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Agronomic traits, secondary metabolites and element concentrations of *Lavandula angustifolia* leaves as a response to single or reiterated drought stress: How effective is the previously experienced stress?

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ABSTRACT

In nature, plants are constantly challenged by an array of drought episodes, which critically affect the distribution of the plants. The drought episodes might occur recurrently, so the plants endure drought by adjusting and shifting their metabolisms. The impacts of subjecting plants to drought stress have been widely investigated, but reports on how reiterated drought stress affects the plants are limited. The present study was designed to investigate the response of lavender, a reputed medicinal and aromatic plant, against single drought, recovery and reiterated drought stress at greenhouse conditions. In this regard, the experimental design was based on three cycles of 11 days of drought by withholding water, followed by subsequent periods of 6 days of recovery, and then double-stressed and single-stressed periods. As expected, the present findings revealed that single stress decreased the fresh and dry weights of the leaf, stem and root. Reiterated drought stress caused critical reductions in the fresh weight of the leaf, stem and root, while the dry weight of stem and root were not significantly affected. Of the estimated traits, only the dry weight of leaf increased with reiterated drought stress. The mineral status of the leaves was adversely affected with single stress, but the effects of recovery and reiterated stress were not in accordance with the improvement in water contents of the leaf and soil. Regarding essential oil compounds, eucalyptol, camphor and endo-borneol were predominant. Single and reiterated drought stress increased camphor percentage, while recovery and full irrigation decreased the percentage. Endo-borneol was decreased under single stress, but reiterated stress increased the percentage of the compound. Considering the phenolic acids, stressed and non-stressed groups were well discriminated and hence, phenolic acids might be useful as good indicators of the stress response in lavender.

Keywords: abiotic stress, lavender, plant stress memory, secondary metabolites, water stress

Abbreviations: DW, dry weight; FW, fresh weight; SWC, soil water content.

INTRODUCTION

Plants, due to their sessile nature, cannot escape from biotic and abiotic stress factors (Atkinson and Urwin, 2012; Gull et al., 2019), but they have tackled the problem through an elaborate system that includes

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signal transductions, regulation of gene expression levels and adjustments in stress-tolerating or -avoiding factors (Chinnusamy et al., 2004). Along with appropriate adjustments as defence strategies, plants are well equipped in order to cope with the harmful impacts of stress (Pastor et al., 2013). In this regard, plasticity in morphological, physiological and chemical composition is an evolved defence mechanism against stressful conditions (Negin and Moshelion, 2020). As in the case of living organisms, stress conditions are also dynamic, and therefore, the relevant stressors might emerge or repeat episodically over periods, such as days, seasons or years (de Freitas Guedes et al., 2019). This situation makes the plants likely to face the same or other stress factors recurrently. However, relative to common stress reports (single-stress-subjected cases), studies on recurrent stress (double stress, reiterated stress) are limited and more specific. In this regard, as highlighted by Fleta-Soriano and Munné-Bosch (2016), recent reports regarding stress memory studies have been oriented on the discovery of epigenomic changes associated with memory in plants. In this context, reports linked to stress memory in plants have been visualised and categorised into clusters to reduce the dimensions of the topics and reveal the hot topics in related studies (Figure 1). Figure 1 was constructed with VOSviewer using 494 documents with criteria of "TITLE-ABS-KEY" (plant AND stress AND memory) AND (LIMIT-TO (SUBJAREA, "AGRI")) on Scopus database (September 4, 2021). As can be clearly deduced from Figure 1, supporting the comments of Fleta-Soriano and Munné-Bosch (2016), the reports are based on molecular approaches. It is worthy to note that physiological and biochemical responses are at their infancy period in plants exposed to double stress, in comparison to the high number of molecular responses. Again, up to our best knowledge, most of the studies have been based on the responses of the plants during various stages in their life cycle, not in trans-generational plants. Some reports even suggest that previous experiences of the stress might prepare the plants or enhance the capacity of plants to retain a memory, which might allow plants to exhibit fast or efficient responses to subsequent stresses (Asensi-Fabado et al., 2013; Nosalewicz et al., 2016); however, the exact impacts of repeated abiotic stresses are still not well and fully understood (Walter et al., 2011).

As reported for quite a number of crop and non-crop plant species ((Farahani et al., 2009; Sharafzadeh and Zare, 2011; Al-Gabbiesh et al., 2015; Wu et al., 2016; Mahajan et al., 2020; Zhang et al., 2022), drought stress limits the uses of arable lands and, consequently, alters the physiological and biochemical attributes of the plants, which are then translated and manifested as reduced crop productivity. In addition, secondary metabolites, especially phenolics and essential oil compounds, have also been monitored in drought-subjected plants due to the postulated roles of secondary metabolites in combating stress (Gao et al., 2020; Jogawat et al., 2021). As clearly reported, secondary

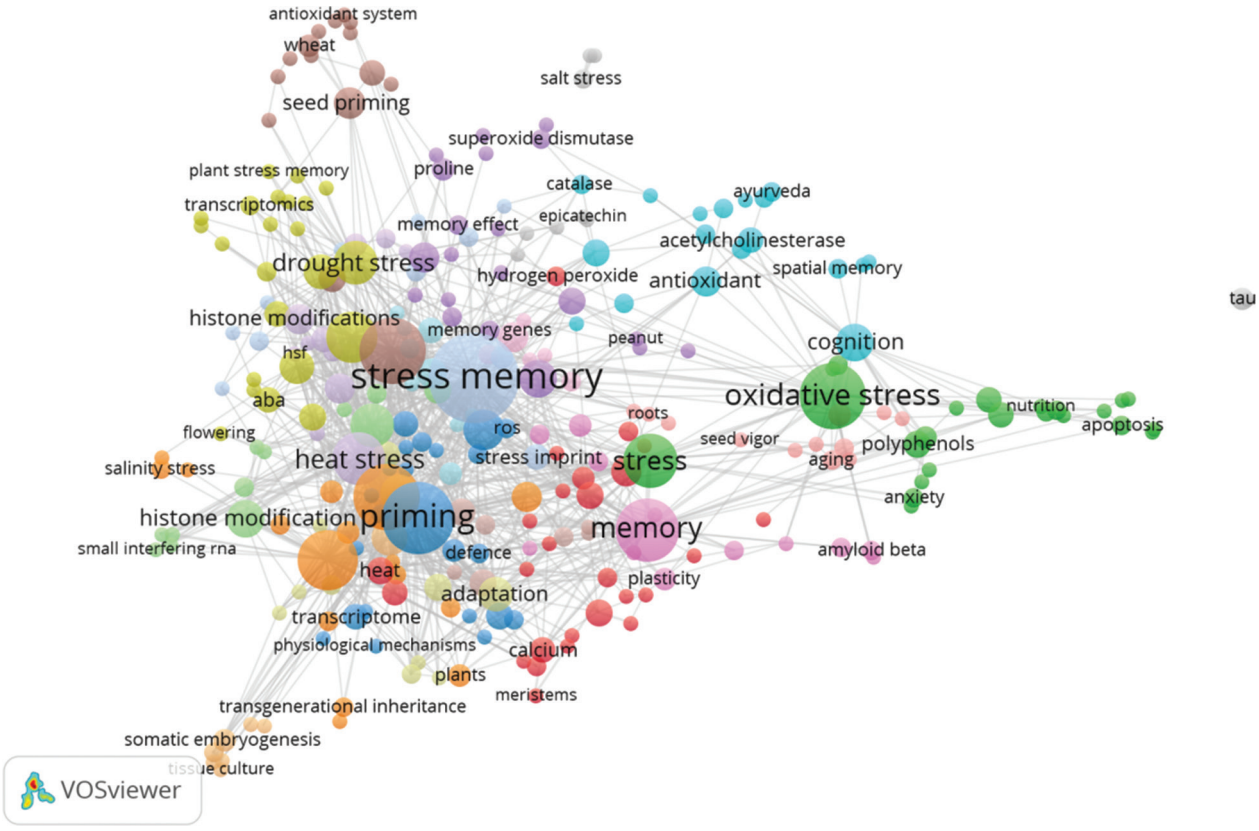

Figure 1. Keywords related to stress memory in the agriculture field.

metabolites, as a part of the non-enzymatic antioxidant defence system, orchestrate the regulatory response of plants with enzymatic antioxidant defence (Khare et al., 2020; Mahajan et al., 2020). In particular, the responses of secondary metabolites have been mostly examined in plants subjected to single stress. However, the composition of essential oils, as a secondary metabolite group, has been, for the first time, monitored in some Lamiaceae plants as a response to single-drought stress, recovery and double-drought stress (Kulak, 2020). Again, in this regard, up to our best knowledge and survey, phenolic compounds have not been hitherto investigated.

Lavender (*Lavandula angustifolia*), belonging to the family Lamiaceae, is a valuable medicinal and aromatic plant native to the Mediterranean region (Détár et al., 2020). It is grown mainly for its essential oil, which is of great interest economic value in the fragrance, flavour, pharmaceutic, perfume and cosmetic industries (Zuzarte et al., 2010; Grant et al., 2011; Salehi et al., 2018; Détár et al., 2020). Regarding the essential oil content and composition, the flowers are considered as the most valuable part of the plant; however, the leaves of the plants are also rich in essential oil (Łyczko et al., 2019). Many biological activities, such as local anaesthetic (Ghelardini et al., 1999), antifungal (D'Auria et al., 2005), antimutagenic (Evandri et al., 2005), antimicrobial (Danh et al., 2013), antioxidant (Danh et al., 2013), antibacterial and anti-inflammatory (Giovannini et al., 2016) effects, have been attributed to lavender essential oil. However, the biosynthesis of the essential oil is not constant but dynamic in response to external stimuli. Although the content and compositional percentage of the essential oil is genetically controlled, environmental conditions and agricultural practices also strongly affect the relevant compounds (Jan et al., 2021).

Furthermore, being widely distributed in the Mediterranean region, it is likely that lavender might be exposed to intensive water deficiency and heat stress in summer. The anticipated potential decreases in natural rainfall in the region might cause severe damage to the plant (Ramos, 2001; Alpert et al., 2002). In this context, understanding the conditions that shift the quality and quantity of volatile and phenolic compounds in plants is essential for the presumed uses of aromatic plants (de Almeida et al., 2016), as in the case of lavender, which is a valued species in rural areas due to its economic importance. In addition, revealing the response of how lavender behaves under stress conditions might be fundamental for yielding the desired volatile and phenolic compounds. Due to the lack of knowledge in this regard, in the present study , for the first time, we investigate the agronomic attributes, phenolic compounds, essential oil compounds and mineral content in the leaves of lavender (*L. angustifolia*) in response to single and reiterated drought stress. We aimed to determine, using agronomic traits and some metabolites of the plants, whether single stress prepared the plant for reiterated stress. This new approach might widen our understanding regarding the irrigation strategies needed to be applied for secondary metabolite production.

MATERIALS AND METHODS

Plant materials and growth conditions

Sixty uniform transplants of *L. angustifolia* were kindly provided by the Agricultural Application and Research Centre, Igdir University, Turkey. The transplants were 1 year old, and the height and life size of the transplants were homogeneous. The selected transplants were grown in a 2-L pot with a mixture of soil/peat/perlite (2:1:1, by volume). Before starting the experiments, all pots were transferred to the greenhouse in order to acclimatise the plants for 15 days. The plants were grown under the following conditions: 14-h photoperiod; mean temperature: 26−30 °C in the day, 16−20 °C in the night; and relative humidity: 60%–70%. Following the acclimatisation period, the lavender transplants were subjected to drought and full irrigations (details are provided in the section on "Application of drought treatments"). The properties of the experimental soils were as follows: saturation: 64.9%; pH: 7.57%; salt: 0.16%; $CaCO_3$: 8.41%; organic matter: 4.29%; K: 670 mg · kg^{-1} and P: 19.87 mg · kg^{-1}.

Application of drought treatments

The experimental design regarding the drought application treatments (single or double stress) are presented in Table 1 and Figure 2. The experimental design of Kulak (2020) and Pintó-Marijuan et al. (2017) with some minor modifications was used for the current study. Changes in the basic growth parameters, mineral content and secondary metabolites of lavender were investigated as the main targets of the present work. In the study, the measurements and sample collections were done on Day 0, Day 11, Day 17 and Day 28. Day 0 was the first day of sampling and measurement of the agronomic attributes, secondary metabolites and mineral content of the leaves of lavender, just before the drought stress was applied. According to the soil water content (SWC), the lavender plants were exposed to three cycles of drought stress for 11 days by completely ceasing irrigation. The lavender plants reached the wilting point after an 11-day drought stress, followed by subsequent drought-stress recovery for 5–6 days. In the work of Kulak (2020), the same lavender species was reported to be tolerant to a 7-day stress period. Due to the differences in experimental soils or background experiences of the lavender, the period of the stress lasted for 11 days. During the period of stress treatment of lavenders, control plants were subjected to full irrigation for 11 days.

This stage of drought application as a *single stress* was termed as the "*stress stage*" (Cycle 1). The "recovery stage" was namely noted as "Cycle 2". After Cycle 2 (recovery stage), the drought-subjected and fully

Table 1. Experimental groups of the study, modified according to Kulak (2020) and Pintó-Marijuan et al. (2017).

Treatments	Experimental groups
Sampling at Day 0	Sampling before treatments at Day 0
Stress after 11 days	Sampling on Day 11 in stressed plant groups
Control after 11 days	Sampling on Day 11 in irrigated plant groups
Stress group: recovery	Samplings on Day 17 after the recovery stage of stressed plant groups
Control group: recovery	Samplings on Day 17 after the recovery stages of irrigated plant groups
Stress group: stress	Samplings on Day 28 in plants, which were subjected to a 11-day stress, a 6-day recovery and a 11-day stress
Stress group: control	Samplings on Day 28 in plants, which were subjected to a 6-day stress, a 11-day recovery and a 7-day irrigation
Control group: stress	Sampling on Day 28 in plants that were irrigated for 17 days and then stressed for 11 days
Control group: control	Sampling on Day 28 in plants that were irrigated for 28 days

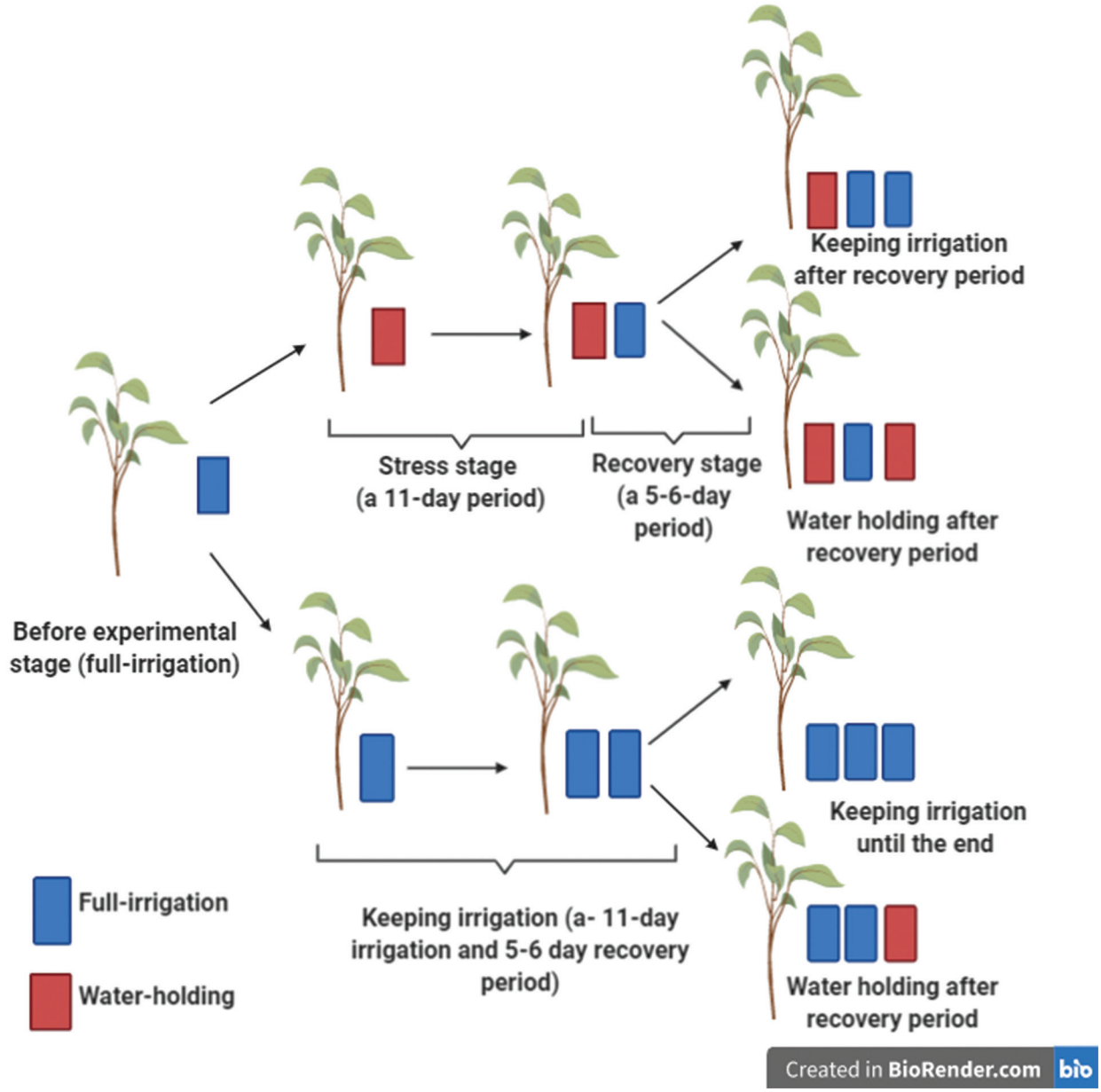

Figure 2. Experimental scheme of the study.

irrigated plants were divided into two more subgroups. In this context, four experimental groups were obtained. For each group, half of the plants were fully irrigated and the remaining half were subjected to drought for 11 days, denoted as Cycle 1 (Figure 2). At the end of each cycle, five plants from each group were randomly selected for the relevant analysis.

SWC and leaf hydration

The gravimetric SWC (SWC_{grav}) was estimated according to Du and Rennenberg (2018). In this regard, SWC_{grav} was calculated gravimetrically after each harvest, being expressed on a dry weight basis using the following formula:

$$SWC_{grav}\,(g\,H_2O \cdot g^{-1}\,DW) = (FW - DW) / DW$$

where FW denotes the fresh weight of the soil before drying, and DW denotes the dry weight of the soil after drying at 105 °C for 48 h.

Leaf hydration (g $H_2O \cdot g^{-1}$ DW) was calculated as (FW – DW)/DW, being expressed on a dry weight basis as in the case of SWC_{grav}, where FW is the fresh mass and DW is the dry mass after drying the samples in an oven at 60 °C for 72 h (Du and Rennenberg, 2018).

Agronomic traits

In order to reveal the changes in basic agronomic traits, we estimated the following in a total of 15 plants, corresponding to five plants for each replicate: leaf fresh weight, leaf dry weight, leaf rehydration, SWC, leaf length, leaf width, stem length, stem fresh weight, stem dry weight, root length, root fresh weight and root dry weight.

Plant sample and extraction

The extraction of leaf samples was carried out according to the modified method used in the study by Celikcan et al. (2021). All chemicals used in the study were obtained from Sigma-Aldrich, St. Louis, MO, USA. In this regard, a shaker-aided sequential extraction was performed at 120 rev · min^{-1} for 24 h at room temperature. Briefly, 3 g of finely dried leaf samples were extracted using 50 mL of methanol. The same extraction follow-up was repeated three times, and the extracts were filtered; the filtrates were collected and evaporated using a rotary evaporator (Heidolph, 94200, Bioblock Scientific, Schwabach, Germany). Until the HPLC analysis of phenolic compounds, the vacuo-dried samples were preserved at +4 °C; samples with 0.5 mg · L^{-1} concentration were prepared.

Quantification of phenolics using HPLC

The methanol extracts of the lavender leaves were filtered through a 0.45-µm disc prior to HPLC analysis. Of the phenolic compounds, ascorbic acid, gallic acid, catechin, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, *o*-coumaric acid, rosmarinic acid, salicylic acid, quercetin and kaempferol were monitored and quantified for each sample corresponding to the treatments. In this context, a (high-performance liquid chromatography (HPLC)) system (Agilent 1260; Agilent, Santa Clara, CA, USA) equipped with a diode array detector was used. The separation of the compounds was done with 10 µL of extract on a column (4.6 × 250 mm, 5 µm; ACE Generix 5C18 (GEN-7444), Scotland) thermostatted at 30 °C. The mobile phases were as follows: (A) 0.1% phosphoric acid in water and (B) HPLC-grade 100% acetonitrile. The phenolics were quantified by comparing the peaks recorded at 300 nm with the standard curves of each acid. The results were expressed as nanograms per microliter (ng · μL^{-1}).

Essential oil extraction and chromatographic analysis of the compounds

For the essential oil extraction, approximately 0.5 g of dried leaf samples was used. Gas chromatography (GC) headspace conditions were as follows: GC cycle time: 50 min; sample volume: 3.0 mL; incubation time: 25 min; incubation temperature: 70 °C; syringe temperature: 70 °C. After optimising the running conditions, the GC apparatus equipped with an HP-5 mass spectrometry (MS) capillary column (30 m × 0.25 µm × 250 µm) and 5977 (Agilent Technologies) with mass selective detector 7890B (Agilent Technologies, Santa Clara, United States) model GC–MS was used for determining the essential oil composition of the leaf samples. An electron ionisation system with ionisation energy of 70 eV was used, and the flow rate of the carrier gas (helium) was set to be 1.0 mL · min^{-1}. Injector and MS transfer line temperatures were set at 250 °C. Column temperature was initially kept at 50 °C for 2 min, then gradually increased to 200 °C at the rate of 5 °C · min^{-1} and ultimately increased to 250 °C at 10 °C · min^{-1}. Samples were injected automatically with split ratio 2:1. Analyses lasted for 35 min. The relevant compounds were identified with electronic libraries using reference compounds from the NIST08, Willey7n.1 and HPCH1607 libraries.

Mineral nutrient content

Leaf mineral content was estimated according to the method of Kaçar and Inal (2010). Briefly, fully developed leaves (from the second or third nodes) were first washed with double-distilled water and then were left for drying at 70 °C for 48 h. One gram of dried and finely powdered leaves was extracted using 3 mL 65% HNO_3 and 1 mL 30% HCl. The obtained solution was digested in a microwave, and the process was terminated by cooling for 45 min. Ultimately, the solutions were filtered, and the filtrates were made up to 50 mL with addition of double-distilled water. Until further analysis using inductively coupled plasma atomic emission spectroscopy (ICP-OES) (Optima 2100 DV; Perkin Elmer Inc., Waltham, MA, USA), the filtrates were preserved at 4 °C.

Experimental design and statistical analysis

For each treatment, three replicates corresponding to 15 plants were used. The experimental data were subjected to one-way variance analysis. The means were separated using Duncan's multiple range test at the 5% probability level ($p < 0.05$) (SPSS22). Due to the high number of dependent and independent variables, principal component analysis (PCA) and heat map clustering were carried out (XLSTAT software and ClustVis, respectively) to visualise, correlate and discriminate the variables.

RESULTS

SWC and leaf hydration

The values of SWC_{grav} significantly decreased from 0.39 g $H_2O \cdot g^{-1}$ DW to 0.11 g $H_2O \cdot g^{-1}$ DW and 0.39 g $H_2O \cdot g^{-1}$ DW to 0.20 g $H_2O \cdot g^{-1}$ DW in the stress and the control groups, respectively, after withholding water supply during the first cycle. In the second cycle, in the recovering stress groups, the SWC_{grav} increased from 0.11 g $H_2O \cdot g^{-1}$ DW to 0.40 g $H_2O \cdot g^{-1}$ DW. Reiterated drought stress sharply decreased the SWC_{grav} from 0.40 g $H_2O \cdot g^{-1}$ DW to 0.048 g $H_2O \cdot g^{-1}$ DW. Moreover, application of a single stress to the control group plants after the second cycle sharply decreased the values from 0.52 g $H_2O \cdot g^{-1}$ DW to 0.058 g $H_2O \cdot g^{-1}$ DW. Leaf hydration was positively correlated with SWC_{grav} ($r = 0.840$, $p < 0.01$) and decreased from 2.08 g $H_2O \cdot g^{-1}$ DW to 0.88 g $H_2O \cdot g^{-1}$ DW during stress (first cycle). During the second cycle, recovery significantly increased the leaf hydration status of the stress group plants. Repeated drought stress, again, caused sharp reductions. Similarly, the lowest values of leaf hydration were obtained with applying single stress to control groups, *i.e.* from 0. g $H_2O \cdot g^{-1}$ DW to 0.058 g $H_2O \cdot g^{-1}$ DW.

Plant growth and biomass production

By exposing lavender plants to reiterated drought under greenhouse conditions (including three cycles of 11 days of drought by withholding water, followed by subsequent periods of 6 days of recovery, and then double-stressed and single-stressed periods), it was observed that stem dry weight and root length did not significantly differ ($p = 0.259$ and 0.169, respectively) among the single-stressed, recovered and double-stressed plants. However, other parameters such as leaf fresh weight, leaf dry weight, leaf length, leaf width, stem length, stem fresh weight, roof fresh weight and root dry weight were significantly affected by the relevant stress treatments (Table 2). Specifically, in the first cycle (single-stressed versus fully irrigated conditions, Cycle 1), drought stress reduced the values of leaf fresh weight, leaf width, leaf length, leaf width, stem length, root fresh weight and root dry weight ($p < 0.05$). It is interesting to note that the responses of leaf dry weight (a decrease) and root fresh weight (an increase) were

Table 2. Plant growth and biomass production traits corresponding to the stressed and non-stressed groups.

Treatments	Leaf (FW; g)	Leaf (DW; g)	Leaf rehydration (g)	SWC (g)	Leaf length (%)	Leaf width (cm)
Sampling at Day 0	24.21 bcd	8.70 c	2.08 b	0.39 b	3.23 c	0.33 bc
Stress after 11 days	20.95 cd	13.88 abc	0.88 c	0.11 d	3.83 bc	0.23 de
Control after 11 days	35.25 b	14.06 abc	2.80 a	0.20 c	4.33 ab	0.33 bc
Stress group: recovery	20.17 cd	9.14 c	2.08 b	0.40 b	4.10 bc	0.27 cde
Control group: recovery	34.87 b	17.06 ab	2.13 b	0.52 a	5.13 a	0.40 b
Stress group: stress	13.79 d	12.07 bc	0.39 cd	0.048 e	3.80 bc	0.25 de
Stress group: control	27.05 bc	13.08 abc	2.85 a	0.37 b	3.77 bc	0.30 cd
Control group: stress	15.81 cd	12.03 bc	0.21 d	0.058 e	4.07 bc	0.20 e
Control group: control	50.36 a	18.42 a	2.38 ab	0.23 c	5.100 a	0.50 a
Significance	$p = 0.000$	$p = 0.026$	$p = 0.000$	$p = 0.000$	$p = 0.007$	$p = 0.000$
Treatments	Stem length (cm)	Stem (FW;g)	Stem (DW;g)	Root length (cm)	Root (FW; g)	Root (DW; g)
Sampling at Day 0	19.83 f	14.82 b	6.24 b	22.67 b	19.53 bc	6.78 b
Stress after 11 days	21.46 ef	12.30 b	7.93 ab	25.00 ab	13.16 cd	6.96 b
Control after 11 days	28.33 bcd	14.77 b	8.52 ab	24.50 b	18.75 bc	10.55 ab
Stress group: recovery	24.00 def	13.15 b	6.58 b	28.67 ab	24.41 ab	7.91 b
Control group: recovery	29.93 bcd	17.09 ab	6.98 b	30.17 ab	28.67 ab	10.41 ab
Stress group: stress	25.67 cde	9.38 b	7.04 b	37.50 a	9.83 d	7.49 b
Stress group: control	30.23 bc	14.40 b	7.14 b ab	31.83ab	28.87 ab	10.48 ab
Control group: stress	31.67 b	8.88 b	7.41 ab	32.00 ab	7.567 cd	6.83 b
Control group: control	43.27 a	23.17 a	11.46 a	38.67 a	31.25 a	13.39 a
Significance	$p = 0.000$	$p = 0.027$	$p = 0.259$	$p = 0.169$	$p = 0.000$	$p = 0.014$

The means in the same column followed by the same letters were not significantly different according to Duncan's test ($p < 0.05$).

DW, dry weight; FW, fresh weight; SWC, soil water content.

only significant after recovery, *i.e.* in the second cycle (Cycle 2), while the other parameters did not differ between single-stressed and recovered plants. In the third cycle, there were no significant differences in leaf fresh weight, leaf dry weight, leaf length, leaf width, stem length, stem fresh weight, root length and root dry weight due to the previously experienced stress periods. Only root fresh weight significantly decreased due to the previously experienced stress period. In the third cycle, it is also interesting to note that applying drought to the fully irrigated plants caused critical reductions in leaf fresh weight, leaf dry weight, leaf length, leaf width, stem length, stem fresh weight, root fresh weight and root dry weight. For these reasons, the differences between irrigated treatments were significant. These findings might suggest that stress-subjected lavender plants had prepared themselves for the reiterated stress (double stress) in the third cycle (Cycle 3).

Heat map clustering and PCA of plant growth and biomass production traits

As a powerful tool for the discrimination of relevant agronomic traits, heat map clustering was used for reducing the dimensions of the variables, visualising and correlating the findings. Accordingly, two major clusters were noted (Figure 3A). Considering the agronomic traits corresponding to the stressed and non-stressed groups, the first one was stem length, root length, leaf length, leaf dry weight and stem dry weight. On the other hand, leaf hydration, SWC, root fresh weight, root dry weight, leaf fresh weight, leaf width and stem fresh weight were classified into the second major cluster. Regarding the clustering of the stressed and non-stressed experimental groups, here also, two major clusters were obtained. In the first major cluster, stress recovery, sampling at Day 0, stress control and control recovery groups were observed, while the other groups were classified under the second major group. However, in a sub-cluster of the second major cluster, control-stress and stress-stress were observed in the same groups. These findings might suggest that post-drought stress in the control (stress to the full irrigated plants in the third cycle) caused damage or exhibited the same effects as in the double-stressed plants, or we might note that stress priming might prepare the plants for the possible emerging stresses. Furthermore,

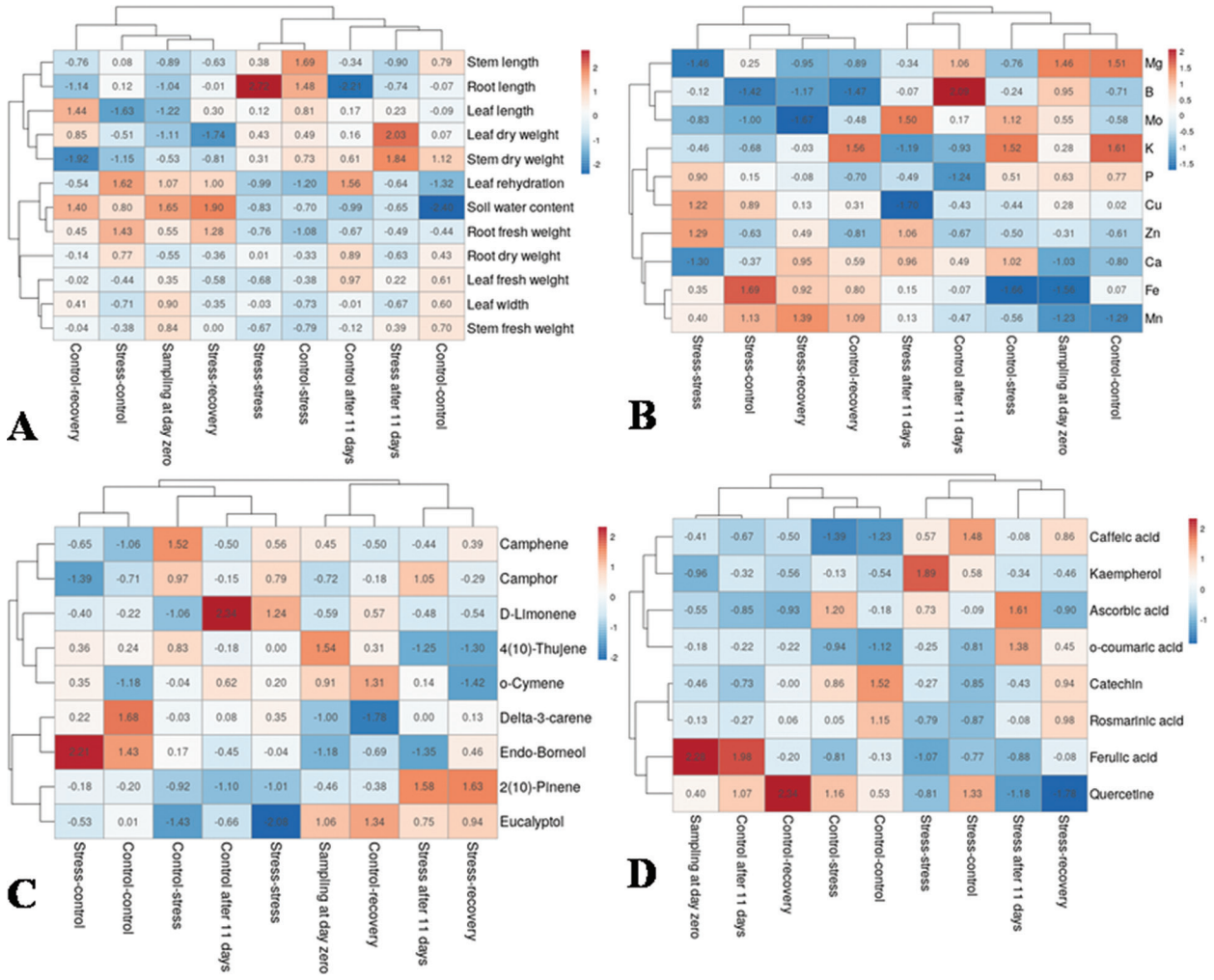

Figure 3. Heat map clustering of (A) plant growth and biomass production traits, (B) mineral contents in leaf, (C) essential oil compounds in leaf and (D) phenolic acids in leaf corresponding to the stressed and non-stressed groups.

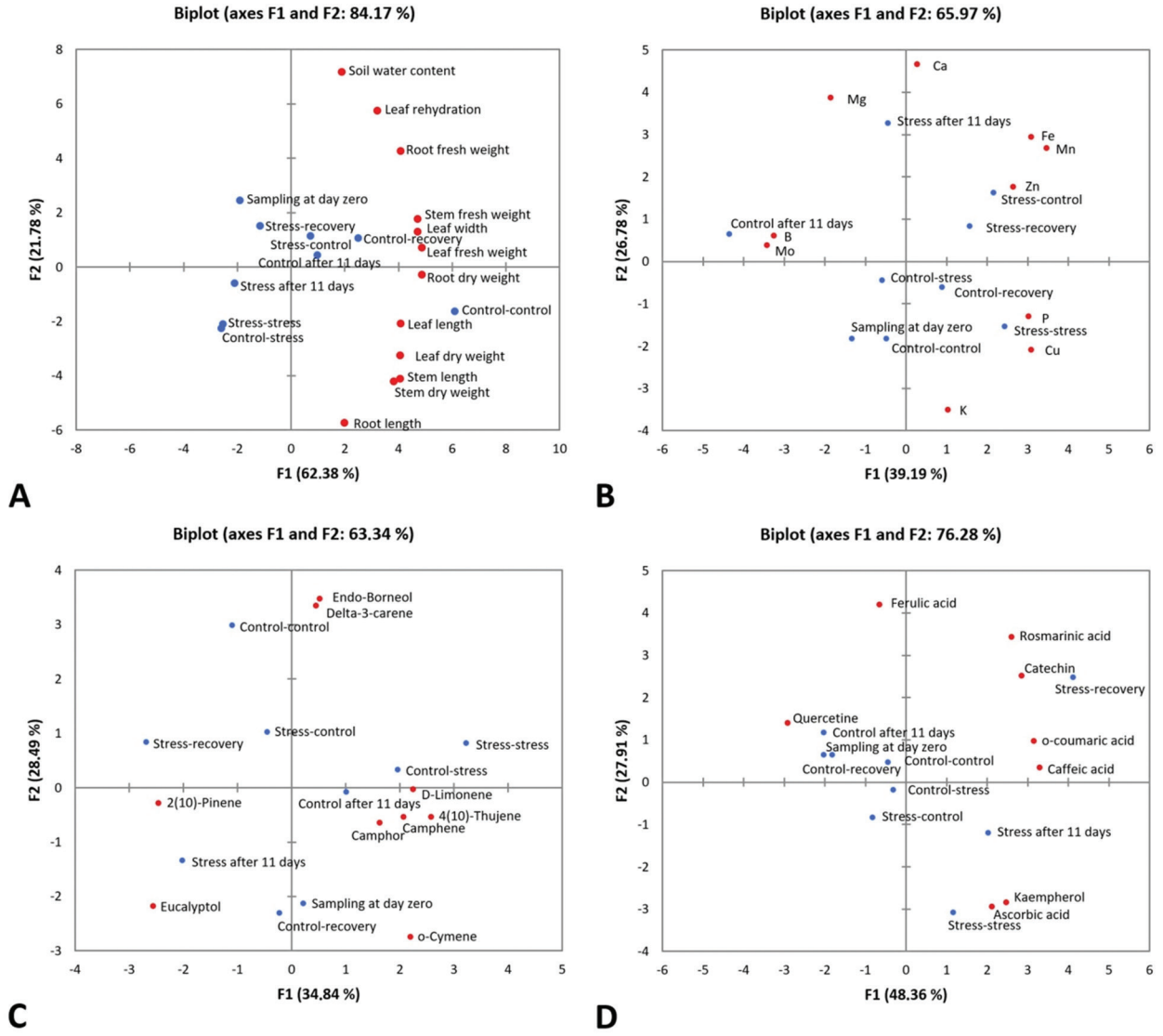

Figure 4. PCA of (A) plant growth and biomass production traits, (B) mineral contents in leaf, (C) essential oil compounds in leaf and (D) phenolic acids in leaf corresponding to the stressed and non-stressed groups. PCA, principal component analysis.

along with PCA, a better and clear discrimination in the growth and biomass production parameters was revealed on the 2D visualisation of the plotted scores, where the two principal components accounted for 84.17% of the total variance (Figure 4A). The first and second axes explained 62.38% and 21.78% of the total variance, respectively. It is worthy to note that retaining irrigation for stressed plants after the recovery stage moved the stressed groups into similar groups with the control plants. Interestingly, withholding water supply to the control plants after the recovery stage moved the control group into a similar group with the stressed plants, as in the case of the sub-cluster of the second major cluster.

Changes in elemental contents

Corresponding to the relevant treatments, it was noted that the responses of the major (Ca, K, Mg and P) and trace elements (B, Mo, Fe, Zn and Mn) in the leaf tissues of lavender were statistically significant (p = 0.000), except Cu (p = 0.121). Especially, drought caused substantial increases in the concentrations of Ca, Mo, Fe, Zn and Mn, while significant reductions in the concentrations of K, P, Mg and B were noted in stress-subjected lavenders at the end of Cycle 1. In Cycle 2, recovery in the stress-subjected plants decreased the concentrations of Ca, B, Mo and Zn, while it increased the concentrations of K, P, Fe and Mn. However, the concentrations of Mg and Cu did not differ between single-stressed and recovered plants. In the third cycle, due to previously experienced stress periods, reiterated stress decreased the concentrations of Ca, Mg, B and Mo, while it increased the concentrations of K, P and Cu. However, among the trace elements, the concentrations of Fe, Zn and Mn did not differ between single-drought stress and reiterated drought

Table 3. Mineral content changes corresponding to the stressed and non-stressed groups (mg · kg^{-1}).

Treatments	Ca	K	P	Mg	B	Mo	Fe	Cu	Zn	Mn
Sampling at Day 0	253.53 e	144.78 c	196.26 ab	542.33 a	21.83 b	3.59 c	307.17 g	11.44 abc	7.90 e	61.34 f
Stress after 11 days	327.05 a	116.15 f	166.33 d	464.33 d	19.43 c	4.92 a	603.00 c	8.21 c	13.30 a	78.26 d
Control after 11 days	282.83 d	103.03 g	117.33 f	498.67 b	23.69 a	2.24 e	423.87 e	8.94 bc	3.39 g	60.21 fg
Stress group: recovery	312.06 b	142.65 cd	182.80 c	465.00 d	18.49 e	2.17 e	669.39 b	11.38 abc	10.56 b	85.96 b
Control group: recovery	290.60 cd	153.83 b	153.50 e	430.33 e	15.72 h	2.01 f	596.30 c	10.70 abc	5.42 f	80.54 c
Stress group: stress	229.85 f	124.78 e	200.97 a	393.00 f	18.53 e	1.50 g	594.60 c	12.86 a	13.54 a	78.62 d
Stress group: control	295.47 c	138.42 d	193.67 b	504.33 b	18.03 f	2.60 d	833.13 a	12.69 ab	8.99 c	90.38 a
Control group: stress	321.58 a	164.02 a	194.00 b	461.67 d	19.49 d	4.12 b	401.57 f	10.79 abc	8.44 d	73.38 e
Control group: control	249.99 e	142.30 cd	170.67 d	481.67 c	16.74 g	1.70 g	454.60 d	9.87 abc	5.30 f	59.45 g
Significance	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.121$	$p = 0.000$	$p = 0.000$

The means in the same column followed by the same letters were not significantly different according to Duncan's test ($p < 0.05$).

stress. Corresponding to the application of stress to the fully irrigated plants after the recovery stage (Cycle 2), drought stress caused substantial increases in the concentrations of Ca, K, P, B, Mo, Zn and Mn, while it decreased the concentrations of Mg and Fe. As in the case of stress-subjected plants from the first cycle, the concentration of Cu did not differ between the full-irrigated and stress-subjected control plants. It is interesting to note that, among the elements quantified, the responses of only K and P were the same against the stress conditions corresponding to the control and stress-experienced plants exposed to the same stress after the recovery stage. The remaining elements behaved relatively differently in stress-experienced and non-stress-experienced plants (Table 3).

Heat map clustering and PCA of element content corresponding to the stress and non-stressed groups

As in the case of agronomic traits, a heat map was constructed for clustering and correlating the mineral content and experimental groups. Regarding the elemental contents corresponding to the experimental groups, two major clusters were observed (Figure 3B). The first one was composed of Mg, Mo and B, while the later included P, K, Ca, Cu, Zn, Fe and Mn. Considering the experimental groups, two major clusters were, again, revealed. The first cluster, except the control-recovery group, was mainly composed of stress-related groups, while the second cluster, except the group subjected to stress after 11 days, was based on the control groups. In relation to mineral content discrimination, a partial and better discrimination was observed for the stress and non-stressed groups. Moreover, the relevant values were subjected to PCA, and according to the PCA, two principal components accounting for 65.97% of the total variance (F_1: 39.19%, F_2: 26.78%) were noted (Figure 4B).

Essential oil composition

The essential oil compounds identified in lavender leaves are listed in Table 4, following their elution order on the HP-5 column. The compounds delta-3-carene, camphene, 2(10)-pinene, 4(10)-thujene, *o*-cymene, D-limonene, eucalyptol, camphor and endo-borneol were screened in the leaves of the stressed and non-stressed groups. In the stress-subjected and fully irrigated treatments, all the identified compounds, except eucalyptol ($p = 0.201$), were substantially affected ($p = 0.01$). Of the identified compounds, eucalyptol, camphene, camphor and endo-borneol were found to be the major constituents based on their percentage. With respect to the treatments, drought caused significant reductions in the percentages of camphene and endo-borneol, while the camphor percentage increased significantly in stress-subjected lavenders at the end of Cycle 1. In Cycle 2, recovery

Table 4. Essential oil compounds corresponding to the stressed and non-stressed groups (%).

Treatments	Delta-3-carene	Camphene	2(10)-pinene	4(10)-thujene	*o*-cymene	D-limonene	Eucalyptol	Camphor	Endo-borneol
Sampling at Day 0	0.690 g	5.300 c	0.000 b	1.790 a	2.570 b	0.000 e	68.970 a	13.280 bc	6.580 ef
Stress after 11 days	1.490 e	4.200 e	0.690 a	0.000 f	1.780 e	0.000 e	68.473 a	17.320 a	6.020 f
Control after 11 days	1.690 d	4.670 d	0.000 b	0.920 d	2.190 c	3.020 b	66.010 a	14.990 b	8.020 cd
Stress group: recovery	1.670 d	5.360 c	0.730 a	0.000 f	0.000 g	0.000 e	69.170 a	14.180 bc	8.900 bc
Control group: recovery	0.050 h	4.160 e	0.000 b	0.990 d	2.783 a	1.450 c	69.200 a	14.220 bc	7.140 de
Stress group: stress	2.360 b	6.210 b	0.000 b	1.270 c	2.530 b	3.720 a	62.670 a	18.030 a	8.940 bc
Stress group: control	1.330 f	3.980 f	0.000 b	0.800 e	1.500 f	0.000 e	64.940 a	12.300 c	9.420 ab
Control group: stress	1.800 c	6.770 a	0.000 b	1.550 b	1.990 d	0.000 e	64.317 a	17.480 a	8.840 bc
Control group: control	2.950 a	3.160 g	0.000 b	0.910 d	0.000 g	0.150 d	65.983 a	12.540 bc	10.060 a
Significance	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.201$	$p = 0.000$	$p = 0.000$

The means in the same column followed by the same letters were not significantly different according to Duncan's test ($p < 0.05$).

in the stress-subjected plants increased the percentage of camphene and endo-borneol and decreased the percentage of camphor. In Cycle 3, being previously stress experienced did not substantially affect the percentage of camphor between single-stress-subjected and reiterated-drought-stress-subjected plants, but prior stress experience increased the percentage of camphene and endo-borneol. With respect to the application of stress in full-irrigated lavenders after the recovery stage, drought stress reduced the percentage of camphene and camphor, in relation to the full-irrigated lavenders, while increasing the percentage of endo-borneol. Similar to the behaviour of elements, relatively, some differences were noted between the plants due to the previously imprinted stress. As reported earlier, it is interesting to note that the predominant compound eucalyptol, with an approximately estimated percentage ranging between 62.67% and 69.20%, was not substantially affected by the treatments.

Heat map clustering and PCA of essential oil compounds corresponding to the stress and non-stressed groups

Heat map clustering analysis separated the essential oil compounds into two major clusters. The first cluster was composed of camphene, camphor, D-limonene, 4(10)-thujene and *o*-cymene, while delta-3-carene, endo-borneol, 2(10)-pinene and eucalyptol were classified in the second cluster (Figure 3C). Regarding the stressed and non-stressed groups, here also, two major clusters were constructed. The first cluster included the following groups: stress-recovery, stress after 11 days, control-recovery and sampling at Day 0. The second cluster was composed of stress-stress, control after 11 days, control-stress, control-control and stress-control. Coupled with heat map clustering, PCA was used to discriminate the essential oil compounds corresponding to the stressed and non-stressed groups. Accordingly, two principal components accounting for 63.34% of the total variance (F_1: 34.84%, F_2: 28.49%) were obtained (Figure 4C).

Individual phenolic acids

Of the large and diverse number of phenolic acids, the contents of ascorbic acid, catechin, ferulic acid, *o*-coumaric acid, rosmarinic acid, quercetin and kaempferol were quantified using HPLC (Table 5). Except kaempferol ($p = 0.201$), the other quantified compounds were significantly affected by the relevant treatments ($p = 0.00$). It is worthy to note that the responses of the compounds against stress conditions were relatively different. Especially, in Cycle 1, drought caused significant increases in the content of ascorbic acid, catechin, caffeic acid, *o*-coumaric acid and rosmarinic acid, in relation to the full-irrigated plants, while the contents of ferulic acid and quercetin decreased. In Cycle 2, recovery in the stress-subjected plants increased catechin, caffeic acid, ferulic acid, *o*-coumaric acid and rosmarinic acid, while decreasing the content of ascorbic acid. Only quercetin content was not affected by the recovery. In Cycle 3, being previously experienced in stress decreased the content of ascorbic acid, catechin, ferulic acid and rosmarinic acid, while increasing the content of caffeic acid and *o*-coumaric acid. However, quercetin content was not affected substantially. Furthermore, subjecting the full-irrigated plants to stress after the recovery stage increased the content of ascorbic acid and *o*-coumaric acid, relative

Table 5. Individual phenolic acids corresponding to the stressed and non-stressed groups (ng · μL^{-1}).

Treatments	Ascorbic acid	Catechin	Caffeic acid	Ferulic acid	*o*-Coumaric acid	Rosmarinic acid	Quercetin	Kaempferol
Sampling at Day 0	0.000 f	2.150 g	1.567 g	3.805 b	1.293 c	6.460 f	0.650 d	1.099 g
Stress after 11 days	12.753 a	10.511 d	5.797 d	1.072 d	5.970 a	11.619 c	0.000 e	1.607 cde
Control after 11 days	0.000 f	2.864 g	1.884 fg	4.970 a	1.933 b	8.121 e	1.599 b	1.462 ef
Stress group: recovery	4.066 d	43.124 a	14.511 a	3.934 b	5.794 a	25.434 a	0.000 e	1.938 b
Control group: recovery	0.000 f	11.412 d	2.703 ef	1.741 c	1.935b	9.941 d	2.399 a	1.374 f
Stress group: stress	7.654 b	8.362 e	7.961 b	0.000 e	1.896 b	3.997 g	0.000 e	2.773 a
Stress group: control	2.737 e	4.849 f	7.172 c	1.253 d	1.367 c	6.330 f	1.236 bc	1.689 c
Control group: stress	5.982 c	17.827 c	2.954 e	1.789 c	1.941 b	10.796 cd	1.278 bc	1.654 cd
Control group: control	3.179 e	21.540 b	2.199 efg	2.001 c	1.379 c	13.427 b	1.025 c	1.500 def
Significance	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.000$	$p = 0.201$

The means in the same column followed by the same letters were not significantly different according to Duncan's test ($p < 0.05$).

to the full-irrigated plants, but it decreased the content of catechin and rosmarinic acid. The other compounds, such as caffeic acid, ferulic acid and quercetin, were not substantially responsive to stress application.

Heat map clustering and PCA of individual phenolic acids corresponding to the stress and non-stressed groups

As in the case of other attributes estimated in the study, the same scattering, clustering and discrimination tools were used for individual phenolic acids. Of these tools, the heat map clustered the relevant acids into two major groups (Figure 3D). Phenolic acids, *viz*. caffeic acid, kaempferol, ascorbic acid and o-coumaric acid, were included in the first major cluster, while the second major cluster included catechin, rosmarinic acid, ferulic acid and quercetin. Considering the experimental groups, the stressed and non-stressed groups were clearly discriminated from each other. In addition, PCA, in addition to the heat map clustering, also explained a high ratio of the total variation (F_1: 48.36% and F_2: 27.91%, with a total variance: 76.28%) (Figure 4D).

DISCUSSION

Drought stress is one of the most devastating abiotic stress factors affecting crop productivity, and its effects have been clearly reported for quite a number of crop plants in general (Sepahvand et al., 2021; Weisany et al., 2021) and, in particular, in lavender (*L. angustifolia*) (García-Caparrós et al., 2019; Szekely-Varga et al., 2020a; 2020b; Mohammadi et al., 2021). The impacts of drought are translated, in general, into retarded growth and reduced crop productivity of most crop plants (reviewed well by Farooq et al., 2012; Anjum et al., 2011). In this context, the complex responses of crop and non-crop plants at the physiological, biochemical and molecular levels have been widely investigated in response to drought stress. To our best knowledge and survey, most of the studies have addressed the topics of single stress and recovery from the single stress, but the impact of reiterated drought stress (with different terms, *viz*. double stress or reiterated drought stress) has not been widely examined or reported, in comparison to the effects of single stress. In recent years, plant researchers have concentrated more on how single-stressed plants respond to reiterated stresses, aiming to reveal the similar or different reactions of plants against stress. Furthermore, plants might experience a different stress factor in the next period of their lives (cross tolerance) (Munné-Bosch and Alegre, 2013; Walter et al., 2013). In this study, we have focussed on the following questions: how does previously experienced drought stress affect the response of plants when subjected to reiterated drought stress? How do agronomic attributes, elements, as well as phenolic and volatile compounds respond to the reiterated drought stress? Is the behaviour of the investigated parameters compatible or parallel to each other in response to the reiterated drought stress? Can a stress-subjected species switch from stress to non-stress conditions or behave similar to control plants after the recovery stage in the context of their metabolites?

In order to test the given questions, an array of parameters – including agronomic traits, mineral contents, essential oils and phenolic acids – were examined in lavender plants. In the current study, drought stress was based on withholding water and lasted for 11 days until wilting point. In the study, successful drought stress was achieved by withholding irrigation as reflected by the largely reduced leaf hydration and SWC, which were subsequently manifested as decreased leaf fresh weight, stem fresh weight, root fresh weight, leaf dry weight, stem dry weight and root dry weight at the end of Cycle 1. As observed for quite a number of plants (Bhusal et al., 2019; Rodríguez-Gamir et al., 2019; Kulak et al., 2021), drought stress causes substantial reduction in the leaf's water content. Corresponding to the lowered water content, turgor status, stomatal adjustments and photosynthesis machinery of the plants are significantly affected. The hampered and retarded physiological and biochemical attributes of

the plants eventually reduce plant growth and biomass production (Amini et al., 2014; Larkunthod et al., 2018). Thus, the decreases estimated in the present study in terms of the agronomic parameters might be explained by the status of leaf hydration and the SWC.

However, the relevant growth and biomass production parameters had not, as expected, completely recovered after re-irrigation, in accordance with the improvement in leaf hydration and SWC at the end of Cycle 2. Of the estimated parameters, root fresh weight approximately doubled, while leaf dry weight decreased. These findings are not relevant from the agronomic and industrial use viewpoints since only the leaves are evaluated as the commercial crop.

Considering the recurring drought stress in Cycle 3, reiterated drought stress caused critical reductions in the fresh weight of the leaf, stem and root, while the dry weight of the stem and root were not significantly affected. Of the estimated traits, leaf dry weight increased as a response to reiterated drought stress. In accordance with the priming or osmo-regulation treatments, we had hypothesised that single stress would prepare and enhance the performance of lavender against possible reiterated drought stress, as in the case of seeds primed with dehydration cycles (Lima and Meiado, 2018): the seedlings of the dehydration-subjected seeds exhibited an enhanced performance, estimated as longer stem, larger stem diameter and higher dry weight values of leaf, stem and roots (Lima and Meiado, 2018). The enhanced performance against stress was attributed to seed hydration memory, which preserved the acquired traits from the previous imprints of the hydration episode (Dubrovsky, 1996; Chen and Arora, 2013; Tabassum et al., 2017). However, the hypothesised outcomes were not confirmed by the values of the agronomic attributes. In accordance with the present findings, it might be deduced that the approach of priming plants with drought stress at the seedling stage is not relevant from the agronomic view. Furthermore, in reputed plants native and common to the Mediterranean region (characterised by mild winter periods and warmer summer seasons), attempts to enhance the potency of lavender seedlings against possible recurring lethal water constraints are required due to reports on the frequency and severity of anticipated droughts (Sun et al., 2020). Similarly, in the present study, subjecting a single-drought stress after the second cycle of the study sharply decreased the fresh weight of leaf, stem and root, as well as the dry weight of the leaf and root. Taking into account these observations, even though promising findings were not noted for subjecting to or priming plants with drought stress, a single stress after the recovery period caused more adverse impacts, in comparison to double-stress. However, it is critical to note that we have observed that maintaining irrigation in stressed plants after the recovery stage moved the stressed groups into similar groups with the control plants. Interestingly, withholding the water supply of the control plants after the recovery stage moved the control group into a similar group with the stressed plants. These findings resemble the report by Cushman and Borland (2002), indicating that species switched their metabolism from Crassulacean acid metabolism (CAM) to C_3 during the recovery stage. As clearly revealed in a large number of reports, the responses of plants regarding their performance or productivity are multidimensional, being dependent on the timing, duration, frequency, severity of drought, as well as developmental stage, variety, or previous imprints (Pastor et al., 2013; Nosalewicz et al., 2016; Lukić et al., 2020). Thus, the present study might be assessed as a preliminary or the first study for medicinal and aromatic plants, up to our best knowledge. More in-depth research is required for the relevant plant groups characterised using their secondary metabolites.

Having great roles in plant metabolism, mineral uptake processes of the plants have been widely examined in plants subjected to water constraints (da Silva et al., 2011; Ahanger et al., 2016). As is clearly well known, nutrient uptake, transport and translocation are significantly restricted under drought as a consequence of the decline in the rate of transpiration (Rennenberg et al., 2006). However, as seen in the case of the above-mentioned attributes, the responses of the mineral content against single stress, recovery or double stress have not been fully known and reported. Herein, up to our best knowledge, we, for the first time, observed the changes in the levels of important minerals. Accordingly, the changes in the contents of minerals were significant corresponding to the drought stress, as expected. Except Ca, the major elements such as K, P and Mg were reduced by drought (Cycle 1), and these elements were recovered after re-watering (Cycle 2), except Mg. In Cycle 3, retaining irrigation after recovery decreased the Mg content, increased the K and P contents and did not affect the Ca content. From the explained ratio obtained from the PCA and heat map clustering, no clear scattering of the minerals corresponding to the stressed and non-stressed groups was observed. These findings suggest that more specific research on element behaviour needs to be conducted. As we observed, the behaviour of K and P was the same against stress conditions corresponding to the control and stress-experienced plants exposed to the stress after the recovery stage.

In order to combat the stress factors, plants have evolved an elaborate defence system, *viz*. enzymatic and non-enzymatic defence systems. As part of the non-enzymatic defence, many secondary metabolites have a substantial role in protecting the plants through combating reactive oxygen species (Bennett and Wallsgrove, 1994; Mazid et al., 2011). Of the secondary metabolites, essential oil compounds and phenolic acids were screened in the leaf tissues of lavender under stressed and non-stressed conditions. The

relevant compounds were well scattered according to the treatments. Regarding essential oil compounds, eucalyptol percentage did not exhibit any plasticity and no differences were noted in response to the treatments. However, drought stress increased eucalyptol percentage in *L. angustifolia* (Chrysargyris et al., 2016; Kulak, 2020), but recovery did not cause significant changes in *L. angustifolia* (Kulak, 2020). Camphor percentage was substantially affected with the treatments, exhibiting significant increases after drought stress and reductions after recovery. These findings are consistent with the report of Chrysargyris et al. (2016). However, camphor percentage decreased with a 7-day water-withholding period (Kulak, 2020). Being inconsistent with the report of Kulak (2020), endo-borneol decreased with single stress, but reiterated drought stress increased the percentage of the compound. These variations might be explained by the differences in experimental soils, previous experiences of the lavender plants and the duration of drought stress.

Phenolic compounds have been considered as indicators of tolerance (Caliskan et al., 2017). Based on the hypothesis linking stress tolerance and phenolics, the relevant compounds have been widely screened in plants subjected to drought stress (Chrysargyris et al., 2016; Gorgini Shabankareh et al., 2021; Mohammadzadeh and Pirzad, 2021). Considering the changes in phenolic acids in the present study, the responses of the compounds were relatively variable but well discriminated corresponding to the stressed and non-stressed groups. This clear scattering, which explained a high ratio of the variation, *i.e.* 76.28%, might be fundamental for further potential studies. For the case of the enzymatic defence system, drought priming enhanced the activities of antioxidant-associated enzymes, which then reduced the damage of the reiterated drought stress in *Alopecurus pratensis* grass, and the stress memory of the plant was correlated with enhanced levels of antioxidant enzymes over long-term drought stress (Lukić et al., 2020). In addition to the partially revealed roles of the enzymatic defence system, in-depth research related to secondary metabolites is required.

CONCLUSION

As clearly reported in quite a number of studies, drought stress adversely affected the plant growth and biomass production in a short period of water-withholding in the present study. However, the relevant agronomic attributes were not completely recovered after re-watering in spite of the improvement in the water contents of the leaf and soil, which were not translated to the other organs of the plant. As in the case of the agronomic traits, the mineral status of the plants was also clearly affected and the mineral uptake was restricted with drought stress; however, the responses of the minerals were not consistent with the water status of the leaf and soil media. In this current model of reiterated drought stress for lavender, the responses of the essential oil compounds and individual phenolic acids were clearly discriminated and might be assessed as indicators for the following researchers due to their well-known properties in the defence system. As mentioned in different sections of the study, there is lack of studies concerned with the secondary metabolite biosynthesis under recurrences of the stress. It is critical to note that a clear match was not observed between agronomic traits, mineral status and secondary metabolites. For this reason, though the present findings will undoubtedly contribute to relevant studies, combined and integrated approaches involving metabolomics and epigenomics are needed to reveal the hidden points.

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AUTHOR CONTRIBUTIONS

All authors have significantly contributed in finalising the research. M.K. and G.G. designed the experimental set-up. M.K., M.Z.K., F.C. and M.G.K. performed the greenhouse experiments and relevant biochemical analyses. M.K. performed the statistical analysis. A.M.K., G.G., M.N. and M.K. analysed the data and wrote the first draft, while the final draft was read and approved by all authors.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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