et al., 2021; Yang et al., 2021). Treated plants pointed out a significant increment in amino acids, such as proline, GABA, pyroglutamic acid, glycine, valine, leucine, and serine, which generally occurs in higher plants in response to environmental stresses (Farooq et al., 2012; Ozturk et al., 2021). Previous studies demonstrated that drought stress upregulated Arabidopsis's branched-chain aminotransferase (BCAT) gene family members, inducing, as also observed in our experiments, an accumulation of valine, isoleucine and leucine (Urano et al., 2009). Also, proline and GABA, significantly accumulated in treated plants, probably playing a protective role against drought stress. Indeed, both molecules increase the osmolality and leaf turgor and reduce oxidative damage via antioxidant regulation (Zulfiqar et al., 2020). Even if in this study- we evaluated the GABA content in the whole leaf, it could be underlined that the increase in this metabolite has been associated with a reduction of stomatal opening, which plays a role in increasing the water-use efficiency and drought tolerance (Hasan et al., 2021; Trovato et al., 2008). The accumulation of several amino acids and soluble sugars has been reported to be implicated in water-status balance maintenance, osmotic homeostasis and/or in the production, within the phloem, of eutectic solvents involved in protecting membranes and enzymes from breaking down (Choi et al., 2011; Gavaghan et al., 2011; Khan et al., 2020). In addition, soluble sugars such as glucose, fructose, galactose etc., could

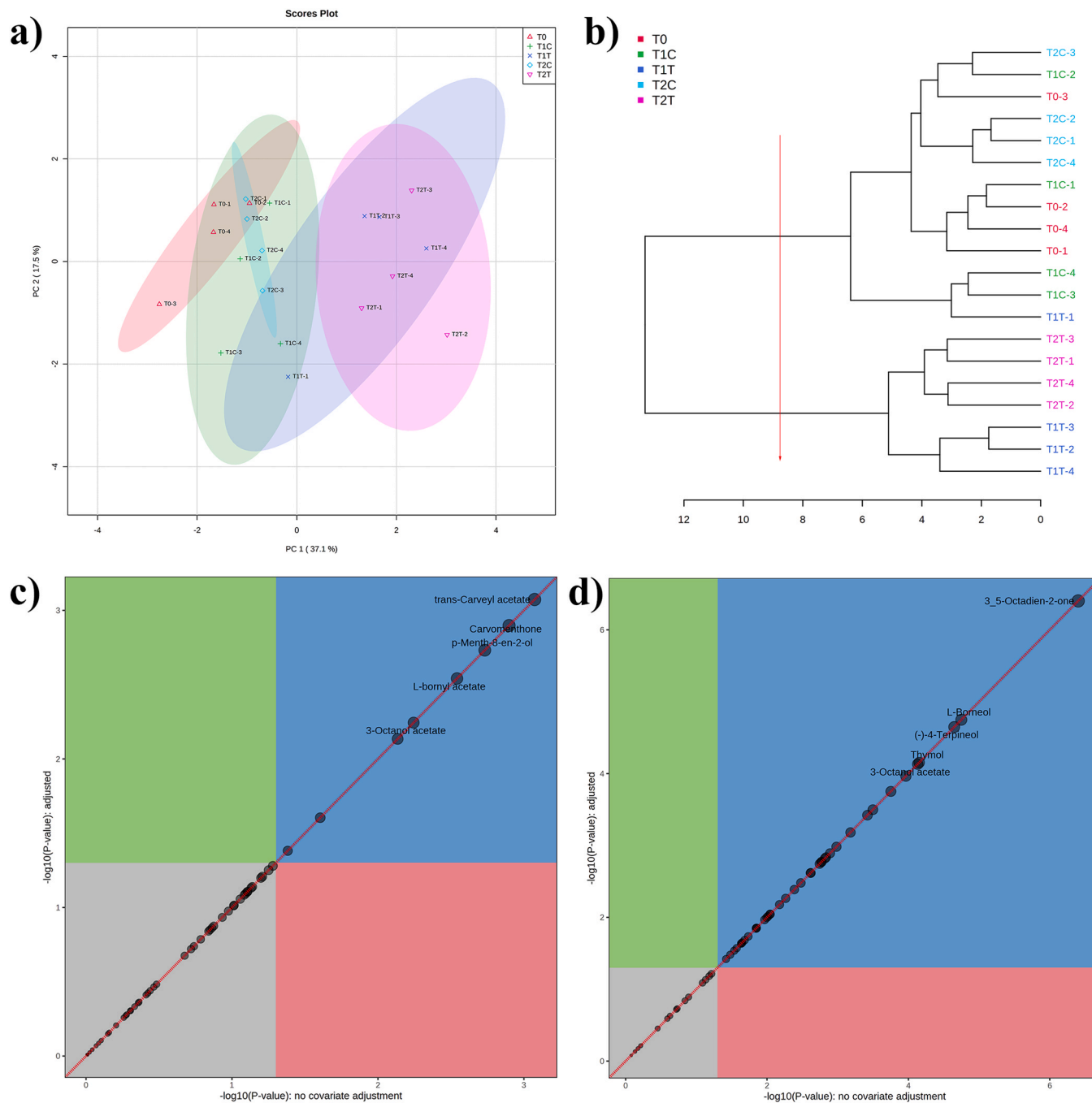


Fig. 9. : Data reporting the effect of cyclic-mild-drought stress along time on plant volatilome. (a) Unsupervised PCA Scores plot between the two selected PCs (the explained variances are shown in brackets); (b) Clustering result shown as a dendrogram (distance measure using Euclidean, and clustering algorithm using ward); (c) Significant metabolites resulting from the linear models with covariate adjustments considering the treatment [control (C) and drought stress (T)] and considering the time (T0-T2). Only the metabolites in the light blue quadrant were characterised by a P value ≤ 0.05 . $n = 4$.

act as signal molecules to regulate the expression of different genes involved in plant resistance or can also be used as a carbon source for plant maintenance during stress or recovery stages (Afzal et al., 2021; Chaves et al., 2002; David et al., 1998; Rosa et al., 2009).

Among the sugars, maltose, whose accumulation is generally related to starch mobilization (resulting significantly affected by the pathway analysis), was one of the most affected metabolites. It has been reported that this metabolite generally accumulates during drought stress and in response to GABA accumulation (Li et al., 2017). Moreover, maltose plays a crucial role in protecting proteins, membranes and the electron transport chain acting as a stabilising factor in the stroma of the

chloroplast (Kaplan and Guy, 2005, 2004). Increased leaf temperatures resulting from reduced transpiration could also induce its accumulation in this context. The potential impact of further maltose metabolism could be linked to the observed buildup of monosaccharides (glucose, fructose, and ribose), essential components for osmotic adjustment. Likewise, enhanced metabolism of these monosaccharides is necessary to support the accumulation of organic acids within the TCA cycle (succinic and malic acid). The treatment significantly influenced this pathway and could subsequently provide the plant with the means to produce energy and carbon skeletons to synthesise various other metabolites (Chia et al., 2000). Specifically, during mild drought

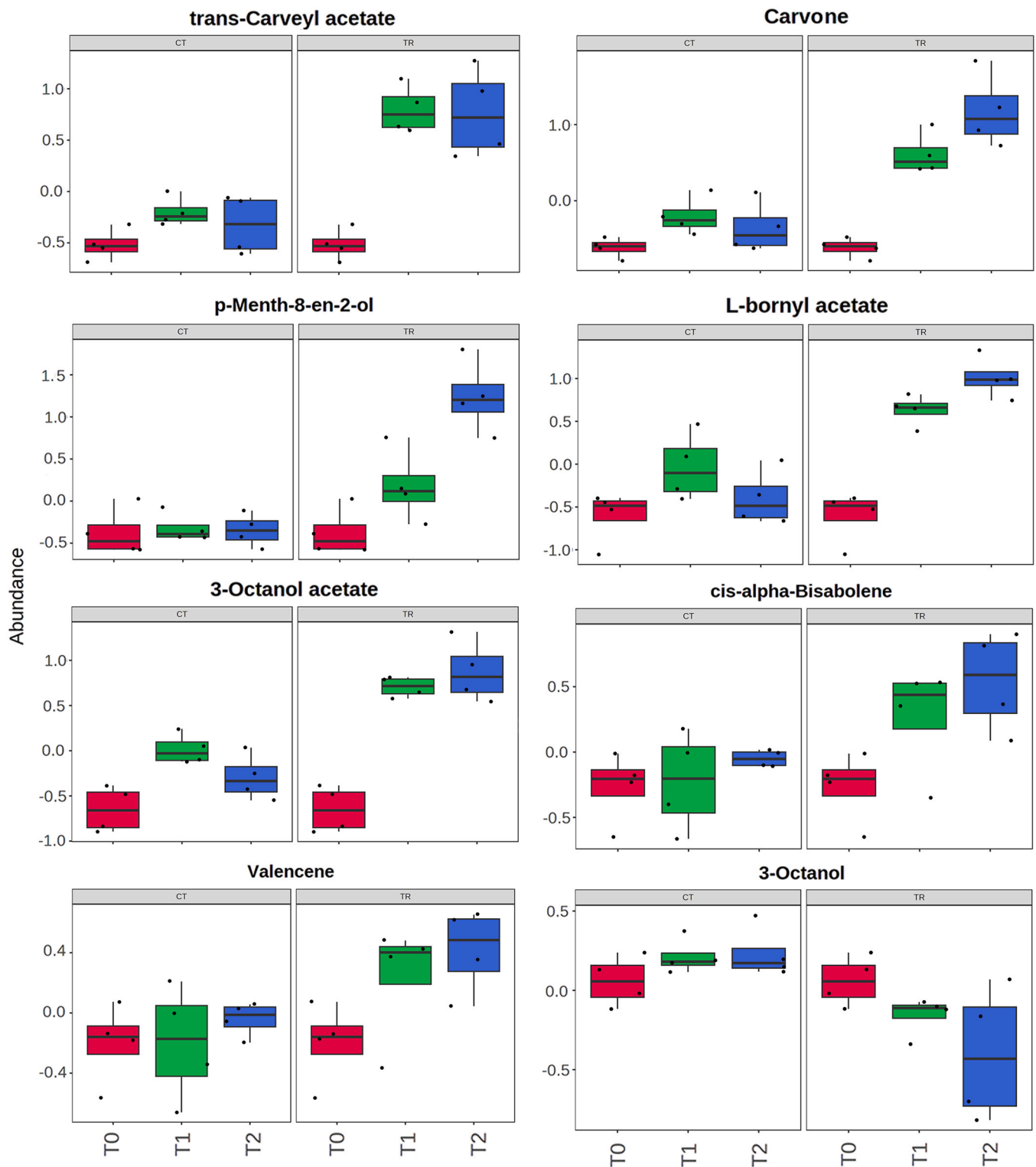


Fig. 10. : Box plots reporting the effects of cyclic mild-drought stress on the relative abundance of the main VOCs identified in *Mentha spicata* plants. The identified metabolites were generated by linear models with covariate adjustments considering the treatment, reporting the trend of the metabolites along the time in control (CT) and mild-cyclically-drought-stressed samples (TR). T0 – beginning of the experiment; T1 – end of the first cycle of the experiment; T2 – end of the second cycle of the experiment. $n = 4$.

conditions, there is a notable increase in malic acid accumulation within plants. Additionally, the enzymes associated with malic acid play a vital role in helping plants adapt to stressful situations by enhancing water use efficiency, optimizing photosynthesis, and supplying the necessary reducing power (Sun et al., 2019a; b; Guo et al., 2017). Evidences

concerning malic acid accumulation in aromatic species during mild-drought stress were also reported by Ashrafi et al. (2018) in drought tolerant thyme species. Also, ascorbic acid, its precursor gulonic acid, and vitamin B5 (pantothenic acid) were accumulated in plants exposed to mild-cyclic drought stress. All those molecules are widely

known to play a pivotal role as antioxidants protecting plants from oxidative damage (Asensi-Fabado and Munné-Bosch, 2010; Valpuesta and Botella, 2004).

Finally, as reported by the pathway analysis, the phenylalanine metabolism involved in phenylpropanoid biosynthesis was significantly impacted by the treatments at both T1 and T2. Concerning plant stress tolerance, phenylpropanoids play a pivotal role in preserving the plant's metabolic machinery and ultrastructure from ROS-mediated oxidative alterations (Dixon and Paiva, 1995; Mayer et al., 2001). Among the molecules significantly accumulated during the experiment, it should be mentioned caffeic acid, a cinnamic acid derivative considered one of the most important phenylpropanoids found in plants, which is involved in plant stress resistance through the induction of lignin production and also involved in the regulation of water flux, turgor pressure, cell expansion, phototropism, and growth (Lattanzio et al., 2009; Riaz et al., 2018).

All these data suggest that spearmint could cope with drought stress by modulating the primary carbon metabolism and the phenylpropanoid pathway to sustain cellular osmoregulation, osmoprotection and antioxidant response.

4.2. Mild-cyclic-drought stress results in *M. spicata* plants with higher functional and nutritional quality

The use of mild drought stress as an abiotic elicitor was adopted in several studies aimed at increasing the nutraceuticals quality of crops (Paim et al., 2020; Thakur et al., 2019). The mild-cyclic-drought stress applied to spearmint positively affected the terpenoid-base aromatic profile of this species and the content of some simple phenolic compounds. Concerning the terpenic profile, it should be highlighted that the treatment significantly affected only 8 out of 55 compounds. All the terpenes significantly accumulated in response to the mild-cyclic-drought stress (*trans*-carveyl acetate, carvone, *p*-menth-8-*e*-*n*-2-*o*l, bornyl acetate, 3-octanol acetate, bisabolene, and valencene) were commonly found as main components in spearmint essential oils (Balla et al., 2017; Salim et al., 2015; Salim, 2016; Zhang et al., 2022). In particular, the monoterpene carvone, which significantly accumulates at both T1 and T2, is the most abundant compound of spearmint essential oils, representing almost 70% of the blend (Balla et al., 2017; Salim et al., 2015; Salim, 2016; Zhang et al., 2022).

This result has certain importance since carvone is an industrially important compound, relevant in the medical field and used as a fragrance and flavour, antimicrobial agent, a building block for the synthesis of other compounds and potato sprouting inhibitor (Bouyahya et al., 2021; Committee, 2014; de Carvalho and da Fonseca, 2006). Moreover, several studies have been focused on finding methods to increase its production in plants (Bulchandani and Shekhawat, 2020; de Carvalho and da Fonseca, 2006; Toxopeus and Bouwmeester, 1992; Yoshida et al., 2021). In addition, the menthanes *p*-menth-8-*e*-*n*-2-*o*l and *trans*-carveyl acetate, significantly accumulated during treatments, are considered industrially important compounds in fine fragrances, decorative cosmetics, several toiletries and in non-cosmetic products such as detergents and household cleaners (Api et al., 2022; Bhatia et al., 2008). Although several manuscripts reported the influence of drought stress on terpene composition, only a few explored the molecular changes that drove their accumulation. For (during severe drought stress, the gene expression levels of *trans*-isopiperitenol dehydrogenase, isopiperitenone reductase, and menthofuran synthase increased, while the expression levels of pulegone reductase and menthol dehydrogenase decreased. Consequently, terpenoid production was positively correlated with gene expression in stressed plants since an increase in pulegone and menthofuran and a reduction in menthol content were observed. We suppose that also carvone accumulation could be driven by an alteration of the expression of genes involved in its biosynthesis, but it is mere speculation since no information is specifically reported in the literature, and we didn't assay the effects of the treatment on gene expression. Phenolic

and other organic compounds, characterised by industrial importance, significantly accumulated in response to the treatments. In particular, nicotinic acid, followed by 2-coumaric acid and caffeic acid, were the most accumulating compounds. Among them, caffeic and nicotinic acid has been proven to be potentially employable in several industrial sectors, being characterised by antioxidant, antimicrobial, cytotoxic, phytotoxic and insecticidal activity, among others (Carlson, 2005; Deshmukh et al., 2012; Elmetwally et al., 2022; Joshi et al., 2014; Magnani et al., 2014; Yu et al., 2021).

5. Conclusions

The results suggest that cyclic-mild-drought stress is a promising approach for promoting a more efficient use of the water required for *Mentha spicata* cultivation, improving its quality traits. Applying cyclic-mild-drought stress has effectively reduced the water required for *M. spicata* cultivation by inducing mechanisms that help plants in preserving water and cope with stress. In particular, plants counteracted the stress, activating several pathways involved in producing primary metabolites with osmoprotectant activity. No significant evidence of osmotic adjustments through ion absorption was observed. Finally, plants treated with the cyclic-mild-drought stress improved their volatile profile, significantly increasing the concentration of carvone, the most representative and industrially important molecule produced by this species.

In summary, the study contributes valuable insights into the physiological, metabolic, and quality-related responses of *M. spicata* to mild cyclic drought stress, offering potential solutions for sustainable and water-efficient cultivation practices. Further research is needed to optimise the application of this approach, also considering other crops, considering that the optimal drought stress treatment will vary depending on factors such as the local climate, pedological conditions, and the plant species. Moreover, the molecular process involved in terpenes accumulation and plant response to mild stress application should be deeply studied to increase our knowledge of plant plasticity.

Supplementary data

The supplementary material, containing all the metabolomic and statistical data, related to this article can be found, in the online Mendeley's repository at the following link: <https://data.mendeley.com/datasets/rsy66stvj6/1> - DOI: 10.17632/rsy66stvj6.2 - The data are under an embargo of six months.

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CRediT authorship contribution statement

Nocito Fabio Francesco: Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Espen Luca:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cocetta Giacomo:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Negrini Noemi:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Prinsi Bhakti:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Araniti Fabrizio:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Supplementary files: Impact of cyclic-mild-drought stress on the metabolism of *Mentha spicata* L.: a strategy to improve quality traits (Original data) (Mendeley Data)

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2024.118129.

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