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Water deficit regimes trigger changes in valuable physiological and phytochemical parameters in *Helichrysum petiolare* Hilliard & B.L. Burtt

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ABSTRACT

The genus *Helichrysum* Miller is a source of many bioactive metabolites commonly used in traditional medicine. In particular, ~~*H.*~~*Helichrysum petiolare* Hilliard & B.L. Burtt shows activities as antiseptic, anti-inflammatory and in the control of anxiety disorder. Biosynthesis and accumulation of secondary metabolites is a defense mechanism of plants and it is strictly influenced by the surrounding environmental conditions. In this study, drought was imposed on *H. petiolare* (HEL008 clone CREA-Sanremo collection) to understand the effect of water stress on the dynamics of plant biomass and secondary metabolites production, and the morphological and physiological mechanisms involved in plant responses. *H. petiolare* was cultivated for 34 days under three water regimes: 100% of container capacity (CC, control), 50% CC (moderate water stress), and 0% CC (severe water stress). Plant growth traits, leaf water potential, gas exchange parameters, phenol, flavonoid, and anthocyanin content, and antioxidant activity changes were determined twice a week, while the volatile organic compounds (VOCs) and essential oils (Eos) at the end of the trial. Severe water stress dramatically reduced aerial and root dry weight, chlorophyll and carotenoid content, leaf water potential, water use efficiency (WUE, *A*/*E*), transpiration rate (*E*), stomatal conductance (*gs*), net photosynthetic rate (*A*) and antioxidant activity. Moderate water stress induced only slight changes and led to an increase of WUE at the end of the experiment. The total amount of VOCs and Eos was not affected by water stress while their quality changed. Moderate water stress increased the main constituents of both VOCs, i.e. the monoterpene hydrocarbons, and Eos, i.e. the oxygenated sesquiterpenes. In conclusion, this *H. petiolare* cultivation under the applied moderate drought condition could lead to a double benefit i.e. water-saving irrigation practice and high quality metabolite production.

Keywords: Essential oils; Gas exchange parameters; *Helichrysum*; Leaf water potential; VOCs; Water stress

1 Introduction

Plants produce a huge and diverse assortment of secondary metabolites. Their biosynthesis is largely influenced by the surrounding environmental conditions (Croteau et al., 2000).

Drought is well known to affect the secondary metabolite content, solute accumulation, and enzymes activities (Bettaieb et al., 2009; Selmar and Kleinwächter, 2013). Accumulation of secondary metabolites is a defense mechanism of plants to adapt to the water stress by altering their cellular metabolism (Gulen and Eris, 2004). Moderate and severe water stress conditions may cause the formation of reactive oxygen species and photoinhibitory damage (Asada, 1996). In the chloroplasts of the plant cells, protection against oxidative damages is provided by both enzymatic and non-enzymatic antioxidants (Asada, 1999). Thus, water stress induces physiological and molecular defense responses by increasing antioxidant concentrations (Eskling et al., 1997) and osmoprotectants in plant tissues.

Many plants have been identified as source of antioxidants and their consumption have been recommended (Liu and Ng, 2000; Lee et al., 2003). Worldwide the consumption of herbal medicines and use of natural antioxidants are continuously increasing with an estimated market value of 700 million US $ (Raut and Karuppayil, 2014). The increase in biosynthesis and accumulation of such metabolites could improve the production of herbal medicines and natural antioxidants for human health (Lubbe and Verpoorte, 2011; Raut and Karuppayil, 2014). Therefore, it is very important to understand the effect of environmental factors on the dynamics of biomass and productivity of secondary metabolites and the morphological and physiological mechanisms involved in plant's innate immune responses (Hsiao, 1973; Levitt, 1980; Davies and Zhang, 1991; Close and Bray, 1993; Kramer and Boyer, 1995).

The beneficial effects on human health of Volatile Organic Compounds (VOCs) and Essential oils (Eos), as herbal remedies produced by aromatic plants, are largely documented, raising interest in the medicinal chemistry community. To date, over 1700 volatile compounds have been identified and characterized for their biological activity (Muhlemann et al., 2014). Therefore, VOCs and Eos are the most studied class of plant secondary metabolites. VOCs emitting and Eos production are influenced by numerous biotic and abiotic factors and researchers are focused to improve their quality through the study of the ecological relevance and the molecular basis involved in plant-environment interactions (Maffei et al., 2011).

Different studies have shown that plants exposed to drought stress produced higher concentration of secondary metabolites than those cultivated under well watered conditions (Selmar and Kleinwachter, 2013; Alinian et al., 2016). In medicinal plants, water stress condition lead to increase the content of artemisinin in *Artemisia annua* L. (Charles et al., 1993), betulinic acid, quercitin, and rutin in *Hypericum brasiliense* Choisy (de Abreu and Mazzafera, 2005), and hyperforin in *Hypericum perforatum* L. (St. John's wort) plants (Zobayed et al., 2007).

In this study we focused our attention on *Helichrysum petiolare* Hilliard & B.L. Burtt. The genus *Helichrysum* Miller, belonging to the family of Asteraceae, consists of approximately 500 species, some of which are endemic to the Mediterranean area. Plants belonging to the *Helichrysum* genus have~~s~~ been traditionally a source of many bioactive compounds (Bremner and Meyer, 2000; Mathekga et al., 2000). Numerous species are commonly used by Mediterranean and South African populations in the treatment of wounds, infections, and respiratory conditions (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996; van Wyk et al., 1997; Scott et al., 2004). Some of them showed also biological activities in ~~in vitro~~in vitro assays. *H. foetidum*, *H. italicum*, and *H. nudifolium* showed anti-oxidant (Czinner et al., 2000; Tirillini et al., 2013), anti-microbial (Meyer and Afolayan, 1995; Taglialatela-Scafati et al., 2013) and anti-inflammatory activity (Jäger et al., 1996), respectively. *H. italicum* ssp. *microphyllum* and *H. zivojinii* appeared to have antifungal, antiviral, anti-HIV, and anti-cancer properties (Angoini et al., 2003; Nostro et al., 2003; Appendino et al., 2007; Matic et al., 2013). Leaves of *H. petiolare* are commonly used to treat coughs, colds, catarrh, headache, fever, menstrual disorders, and urinary tract infections (Lourens et al., 2008). Moreover, *H. petiolare* showed activities as antiseptic, anti-inflammatory and in the control of anxiety disorder (Eliovson, 1984; Arnold et al., 2002; Lourens et al., 2004, 2008, 2011).

The present study aimed to understand the morphological, physiological, and biochemical changes in *H. petiolare* plants induced by different water irrigation regimes and to evaluate their influence on the so-called volatilome (VOCs emitted) and Eos profiles.

2 Materials and methods

2.1 Plant material, experimental design and treatments

Plants of *H. petiolare* Hilliard & B.L. Burtt were provided by the CREA-FSO collection (clone ID = HEL008; located in Sanremo, Imperia, Italy~~ 43°81′60.28″N Lat, 7°76′67.38″~~—43°81′60.28″N Lat, 7°76′67.38″E Long), grown in the glasshouse of the University of Torino (Italy, 45°06′23.21~~″N Lat, 7°57′82.83″~~″N Lat, 7°57′82.83″E Long), clonally multiplied by cuttings, and placed in pots of 9 cm in diameter (one plant per pot) filled with peat (Silver Torf, Agrochimica, Bolzano, Italy) and Agriperlite® (70:30). Fertilization took place with a slow-release fertilizer (Osmocote 15:11:13; Scotts Europe, The Netherland). When plants reached at least 20 cm in height were transferred in a climatic chamber with controlled growth conditions (25 °C, 60% air humidity, 300 µmol m^{-2} s^{-1} and 16/8 h photoperiod) for all the experiment.

The experimental design was a split-plot design with three treatments and four replications per treatment. A total of 120 plants were randomly divided in three groups and subjected to irrigation at 100% of container capacity (CC, control), 50% CC (moderate water stress), and 0% CC (severe water stress). The value of 50% CC was set to determine moderate water stress in *H. petiolare* plants in a previous study (Caser et al., 2012). No irrigation treatment (0% CC) was applied to induced severe water stress. All the water contents were kept constant throughout the experiment. Gravimetric determinations of water contents were made by weighing soil samples before and after oven-drying to constant weight at 80 °C for one week. These values were used to calibrate all measurements of the moisture content of the substrate in the container. Container capacity was determined 48 h after irrigation and was calculated according to the equation of Paquin and Mehuys (1980). The soil moisture levels were maintained by manual irrigation and checked by weighing individual container every two days. The experiment lasted for a total of 34 days.

2.2 Plant growth parameters

Height and diameters of each plant were measured twice a week and used to calculate the growth index (GI; Π*{[(D' + D")/2]/2}2*H, where D' + D″)/2]/2]^2 × H, where D′ is the widest width, D″ is the perpendicular width and H is the height; Hidalgo and Harkess, 2002). At the end of the experiment, ten plants per treatment were harvested and roots and aerial parts were separated. After recording their fresh biomass, they were oven-dried at 65 °C for one week and dry biomass was weighted.

2.3 Pigments analysis

The concentration of pigments in 50 mg of fresh fully formed leaflets per treatment was evaluated twice a week. The chlorophyll and carotenoids were over night extracted in 5 ml of pure methanol at 4 °C in the dark. The absorbance of the extracts at 665, 652, and 470 nm was spectrophotometrically determined using a Ultrospec 2100 pro (Amersham Biosciences, UK), and the content of Chl a, Chl b, and carotenoids, respectively, were determined using the method described by Lichtenthaler (1987). The relative quantity of chlorophyll present in leaf tissue was also measured twice a week on 10 randomly selected leaves per treatment using the Chlorophyll Meter SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan).

2.4 Leaf water potential and gas exchange parameters

One hour before the beginning of the measurements (10 to 12 a.m.), the plants were transferred in lab for adaptation to ambient light intensity and temperature.

Midday leaf water potentials (LWP, Ψw) were determined twice a week, in three fully expanded leaves of six plants per treatment using a Scholander-type pressure chamber (Soil Moisture Equipment, Santa Barbara, CA, USA) (Scholander et al., 1965).

The measurement of internal CO_2 concentration (C_i), transpiration rate (E), stomatal conductance (g_s), and net photosynthetic rate (A) were performed on adult leaves twice a week, using a portable infrared gas analyzer ADC-LCPro+ (The Analytical Development Company Ltd., Hoddesdon, UK). Instantaneous water use efficiency (WUE) was calculated as the ratio between A and E. Most apical leaves of a shoot on six plants per treatment were clamped in the leaf chamber, where light source was set at 1200 µmol photons m^{-2} s^{-1} and temperature (25 °C) was kept constant. Environmental concentration of CO_2 (450–470 ppm) and vapour pressure deficit (2.3 kPa) were maintained during the experiments.

2.5 Total phenol content

The total phenol content was evaluated twice a week in 100 mg of fresh leaves per treatment. Leaves were pulverized and homogenized in a mortar with 1 ml of 70% (v/v) methanol. After 30 minutes of incubation at 4 °C, the samples were centrifuged at 15000 g for 10 minutes × g for 10 min, then the supernatants were utilized for the further analyses (Bretzel et al., 2014). Total soluble phenol compound content was assayed with the method based on Folin-Ciocalteau's phenolic reagent and spectrophotometrically determined (Singleton and Rossi, 1965). Twenty µl microliter of methanol extracted samples were added and mixed with 0.5 ml of Folin-Ciocalteau's reagent and 0.45 ml of 7.5% (w/v) of saturated sodium carbonate solution. After the incubation at room temperature for 2 h, the absorbance at 765 nm of the samples was detected in UV–vis spectrophotometer (Cintra 101, GBC Instruments, Australia). The presented data are the mean of three independent replications.

2.6 Flavonoid content

The total flavonoid content was determined twice a week on 100 mg of fresh leaves per treatment by using the colorimetric method of Kim et al. (2003). Twenty-five µl microliter of methanolic extract were added to 225 µl of distilled water and to 75 µl of 5% (w/v) sodium nitrite ($NaNO_2$). After 5 minutes of incubation were added 75 µl of 10% (w/v) of aluminum trichloride ($AlCl_3$) and after 5 minutes were added 500 µl of 1 M sodium hydroxide (NaOH). The absorbance of the samples was read at the UV–vis spectrophotometer (Cintra 101, GBC Instruments, Australia) after 15 minutes at 415 nm. The quantitative determination was made using a calibration curve with, as standard, quercetin 1:1 (w/v) dissolved in absolute methanol. Each analysis was repeated three times.

2.7 Anthocyanin content

Fresh leaves (200 mg) per treatment were collected twice a week and grounded in a volume of methanol/HCl (v/v 99/1%) with the addition of 2/3 volume of distilled water. Extracts were recovered, and a volume of chloroform was added to remove chlorophylls through mixing and centrifugation (1 min at 14,000 g × g). Anthocyanins contained in the aqueous phase were recovered and absorption was determined spectrophotometrically at 535 nm (Cheng and Breen, 1991). Calculation of anthocyanins was based on the standard curve prepared using cyanidine chloride. The contents were expressed as milligram per gram dry weight. Mean values were obtained from three independent replicates.

2.8 Determination of antioxidant activity

The antioxidant activity per treatment was determined twice a week on one hundred mg of fresh leaves per treatment by using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging method (Brand-Williams et al., 1995). One milliliter of sample at different concentrations (0.25, 0.50 and 1 mg ml^{-1}) was added to 0.500 ml of a DPPH methanol solution 0.25 mM (w/v) and incubated at room temperature in the dark for 30 min. The activity was measured as a decrease in absorbance at 517 nm using the spectrophotometer Cintra 101 (GBC Scientific Instrument, Australia). The percent inhibition of the DPPH radical by the samples was calculated according to the formula: % inhibition = (Ablank - Asample/Ablank) x 100, where A %inhibition = $(A_{blank} - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the DPPH radical without the antioxidant and A A_{sample} is the absorbance of the samples. The extract concentration (µg ml^{-1}) providing 50% of antioxidant activities (IC50) was calculated by plotting in a graph inhibition percentage against extract concentration. All determinations were performed in triplicate.

2.9~~8.1~~ (no italics) Analysis of VOCs

Emitted VOCs were analyzed using a Supelco solid phase microextraction (SPME) (Supelco, Bellefonte, PA, USA) device coated with polydimethylsiloxane (PDMS, 100 μm) in order to sample the headspace of 3 g of fresh twig. Each sample was introduced into a 100 ml glass conical flask and allowed to equilibrate for 30 min. After the equilibration time, the fiber was exposed to the headspace for 15 min at room temperature; once sampling was finished the fiber was withdrawn into the needle and transferred to the injection port of the Gas Chromatography–Electron Impact Mass Spectrometry (GC–EIMS) system where the fiber was desorbed. GC–EIMS analysis was performed with a Varian CP 3800 gas chromatograph chromatograph (Varian, Inc., Palo Alto, CA) equipped with a DB-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector chromatograph (Varian, Inc., Palo Alto, CA). Analytical conditions were as follows: injector and transfer line temperature were 250 °C and 240 °C, respectively; oven temperature was programmed from 60 °C to 240 °C at 3 °C min^{-1}; helium as carrier gas was set at 1 m~~l~~ min^{-1}; and the injection was in splitless mode. Identification of the constituents was based on comparison of retention times with those of authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons, and on computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra built from pure substances and components of known mixtures and MS literature data (Swigar and Silverstein, 1981; Davies, 1990; Adams, 1995). Moreover, the molecular weights of all the identified substances were confirmed by gas chromatography-chemical ionization mass spectrometry (GC–CIMS), using MeOH as CI ionising gas. The relative proportions of the volatile constituents were percentages obtained by peak-area normalisation, and all relative response factors were taken as one.

2.10~~8.2~~ (no italics) Extraction and analysis of essential oils

The essential oils (EOs) were obtained from 7– to 20 g of dried leaves and distilled by Clevenger-type apparatus (2 h), in according to the procedure described in the Italian Pharmacopoeia (Farmacopea Ufficiale della Repubblica Italiana, vol I. IXth edn. Zecca dello Stato: Rome, 1991). The essential oils were dissolved in diethyl ether (Et_2O), dried over anhydrous magnesium sulfate ($MgSO_4$), filtered and the solvent removed by evaporation on a water bath. The resulting essential oils were stored in a glass vial at 4 °C until use. All essential oils (injection of 0.2 μL, 10% hexane solution) were analysed by GC–EIMS as described in the previous paragraph.

2.11~~9~~ Statistical analysis

All measured and derived data were firstly subjected to the homogeneity of the variances and then post-hoc tested using Ryan-Einot-Gabriel-Welsch-~~F~~ *F* test (REGW-F). The critical value for statistical significance was ~~F~~ *P* < 0.05. All the data were computed by means of the SPSS statistical package (version 19.0; SPSS Inc., Chicago. Illinois).

3 Results and discussion

3.1 Effect of water stress on growth parameters

As well known, water shortage in plants cause the decrease of growth and cell development, especially in stem and leaves (Shao et al., 2008). In this study, the applied water stress did not affect the growth index of *H. petiolare* plants (data not shown) in agreement with previous findings in the genus (Pellizzaro et al., 2004; Caser et al., 2012). These authors observed that *H. italicum* and *H. petiolare* plants maintained their morphological and aesthetic values under severe and moderate drought condition, respectively. Conversely, in other medicinal and aromatic plants such as *Origanum majorana* (Rhizopoulou and Diamantoglou, 1991), *Mentha arvensis* (Misra and Srivastava, 2000), *Salvia officinalis* (Bettaieb et al., 2009) and *Ocimum basilicum* (Ekren et al., 2012) plant growth was negatively affected by moderate water stress condition. Thus, our data highlight that *H. petiolare* seems to better tolerate drought effects.

Generally, in drought conditions plants optimize the water uptake by improving the root system by means of growing to deeply explore soil area and by activate molecular and biochemical pathways to modulate water transport and metabolism (Chaves et al., 2003; Comas et al., 2013). Here, at the opposite the absence of irrigation (0% CC) reduced the dry weight more in the roots (−91.4%) than in the aerial parts (−45.1%), while moderate stress (50% CC) reduced only the root dry weight (−35.3%) (Fig. 1) by modifying the shoot:root ratio in favor of the shoot as previously reported for the other plants (Comas et al., 2013). Since the largest amount of Eos is accumulated in leaves and stems (Perrini et al., 2009), these results suggest that the imposed moderate stress did not influence the biomass production useful for extraction procedures.

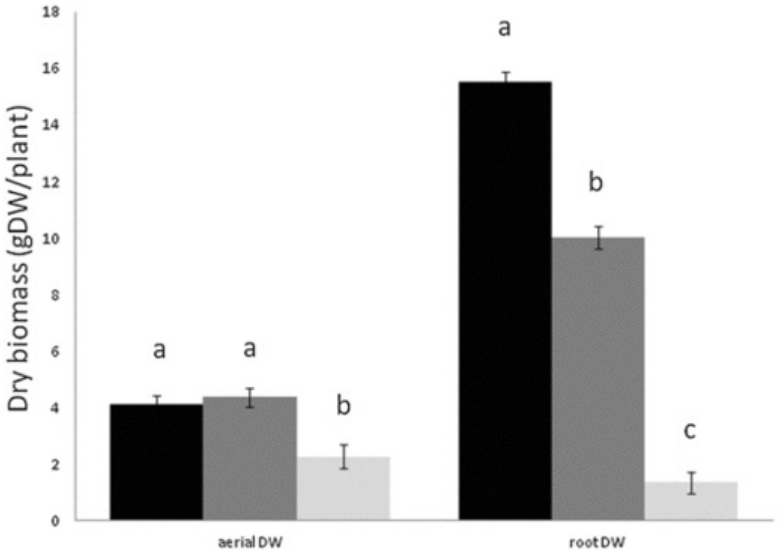

Fig. 1 Dry biomass (g DW/plant) of *Helicrhysum petiolare* aerial and root sections as influenced by different water regimes (black bar refers to 100% container capacity, 100% CC; dark grey bar refers to 50% CC; light grey bar refers to 0% CC). Values with different superscripts (a–c) are significantly different at $P < 0.05$ according to the REGW-F post-hoc test.

3.2 Effect of water stress on pigment content

Several studies reported that water stress leads to a decreased level of chlorophylls and carotenoids in plant leaves (Reddy et al., 2004; Kaminska-Rozek and Pukacki, 2004; Guerfel et al., 2009). Reduction in chlorophyll and carotenoids content is identified as a drought response mechanism in order to minimize the light absorption by chloroplasts (Pastenes et al., 2005). SPAD chlorophyll meter is frequently used as a quantitative measure of the severity of leaf damages associated with different biotic and abiotic stresses (Barraclough and Kyte, 2001; Caser et al., 2013) and, to a limited extent of leaf photosynthetic capacity (Castelli et al., 1996). In this study, no differences among treatments were observed (data not shown).

Prolonged severe water stress (0% CC) significantly reduced the total chlorophyll and carotenoid content already at the day 4 (Fig. 2). While, 50% CC irrigation induced a reduction of only the chlorophyll content (~~-21% and -~~21% and –30% at the day 32 and 34, respectively). A reduction in chlorophyll content was previously reported in other moderate drought stressed plants such as *Catharanthus roseus* (Jaleel et al., 2008) and severe drought stressed plants such as *Helianthus annus* (Kiani et al., 2008). As reported by Jaleel et al. (2009), carotenoids act as antioxidant and play fundamental role in protecting photochemical processes from oxidative damages. Here, carotenoids were not affected in moderate stressed plants, suggesting their additional role in helping plants to withstand adversaries (i.e., oxidative damages).

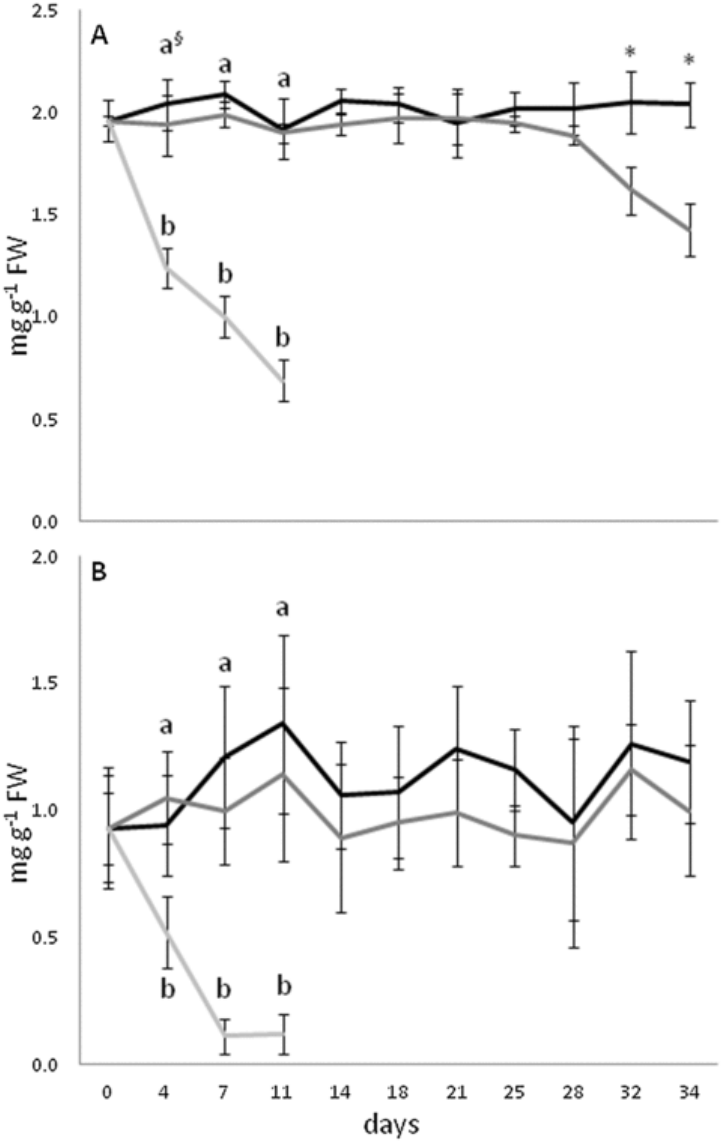

Fig. 2 Total chlorophyll (A) and total carotenoid (B) measured on *Helichrysum petiolare* plants during the experiment. Plants were subjected to control irrigation (100% container capacity, 100% CC-black line), moderate drought stress (50% CC-dark grey line), and no irrigation (0% CC-light grey line). [§]Mean values showing the same letter are not statistically different at $P \leq 0.05$ according to the REGW-F post-hoc test. The statistical relevance of 'Between-Subjects Effects' tests (* = $P<0.05$) was evaluated.

3.3 Effect of water stress on leaf water potential and gas exchange parameters

It is well known that drought-induced decreases in photosynthesis are due primarily to stomatal closure by means hydraulic and hormonal signals, which decreases CO_2 availability in the mesophyll, rather than to the direct effect on the capacity of the photosynthetic apparatus (Genty et al., 1987; Cornic, 1994). Clearly, stomatal closure is one of the first responses to soil drying, and a parallel decline in photosynthesis and stomatal conductance (*gs*) under progressive water stress has been reported (Chaves et al., 2009).

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In this study, water stress affected leaf water potential, stomatal conductance rates, and photosynthetic performance of the studied *H. petiolare* plants (Fig. 3). The LWP in the control plants kept constant during all the experiment with a mean value equal to -0.64 MPa (Fig. 3A). In plants irrigated at 50% CC, LWP was significantly lower at the days 32 and 34 (~~-1.13 MPa and~~ -1.13 MPa and -1.22 MPa, respectively). While the absence of irrigation (0% CC) reduced LWP already at the day 7 (-0.79 MPa) and then, a dramatically decreased occurred at the day 11 (-1.16 MPa) with the complete leaf withering. Within the genus *Helichrysum* Innes and Kelly (1992) showed that *H. aggregatum* plants during a drought season were tolerant, surviving at very low water potential, even if were severely visual affected. Thus, interspecific variability in drought tolerance seems to exist.

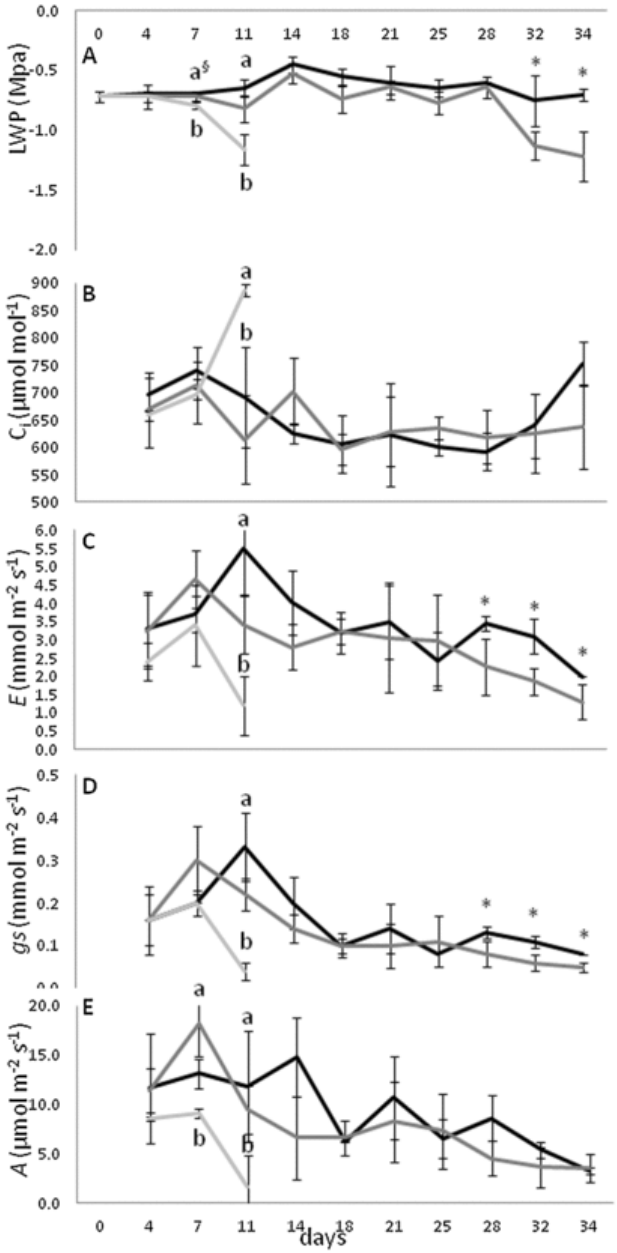

Fig. 3 Leaf water potential (LWP - A) and gas exchange parameters (internal CO_2 concentration, Ci - B; transpiration rate, E - C; stomatal conductance, g_s - D; net photosynthetic rate, A - E) measured on *Helichrysum petiolare* plants subjected to control irrigation (100% container capacity, 100% CC-black line), moderate drought stress (50% CC-dark grey line), and no irrigation (0% CC-light grey line). §Mean values showing the same letter are not statistically different at $P \leq 0.05$ according to the REGW-F post-hoc test. The statistical relevance of 'Between-Subjects Effects' tests (* = $P \leq 0.05$) was evaluated.

During the experiment no differences in internal CO_2 concentration were observed for irrigated and irrigated at 50% CC plants (Fig. 3B). The transpiration rate (*E*) and stomatal conductance (*gs*) followed the same trend (Fig. 3C and D). They decreased in plants under 0% CC at day 11 while in plants under 50% CC from day 28 onward. During drought stress processes, stomata are induced to close as leaves sense water deficit, especially after leaf water potential become more negative. Regarding the net photosynthetic rate, only the severe water stress condition induced a significant reduction, already at the day 7 showing a net decreasing trend (Fig. 3E). Taken together these results suggest that photosynthetic apparatus tolerates moderate water deficits and that stomata constitute the main limiting factor of carbon uptake under drought stress (Medrano et al., 1997; Petridis et al., 2012). Fig. 4 shows how instantaneous water use efficiency (WUE, *A*/*E*) changed during the experiment. As well as for the previous analyses, plants subjected to severe water stress presented the lower WUE, at day 11. Plants well irrigated (100% CC) showed a significant increase in WUE during the mid-time period from the day 14 to the day 18 (+35.6% and +40.6%, respectively) compared to 50% CC, followed by a slight decrease until the end of the experiment. While plants subjected to moderate water stress showed an increase in WUE at the end of the experiment (+42.7%). These results confirm that *H. petiolare* is able to efficiently use water resources. In fact, drought-stressed plants with higher WUE are more efficient in utilizing energy captured by photosynthesis per unit of water transpired. Similar trends were observed in *Callistemon* (Alvarez et al., 2011), *Hybanthus floribundus* (Kachenko et al., 2011), *Rosa* x *hybrida* 'RADrazz' (Cai et al., 2012), and in the grapevine cultivars 'Grenache' and 'Tempranillo' (Medrano et al., 2015). In all these cases, higher values of WUE corroborated their drought-resistant attitude.

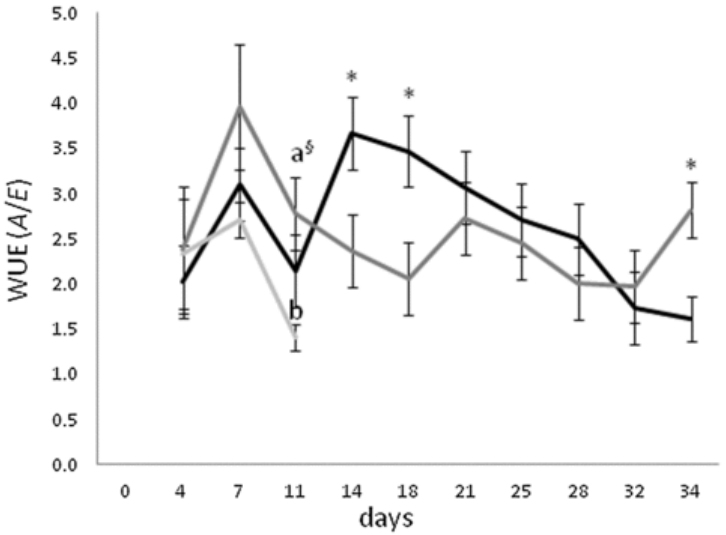

Fig. 4 Evolution of the instantaneous water use efficiency (WUE) in *Helichrysum petiolare* plants subjected to control irrigation (100% container capacity, 100% CC-black line), moderate drought stress (50% CC-dark grey line), and no irrigation (0% CC-light grey line) during the experimental period. §Mean values showing the same letter are not statistically different at $P \leq 0.05$ according to the REGW-F post-hoc test. The statistical relevance of 'Between-Subjects Effects' tests (* = $P \leq 0.05$).

3.4 Effect of water stress on phenol, flavonoid, and anthocyanin contents and antioxidant activity

H. petiolare plants subjected to severe drought conditions sharply increased the polyphenols, flavonoids, and anthocyanins content after few days of stress, while 50% CC let at final stage of treatment to a slight change in polyphenol content in comparison to the control plants (Fig. 5A - B - C). Some authors reported similar trends for other aromatic species. In *Salvia officinalis*, moderate drought condition increased the level of total and individual polyphenols (Bettaieb et al., 2011). Manukyan (2011) reported difference in productivity and secondary metabolites content in *Nepeta cataria* L. and *Melissa officinalis* L. subjected to drought stress. Polyphenols are probably the most versatile secondary metabolites, thus allowing plants to respond promptly to unpredictable stress agents of different origin (di Ferdinando et al., 2014) and also flavonoids play a role as UV-screening, antioxidant, and developmental regulators, such as in the acclimation/adaptation of plants to severe constrains of the Mediterranean climate (di Ferdinando et al., 2014). Polyphenols, such as phenolic glucosides, hydroxycinnamic acid derivatives, and flavonoids are involved in secondary cell wall thickening (Gunnaiah et al., 2012), thus mechanically increasing reinforcement of tissues, considered a key anatomical feature conferring drought tolerance (di Ferdinando et al., 2014). More differences were highlighted in the antioxidant activity (Fig. 5D). Plants under 0% CC showed the lowest antioxidant power (0.02 mg ml^{-1}FW) at the day 11. Plants at 50% CC showed higher values than control plants at days 14, 18 and 21, then a lower value at day 34. The antioxidant defence system is responsible for increasing tolerance in plants exposed to various environmental stress conditions (Ahmad et al., 2010). In the present study, an intensification of antioxidant content was observed only in plants subjected to moderate stress at mid-time of the experiment, highlighting that the effect of water stress is quickly restored in one week. As reported by Saraim and Srivastava (2001) water stress tolerant wheat genotypes showed

higher levels of antioxidants, but did not show uniform increase. Similarly, drought stress resulted in a significant increase in antioxidant concentration also in turf grass (Vranova et al., 2002) and *Catharanthus roseus* (Jaleel et al., 2007).

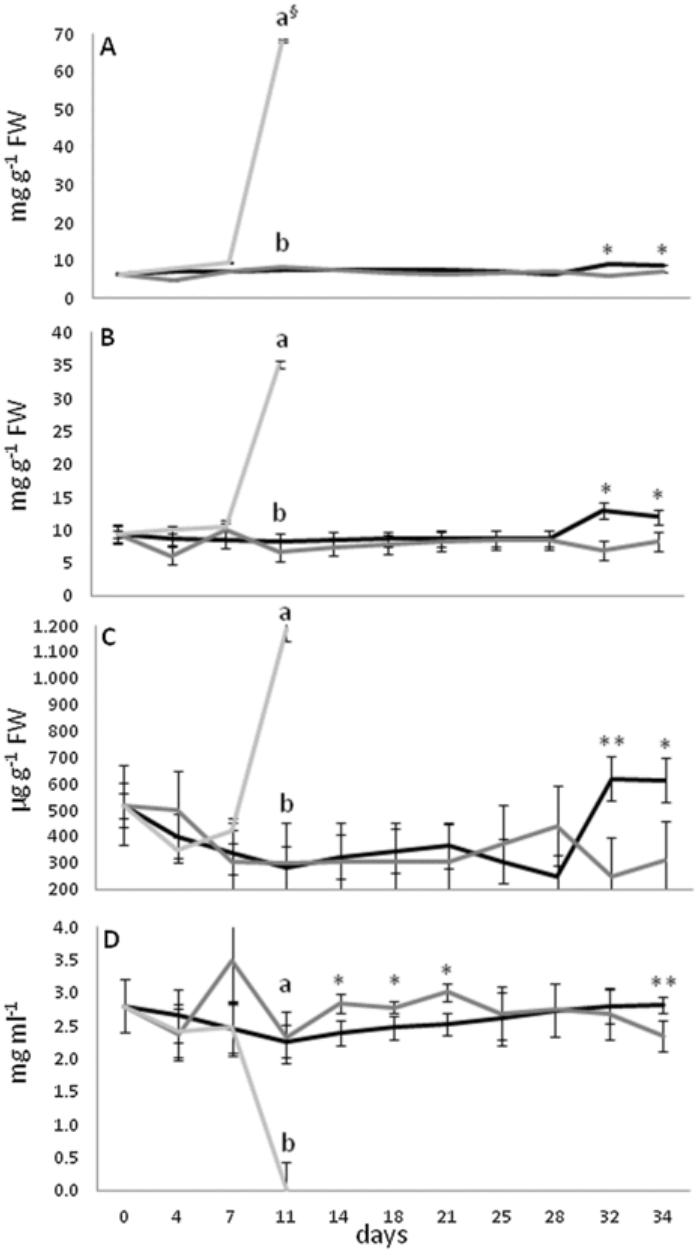

Fig. 5 Total polyphenols (A), flavonoids (B), anthocyanins (C), and antioxidant activity (D) measured on *Helichrysum petiolare* plants during the experiment. Plants were subjected to control irrigation (100% container capacity, 100%CC-black line), moderate drought stress (50% CC-dark grey line), and no irrigation (0% CC-light grey line). $Mean values showing the same letter are not statistically different at $P \leq 0.05$ according to the REGW-F post-hoc test. The statistical relevance of 'Between-Subjects Effects' tests (* = $P < 0.05$, ** = $P \leq 0.001$).

3.5 Effect of water stress on headspace components

The volatiles emitted from the analysed twig plants and identified by GC–MS are reported in Table 1. In total 41, 45, and 45 compounds were identified in plants treated with 100% CC, 50% CC, and 0% CC, respectively, corresponding to the total amount of constituents of the headspace (98.9%, 99,8%, and 99.9% in 100% CC, 50% CC and 0% CC, respectively). In general, water stress conditions reduced the content of the main constituents of volatile fractions, the monoterpene hydrocarbons (65.8, 66.4 and 55.7% in 100% CC, 50% CC and 0% CC, respectively) and its typical compound detected as Sabinene (51.1, 47.9 and 40.4% in 100% CC, 50% CC and 0% CC, respectively). Moreover, plants subjected to 0% CC showed an increase in non terpene derivates (8.2%, (*E*)-3-hexanol and (*N*)-hexanol the most representative) and in oxygenate monoterpenes (9.8%, Lavandulyl acetate the most representative) content. Only in plants under severe drought condition was induced the production of phenylpropanoids (0.2%, Dillapiole the only representant). In evergreen Mediterranean woody plants, monoterpene emission is frequent (Loreto et al., 2014). The idea is that volatile isoprenoids, and particularly monoterpenes, increase plant fitness to stressful environments. Indeed, Llusià et al. (2010) observed that plant species invading the Hawaii were characterized by a higher number of monoterpene emitters compared to native vegetation, possibly demonstrating a higher competitive or adaptive capacity (Loreto et al., 2014). The impact of drought stress on volatile isoprenoids is more controversial. Isoprene emitters are not widespread among plants adapted to arid environments, being more common in fast-growing species of families adapted to environments where water is not a limiting factor, e.g. Salicaceae and Arundinaceae (Fineschi and Loreto, 2012). Moreover, while mild droughts may stimulate isoprene emissions (Loreto and Sharkey, 1990; Brilli et al., 2007), severe and prolonged droughts heavily inhibit isoprene (Brilli et al., 2007). Although all investigated headspaces contain a similar array of constituents, the relative contribution of them varies in plants subjected to prolonged drought condition. If in plants under control and moderate stress was observed a chemical profile composed by Sabinene~~>α-Pinene>Longipinalol>~~ > α-Pinene > Longipinalol > Dihydroagarofuran, in plants under 0% CC the profile was Sabinene~~> Longipinalolo>α-Pinene>~~ > Longipinalolo > α-Pinene > Lavandulyl acetate.

Table 1 Effect of different water irrigation regimes (100% container capacity, CC; 50% CC and 0% CC) on the main volatile constituents of *Helichrysum petiolare* plants headspace. The constituents for each category (nt, non terpene derivates; mh, monoterpene hydrocarbons; om, oxygenated monoterpene; sh, sesquiterpene hydrocarbons; os, oxygenated sesquiterpene; pp, phenylpropanoids) were identified by HS-SPME and expressed in relative percentage of total compounds. Compounds are listed in order of their elution (Linear retention index, I*RI*) from apolar column. Percentage values are means of three values with standard error for the components below 2% in all cases.

Category	Constituents	IRI	100% CC	50% CC	0% CC
nt	(*E*)-3-hexan-1-ol	851	0.3	0.5	3.4
nt	(*N*)-hexanol	875	–	–	3.0
nt	1- Nonene	889	0.3	0.2	0.1
mh	Tricyclene	926	–	0.2	–
mh	α-Tujene	931	–	0.1	–
mh	α-Pinene	939	9.0	10.4	8.1
mh	Camphene	953	–	0.1	0.1
mh	Sabinene	976	51.1	47.9	40.4
mh	Myrcene	991	–	–	0.2
nt	Hexenol-(*Z*)-3- acetate	1004	0.7	1.0	0.3
mh	α-Terpinene	1018	1.1	1.6	0.7
mh	*p*-Cymene	1022	0.2	0.2	0.2
mh	Limonene	1031	3.6	4.0	4.0
mh	(*Z*)-β-Ocimene	1040	0.2	0.8	0.9
mh	(*E*)-β-Ocimene	1050	–	0.1	0.2
mh	γ-Terpinene	1062	0.4	0.6	0.6
mh	Terpinolene	1088	0.2	0.4	0.3
nt	1-Undecene	1086	0.3	0.1	0.2
nt	Nonanal	1104	–	–	0.2
om	Allo-ocimene	1129	0.2	0.1	0.2

om	Pinocarvone	1162	0.2	0.1	0.2
om	Lavandulol	1166	0.4	0.6	0.6
om	*Cis*-pinocamphone	1173	–	0.1	–
om	Myrtenal	1193	0.2	0.3	0.7
nt	*n*-Dodecane	1199	0.1	0.1	0.3
nt	(*N*)-decanale	1204	–	0.1	0.1
nt	3-Methyl-3hexen-1-yl-ester butanoate	1237	–	–	0.4
om	Bornylacetate	1285	0.2	0.1	0.3
om	Lavandulyl acetate	1289	1.5	1.2	5.1
om	Trans-pinocarvylacetate	1297	0.4	0.2	0.4
om	Myrtenylacetate	1326	0.4	0.3	0.7
sh	α-Copaene	1376	1.9	1.6	1.1
om	*Trans*-myrtenol acetate	1381	–	0.8	0.4
sh	Cyperene	1398	–	0.5	–
nt	(*N*)-tetradecane	1399	0.2	–	–
sh	α-Gurjunene	1409	0.6	–	0.6
sh	β-Caryophyllene	1418	0.9	0.8	0.8
sh	α-Guaiene	1439	0.2	0.3	0.3
sh	α-Humulene	1454	3.8	4.5	4.1
sh	Alloaromadendrene	1461	0.7	0.7	0.7
sh	γ-Himachalene	1476	0.6	0.8	1.0
sh	γ-Muurolene	1477	–	0.4	–
sh	α-Selinene	1494	0.7	0.9	0.7
os	β-Dihydroagarofuran	1496	4.6	4.9	4.9
sh	α-Bulnesene	1505	0.1	0.1	0.1
om	Lavandulyl isovalerate	1505	1.3	1.3	1.2
sh	γ-Cadinene	1524	2.3	2.3	3.0
sh	α-Calacorene	1542	–	0.1	0.2
os	Longipinanol	1575	7.1	8.1	8.4
os	*Cis*-β elemenone	1590	0.1	–	–
nt	Hexadecene	1593	0.1	–	–
os	Eudesmol 5-epi-7-epi	1603	0.5	0.1	–
pp	Dillapiole	1623	–	–	0.2

os	Bisabolol-epi-α	1685	0.2	–	–
nt	Salicyl-2-ethylhexy-l	1807	1.6	0.3	0.2
nt	Isopropyl tetradecanoate	1828	0.4	–	–
	Total		98.9	99.8	99.9
	Non Terpene Derivates (nt,%)		4.0	2.3	8.2
	Monoterpene Hydrocarbons (mh,%)		65.8	66.4	55.7
	Oxygenated Monoterpene (om,%)		4.8	5.1	9.8
	Sesquiterpene Hydrocarbons (sh,%)		11.8	13.0	12.6
	Oxygenated Sesquiterpene (os,%)		12.5	13.1	13.3
	Phenyl Propanoids (pp,%)		–	–	0.2

3.6 Effect of water stress on essential oil composition and yield

Overall, a total number of 45 different constituents of Eos were detected (44, 37 and 40 in plants irrigated with 100% CC, 50% CC and 0% CC, respectively) (Table 2). Regarding the constituent category, the oxygenated sesquiterpenes (os) equally increased in 50% CC and 0% CC (+9.1%), while the sesquiterpene hydrocarbons (sh) decreased (-28.5%). The Eo components were mainly constituted of Longipinalol (an oxygenated sesquiterpene) that remained the chief constituent of *H. petiolare* essential oil in all the studied plants. This constituent slightly increased under water stress conditions (37.2%, 41.9% and 40.8% in 100% CC, 50% CC and 0% CC, respectively) as well as the other two main components, Dihydroagarofuran (18.3%, 18.6%, 19.5% in 100% CC, 50% CC and 0% CC, respectively), and α-Pinene (5.2%, 6.5% and 6.2% in 100% CC, 50% CC and 0% CC, respectively). No information about the longipinalol acticity are present in literature. In a previous study, Lourens et al. (2004) extracted and characterized the Eos of *H. dasyanthum*, *H. excisum*, *H. felinum* and *H. petiolare*. These authors showed a chemical profile of *H. petiolare* composed by 1,8-Cineolo>β-Caryophyllene>p-Cymene> > β-Caryophyllene > p-Cymene > α-Pinene (22.4%, 14.0%, 9.8% and 6.8%, respectively). Differences with our results could be due to age of plants, cultivation practices and controlled growth conditions. The Eos profile demonstrates a high level of variability in terms of yield and composition and this has been attributed to the interactions between factors such the geographic origin, edaphic and climate features, genetic variability and phenological phase of the plants. In addition, the used plant material type and extraction method can determine the yield and constituents of Eos (Hussain et al., 2008). Our data resulted similar to the profile obtained by Lourens et al. (2004) in *H. felinum* with the monoterpenes largely absent. This chemical profile could be correlated with results obtained with the Greek species *H. orientale*, *H. heldreichii*, *H. italicum* ssp *microphyllum* and *H. doerfleri* by Roussis et al. (2000) where sesquiterpenes dominated.

Table 2 Effect of different water irrigation regimes (100% container capacity, CC; 50% CC and 0% CC) on the essential oil components of *Helichrysum petiolare* plants. The constituents for each category (nt, non terpene derivates; mh, monoterpene hydrocarbons; om, oxygenated monoterpene; sh, sesquiterpene hydrocarbons; os, oxygenated sesquiterpene) were identified by GC–MS and expressed in relative percentage of total compounds. Compounds are listed in order of their elution (Linear retention index, I*RI*) from apolar column. Percentage values are means of three values with standard error for the components below 5% in all cases.

Category	Constituents	IRI	100% CC	50% CC	0% CC
mh	α-Tujene	931	0.1	1.1	1.0
mh	Camphene	953	0.1	–	–
mh	α-Pinene	980	5.2	6.5	6.2
mh	3-*p*-Menthene	1004	0.7	0.2	0.2
mh	Limonene	1031	0.3	0.3	0.3
om	1,8-Cineolo	1033	0.5	0.3	0.3
mh	*z*-β-Ocimene	1040	–	0.2	0.2
mh	γ-Terpinene	1062	0.1	–	0.1
om	Camphor	1143	0.4	–	–

om	Lavandulol	1166	0.2	0.4	0.3
om	Myrtenol/Myrtenal	1193	0.4	0.5	0.5
om	Safranal	1200	0.1	0.2	0.2
om	Lavandulylacetate	1289	1.2	1.4	1.3
om	*Trans*-pinocarvylacetate	1297	0.2	0.2	0.2
om	Myrtenylacetate	1326	0.6	0.8	0.8
sh	α-Copaene	1376	0.4	0.5	0.4
om	*Trans*-myrtenolacetate	1381	0.1	0.2	0.2
sh	Cyperene	1398	0.2	0.2	0.2
sh	β-Caryophyllene	1418	0.4	0.6	0.6
om	Lavandulyl isobutyrate	1423	0.1	0.2	0.2
sh	α-Guaiene	1439	0.1	0.2	0.2
sh	α-Humulene	1454	3.4	3.8	3.6
sh	Alloaromadendrene	1461	0.5	0.5	0.5
sh	γ-Himachalene	1476	2.2	0.6	0.6
sh	γ-Muurolene	1477	1.7	–	–
sh	β-Selinene	1485	1.9	0.7	1.0
sh	α-Selinene	1494	1.4	–	–
os	Dihydroagarofuran	1496	18.3	18.6	19.5
sh	α-Bulnesene	1505	0.5	–	–
sh	Lavandulyl isovlerate	1510	1.5	1.7	1.6
sh	δ-Cadinene	1524	2.9	3.1	3.1
sh	α-Calacorene	1542	1.3	1.2	1.2
os	Longipinalol	1575	37.2	41.9	40.8
os	Prenopsan-8-ol	1575	1.3	1.5	1.4
nt	(*N*)-Hexadecane	1600	0.2	0.3	0.5
os	Humulene epoxide II	1606	0.9	1.0	1.2
os	1,10-di-epi-cubenol	1614	0.8	1.0	1.0
os	1-epi-cubenol	1627	0.5	0.7	0.7
os	epi-a-muurolol	1641	0.7	0.9	0.8
os	α-Muurolol	1645	0.3	0.4	0.3
os	Selin-II-en-4-α-ol	1652	1.9	2.2	2.4
sh	Cadalene	1674	0.2	0.2	0.3

os	Bisabolol-epi-α	1683	0.1	–	0.1
os	g-(*E*)-Atlantone	1706	0.3	0.3	0.3
nt	Isopropyl tetradecanoate	1828	1.8	–	1.3
	Total		93.2	94.6	95.6
	Non Terpene Derivates (nt,%)		2.0	0.3	1.8
	Monoterpene Hydrocarbons (mh,%)		6.5	8.3	8.0
	Oxygenated Monoterpene (mo,%)		3.8	4.2	4.0
	Sesquiterpene Hydrocarbons (sh,%)		18.6	13.3	13.3
	Oxygenated Sesquiterpene (os,%)		62.3	68.5	68.5

4 Conclusions

To the best of our knowledge, this is the first report showing a detailed characterization of the main physiological and chemical parameter changes under different water stress conditions in the genus *Helicrhysum*.

The present study shows that severe and moderate water stress generally induced changes in the most part of the studied parameter. While they did not affect modifications in plant growth, and the total amount of volatile and essential oil constituents. In our experimental condition *H. petiolare* resulted tolerant to moderate stress conditions and could be grown under moderate deficit irrigation (50% CC) for essential oil production, highlighting the possible sustainable use of water during their cultivation. Under a severe water stress condition, photosynthesis was limited as a consequence of the low CO_2 uptake due to reduced stomatal conductance under water deprivation. While, plants subjected to moderate stress showed higher water use efficiency, highlighting that the limited CO_2 assimilation not resulted in lower carbon availability to allocate for photosynthesis, for secondary metabolite production, and to maintain the biomass under such a stress condition.

In the Mediterranean environment, irrigation at 100% CC is rarely realized in industrial production conditions. However, no previous detailed information on irrigation regimes for *H. petiolare* cultivation were available. Our data indicated that moderate drought condition (50% CC) could be applied during the cultivation of *H. petiolare* to change metabolites concentration and improve plant quality. The present study could represent a source of information for different industries involved in the medicinal and aromatic product production.

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Highlights

- The first report on physio-chemical trait changes under water stress in *Helicrhysum*.
- Moderate (50% container capacity) and severe (0% CC) water stress were applied.
- Moderate water stress induced slight changes and led to an increase of WUE.
- Moderate water stress modified VOCs and Eos constituents.
- *Helicrhysum* can be irrigated at 50% CC, saving water.

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