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Ecophysiological and phytochemical responses of *Salvia sinaloensis* Fern. to drought stress

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Abstract

Salvia sinaloensis Fern. (sage) is a medicinal plant containing plant secondary metabolites (PSMs) with antioxidant properties. The current study investigated the effects of drought stress on *Salvia sinaloensis* morphological and ecophysiological traits, and active constituent production. Sage plants were cultivated in controlled conditions for 34 days and exposed to full irrigation as control, half irrigation, and no irrigation. Changes in growth index (G.I.), dry biomass, leaf water potential (LWP), physiological parameters, active compounds, volatilome (BVOCs) and essential oils (EOs) were determined. Not irrigated plants showed a decrease in total chlorophyll content (~-14.7%) and growth (G.I., ~-59.4%) from day 18, and dry biomass at day 21 (-56%), when the complete leaf withering occurred (LWP, -1.10 MPa). Moderate drought stressed plants showed similar trends for chlorophyll content and growth but kept a constant LWP (-0.35 MPa) and dry biomass throughout the experiment, as control plants. Carotenoids were not affected by water regimes. The photosynthetic apparatus tolerated mild to severe water deficits, without a complete stomatal closure. Plants under both stress conditions increased the percentage of phenols and flavonoids and showed altered BVOC and EO chemical profiles. Interestingly Camphor, the main EO oxygenated monoterpene, increased in moderate stressed plants while the sesquiterpene hydrocarbon Germacrene D decreased. The same trend was seen in the headspace under stress severity. The data evidenced a possible role of the active molecules in the response of *S. sinaloensis* plants to drought stress. Taking together, these findings point at *S. sinaloensis* as a potential drought adaptive species, which could be used in breeding strategies to obtain sages with high quality PSMs, saving irrigation water.

Keywords: Antioxidant activity, BVOCs, Drought stress, EOs, Monoterpenes, Sage

Abbreviations

BVOCs	Biogenic volatile organic compounds
EOs	Essential pils
PSMs	Plant secondary metabolites
MAPs	Medicinal and aromatic plants
CC	Container capacity
LWP	Leaf water potential
Ci	Internal CO_2 concentration
E	Transpiration rate

52	g_s	Stomatal conductance
53	A	Net photosynthetic rate
54	G.I.	Growth index
55	FRAP	Ferric reducing antioxidant power
56	Fe^{3+}-TPTZ	Ferric tripyridyl triazine
57	SPME	Solid phase micro extraction
58	GC-EIMS	Gas chromatography–electron impact mass spectrometry
59	WUE	Water use efficiency
60	FW	Fresh weight
61	DW	Dry weight

Introduction

Biogenic volatile organic compounds (BVOCs) and essential oils (EOs) are plant secondary metabolites (PSMs), with important ecological functions within defence, protection, and signalling mechanisms (Loreto and Schnitzler 2010; Raut and Karuppayil 2014). Plants modulate their concentration to withstand stress-related conditions (Fleta-Soriano and Munné-Bosch 2016), such as those provoked by drought (Selmar and Kleinwachter 2013; Kleinwachter et al. 2015). Water deficit affects many aspects of plant physiology and biochemistry, activating root to shoot signalling hydraulic and hormones-mediated processes (Gulen and Eris 2004; Jaleel et al. 2008; Schachtman and Goodger 2008; Pirbalouti et al. 2014; Fleta-Soriano and Munné-Bosch 2016; Ali et al. 2017; Caser et al. 2017). Plants respond by closing their stomata and accumulating compatible solutes to maintain a low water potential and avoid dehydration (Skirycz and Inze 2010). The increased diffusion barrier impairs influx of carbon dioxide (CO_2) into leaves. In consequence, lower amounts of CO_2 are fixed via the Calvin Cycle, and then less reduction equivalents ($NADP^+$) are available as electron acceptors (Kleinwachter et al. 2015). In this state, all reactions must be promoted to consume $NADPH + H^+$ such as the biosynthesis of highly reduced secondary compounds, i.e. phenols, terpenoids and alkaloids.

BVOCs and EOs are also sources for pharmaceutical, food additives, flavours, and fine chemicals (Zhao et al. 2005). In the last years the global EO production was around 50 to 100 tonnes per annum (Lubbe and Verpoorte 2011; Rauter et al. 2012; Ben Farhat et al. 2013). In medicinal and aromatic plants (MAPs), drought stress enhanced total phenolics and flavonoids production in *Labisia pumila* Benth. & Hook. (Jaafar et al. 2012) and monoterpene hydrocarbons and oxygenated sesquiterpenes in *Helichrysum petiolare* Hilliard & B.L. (Caser et al. 2016).

Salvia species is well known for possessing numerous secondary metabolites with significant benefits to human nutrition and health, used in folk medicine for a long time and therefore many species were listed in the official Pharmacopoeias. Antiinflammatory and antioxidant properties were observed in leaf extracts of *S. officinalis* L. (Munné-Bosch et al. 2001), anticonvulsant and sedative properties in leaves and seed extracts of *S. leriifolia* Benth (Hosseinzadeh and Arabsanavi 2001), and analgesic, neuroprotective and antiparkinson properties in roots of *S. miltiorrhiza* Bunge (Du and Zhang 2004). However, other *Salvia* species, such as in *S. sinaloensis* Fern, had beneficial properties (Abreu et al. 2008) but little information is available.

Despite the enormous richness of MAP species, breeding activities are advanced only for those plants with high demand and cultivation area (e.g. *Origanum vulgare* L. and *Matricaria recutita* L.). Wild collection and simple domestication are therefore still the main production strategies (Novak 2017).

The need of support and speed up MAPs breeding also for minor crops impelled us to deepen the knowledge about *S. sinaolensis* characteristics in relation to cultivation practices.

In sage, imposed drought stress already proved to increase EOs, total polyphenols content and monoterpenes in *S. officinalis* (Abreu and Munne-Bosch 2008; Bettaieb et al. 2009; Bettaieb et al. 2011; Radwan et al. 2017) and active constituents in *S. miltiorrhiza* (Liu et al. 2011).

Here we investigated *S. sinaloensis* dynamics in response to water limitation through morphological, ecophysiological and phytochemical analyses, contributing to our understanding of the mechanisms underlying plant adaptation and PSMs production.

Materials and methods

Plant material and treatments

Three year old plants from the CREA-FSO collection (Code database RGV FAO Salv095, Sanremo, Imperia, Italy - 43°81'60.28''N Lat, 7°76'67.38''E Long) were cloned by cuttings in the glasshouse of Dept. of Agricultural, Forest and Food Sciences of the University of Torino (Italy, 45°06'23.21''N Lat, 7°57'82.83''E Long), and cultivated in vases (9 cm in diameter – one plant per vase) containing peat (Silver Torf, Agrochimica, Bolzano, Italy) and Agriperlite® (70:30), and fertilized with 4 g L^{-1}, Osmocote (15:11:13; Scotts Europe, The Netherland). When plants reached 20 cm in height, one hundred and twenty pots were transferred in a growth chamber at 20 °C, 60% relative humidity and 16 h photoperiod (constant average PAR of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

The experimental design was a split-plot design with three treatments and four replications per treatment. Plants were randomly divided in three groups and subjected to irrigation at 100% of container capacity (CC, control), half of the irrigation volume provided to CC controls (50% CC - moderate drought stress), or no irrigation (0% CC). The value of 50% CC was set to determine moderate drought stress in *S. sinaloensis* plants in a previous study (Caser et al., 2012). 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 weighting individual container every two days. The experiment lasted for a total of 34 days.

Ecophysiological measurements

Leaf water potential (LWP, Ψw), internal CO_2 concentration (C_i), transpiration rate (E), stomatal conductance (g_s) and net photosynthetic rate (A) were measured twice a week (10-12 a.m.). In each treatment, LWP was counted in three leaves of five plants with a Scholander chamber (Soil Moisture Equipment, Santa Barbara, CA, USA) (Scholander et al. 1965).

C_i E, g_s and A were measured through a gas analyzer ADC-LCPro+ (The Analytical Development Company Ltd, Hoddesdon, UK). For each treatment three apical leaves of five plants were clamped in the leaf chamber, and routines of measurements were performed according to Caser et al. (2016). The CO_2 concentration (450-470 ppm) and vapour pressure deficit (19.46 ± 1.67 Pa/KPa) were kept constant during the experiment. The ratio between A and E was used to calculate the instantaneous water use efficiency (WUE).

Determination of pigment content

Fifty mg from four fresh fully formed leaves per treatment were used to determine chlorophyll and carotenoids twice a week. After an over-night extraction in 5 ml of methanol at 4 °C in the dark, pigments were spectrophotometrically determined at 665, 652, and 470 nm using a Ultrospec 2100 pro (Amersham Biosciences, UK) as described by Lichtenthaler (1987).

The Chlorophyll Meter SPAD-502 (Konica Minolta Sensing Inc., Osaka, Japan) was used to measure the relative quantity of chlorophyll present in five randomly selected leaves per treatment.

Determination of growth index and biomass

Height and diameter of each plant per treatment were measured to calculate the growth index (G.I.; Π*{[(W'+W")/2]/2}^{2}*H, W' is the broadest diameter, W" is the perpendicular diameter and H is the height; Hidalgo and Harkess 2002) . At day 34, roots and aerial parts of ten plants per treatment were separated and weighed. After recording their fresh biomass, they were oven-dried at 65 °C for one week and dry biomass was measured.

Determination of phenol and flavonoid content, and antioxidant activity

Fresh leaves (100 mg per treatment) were pulverized and homogenized in a mortar with 1 ml of 70% (v/v) methanol to facilitate the extraction. After 30 minutes of incubation on ice, the extracts were centrifuged at 10.000 g for 10 minutes at room temperature to collect the supernatant (methanol extract) to be used for the determination of phenol and flavonoid content, and the antioxidant activity. The content of total phenols was measured by using the Folin-Ciocalteau's phenolic method and determined

as reported by Singleton and Rossi (1965). Twenty µl 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).

Total flavonoid content was determined by applying the colorimetric method of Kim et al. (2003). Twenty-five µl 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 1M sodium hydroxide (NaOH). The absorbance of the samples was detected 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.

The antioxidant activity was determined by using the ferric reducing antioxidant power (FRAP) method with minor modification (SzőllősiandSzőllősiVarga 2002). The FRAP procedure is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. An intense blue color complex was formed when ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex was reduced to the ferrous (Fe^{2+}) form and the absorption at 593 nm was recorded. The calibration curve was plotted with absorbance at 593 nm versus concentration of Fe^{2+} solution which in turn plotted against concentrations of standard antioxidant. A total of 50 µl samples extract were added to 1.5 ml of the FRAP reagent and mixed well. The absorbance is measured at 593 nm using UV-VIS spectrophotometer (Cintra 101, GBC Instruments, Australia). Samples are measured in three replicates.

At the end of the experiment, the total amount of total phenols, flavonoids and antioxidant activity per plant (mg/plantFW) was estimated on the basis of the plant fresh biomass.

BVOC analysis

The evaluation of biogenic volatile organic compounds (BVOCs) was conducted at day 14. The headspace of 3 g of twig was measured by applying a Supelco Solid Phase Micro Extraction (SPME) (Supelco, Bellefonte, PA, USA) with polydimethylsiloxane (PDMS, 100 µm). Each sample was introduced into a 100 ml glass conical flask and equilibrated 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 (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 mL min^{-1}; and the injection was in splitless mode. Identification of the constituents was based on comparison of their retention times with those of authentic samples, and on computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra built from pure substances and MS literature data (Swigar & Silvestein, 1981; Davies, 1990; Adams, 1995).

The relative proportions of the volatile constituents were percentages obtained by peak-area normalisation, and all relative response factors were taken as one.

EO analysis

Twenty g of dried leaves were used for the distillation of essential oils (EOs) by using a Clevenger-type apparatus (2 h) (Farmacopea Ufficiale della Repubblica Italiana, vol I. IXth edn. Zecca dello Stato: Rome, 1991). The yields of distillation were not determined due to the low amount of the starting plant material. The EOs obtained were solubilized in *n*-hexane, filtered over anhydrous sodium sulphate and stored in a vial at 4 °C in the dark until use. GC-EIMS was used to analyse all EOs (injection of 0.2 μL, 10% hexane solution) as reported above.

Statistical analysis

Data were previously tested for the variance homogeneity. Ryan-Einot-Gabriel-Welsch-F post-hoc test (REGW-F) ($P < 0.05$) was applied by means of the SPSS statistical package (version 19.0; SPSS Inc., Chicago. Illinois).

Results and discussion

Changes in ecophysiological parameters

In this study, moderate drought stress did not affect LWP compared to control (Fig. 1). In both cases it was constant throughout the experiment, with a mean value of -0.35 MPa. On the opposite, no irrigation significantly reduced LWP to -0.64 MPa at day 7. Then, a decreasing trend was observed till day 21 (-1.10 MPa) when the complete leaf withering occurred. Within the genus, similar results were found by Eakes et al. (1991) in *S. splendens* Sellow 'Bonfire' whose leaves reached a LWP ranging between -1.10 and -1.40 MPa when exposed to episodic drought (moisture stress conditioned). In other xerophytic sage

species, drought induced much lower LWP. *S. officinalis* plants subjected to 50% CC and 25% CC reached LWP equal to -3.00 and -4.80 MPa, respectively (Bettaieb et al. 2011), and *S. mellifera* Greene under severe drought condition reached LWP equal to -8.0 MPa, even if severely affected by embolism (Hargrave et al. 1994).

Stomatal closure is the primary mechanism by which plants regulate water loss. It is widely observed that plants reduce stomatal conductance of their leaves in response to declining water potential (Oren et al. 1999). Here, drought did not affect internal CO_2 (C_i) concentration with the exception for the 0% CC treatments that led to increase C_i at day 21(Fig. 2A). More differences were found for *E*, *gs* and *A*, showing similar trends (Fig. 2B-C-D). Not irrigated plants showed lower values from day 14, shifted to day 21 (as regarding *gs* and *A*) and from day 25 (*E*). At the end of the experiment no differences were observed between control and half irrigation for all measured traits. Thus, drought stress led to partial stomata closure (Fig. 2B). As result, the influx of CO_2 is diminished, visible by the strongly decreased rate of photosynthesis in the stressed plants (Fig. 2C) and the lower growth rate (Fig. 3). However, as the stomata closure is not very distinct, the actual CO_2 concentration in the stressed plants is not significantly lower than in the controls. Obviously, the steady state concentration, at least in part, is determined by the photosynthetic action (i.e. the affinity of RuBisCO). This is confirmed by the fact that in the severely stressed plants, the internal CO_2 concentration increased, when CO_2 consumption is stopped due to the lacking photosynthetic activity after 21 days. Results suggested that the photosynthetic apparatus of *S. sinaloensis* could tolerate mild to severe water deficits and decreasing in photosynthesis couple with reduced stomatal function (Eakes et al. 1991; Galmes et al. 2007).

Instantaneous water use efficiency (WUE, *A*/*E*) is used as an indicator of the water amount applied for growing by plants. Different authors indicated that stressed plants showing higher *A*/*E* are more able to utilize energy obtained by photosynthesis per unit of water transpired (Liu and Stützel 2004; Monclus et al. 2006). In this study, drought did not affect WUE except for the 0% CC treatment at day 21 (Fig. 2E). This finding indicates that *S. sinaloensis* could efficiently use water resources under moderate and severe drought stress condition at least under control conditions. In many species WUE was improved under water limitation and low *gs* (Liu et al. 2005). Among these, *Trifolium alexandrinum* L. (Lazaridou and Koutroubas 2004), *Spartina alterniflora* Loisel (Hessini et al. 2009), *Callistemon* (Alvarez et al. 2011), *Hybamthus floribundus* Lindl. (Kachenko et al. 2011), *Rosa hybrida* L. (Cai et al. 2012), *Vitis vinifera* L. 'Grenache' and 'Tempranillo' (Lovisolo et al. 2010; Medrano et al. 2015), and *Helichrysum petiolare* (Caser et al. 2016). In sage, Lambrecht et al. (2011) observed that leaf physiological activity of *S. mellifera* was not limited by imposed water deficits as well as indicated in

our study. The photosynthetic apparatus was not completely affected by drought stress also in *S. splendens* 'Bonfire' (Eakes et al. 1991) and in *S. pitcheri* L. (Hamerlynck et al. 1997), highlighting the positive water use efficiency attitude of different species within the genus *Salvia*.

Changes in chlorophyll, carotenoids and plant growth

The decrease of chlorophyll and carotenoids in drought stressed plants is reported by several authors (Kaminska-Rozek and Pukacki 2004; Pastenes et al. 2005; Guerfel et al. 2009). Here, moderate and severe drought stress reduced the total chlorophyll content starting from day 18 up to the end of the experiment (Supplementary Table 1), while carotenoids and Chl:Car ratios were not affected by the imposed irrigation regimes. Plants of *S. officinalis* treated with 50% CC and 25% CC presented similar values and trends (Bettaieb et al. 2011). Similarly, a chlorophyll decrease was observed also in plants of *Catharanthus roseus* L. (Jaleel et al. 2008) and *Helichrysum petiolare* (Caser et al. 2016). Jaleel et al. (2009) indicated that carotenoids play an important role in protecting different processes from reactive oxygen species damages. Here, the presence of constant amount of carotenoid during the experiment suggests a possible role to cope with oxidative damages. Similar results were observed by Caser et al. (2016) in *H. petiolare* plants subjected to 50% CC water regime.

The evaluation of SPAD units by the use of the chlorophyll meter is commonly used to associate leaf damages and abiotic stresses (Caser et al., 2013) and leaf photosynthesis (Castelli et al. 1996). Here, no significant results were highlighted (data not shown). Similarly, Caser et al. (2012) showed no differences of SPAD values in *S. dolomitica* Codd and *S. sinaloensis* plants under drought conditions with the exception for those irrigated with 20% CC in which was observed an increase.

The decrease of chlorophyll content might cause a reduction in growth parameters of plants under water stress conditions (Viera et al. 1991). In this study, all the treated plants grew similarly till day 11 (Fig. 3). Then, drought stressed plants significantly reduced their growth. Not irrigated plants reached the lowest G.I. at day 21 (2654.00 cm^3), while plants under 50% CC kept constant G.I. from day 18 onward, ranging between 6908.73 cm^3 and 5686.40 cm^3. These results are in agreement with a preliminary study on *S. sinaloensis* plants treated with different irrigation regimes where irrigation with 20% CC and 40% CC significantly reduced the plant growth compared to 80% CC and controls (Caser et al. 2012). However, as shown in Table 1, here only severe drought stress reduced drastically the total dry mass of plants (-56%), affecting particularly the roots (-67%). Thus, the R:A ratio (-55%), favoring the shoot as showed in other species under severe drought stress conditions (Comas et al. 2013; Caser et al. 2016). A reduction of shoot dry weight, leaf area, stem length and root length was similarly seen in

S. splendens (Eakes et al. 1991; Burnett et al. 2005). On the opposite, half irrigated plants showed an increase in root dry biomass (+14%) and consequently in R:A ratio (+37%) compared to control. Since roots are the only source to acquire water from soil, the root growth, its density, proliferation and size are key responses of plants to drought stress (Kavar et al. 2007). The drought tolerance of tea, onion and cotton was increased by improved root growth and root functioning (Farooq et al. 2009).

Changes in phenols and flavonoids and antioxidant activity

Polyphenols and flavonoids are among the most adaptable PSMs, helping plants to cope with different stress conditions (Di Ferdinando et al. 2014). In this study the total content of phenols in full-irrigated plants decreased during the whole experiment, reaching the lowest value between days 21 and 25 (0.98 mg/g FW) (Fig. 4A). Conversely, at the same data point moderate stressed plants exhibited the highest amounts (14.26 mg/g FW and 13.16 mg/g FW at the days 21 and 25, respectively). A different behavior was also observed in severe stressed plants, that showed higher content of phenols from day 11 to day 18 with the highest value at day 11 (15.74 mg/g FW). A similar dynamic was observed for the total flavonoids accumulation (Fig. 4B). In control plants, flavonoids increased during the first 7 days of cultivation. Subsequently, they dramatically decreased reaching minimum values between days 21 (1.19 mg/g FW) and 25 (1.16 mg/g FW). By contrast, in moderate stress condition flavonoid content kept constant until day 21, later increased with higher values from day 18 till the end of the experiment, reaching the maximum content at day 25 (2.89 mg/g FW). In the not irrigated plants, the total content of flavonoids peaked at day 11 (3.73 mg/g FW). In literature, different works showed that in drought stressed plants was observed an increase in secondary metabolites content. Plants of *Prunus persica* L., *Echinacea purpurea* L., *Hypericum brasiliense* Choisy, *Trachyspermum ammi* L. and *Labisia pumila* Benth. & Hook. subjected to drought stress showed significant increase in total phenols (Kubota et al. 1988; Gray et al. 2003; de Abreu and Mazzafera 2005; Azhar et al. 2011; Jaafar et al. 2012) and *Pisum sativum* in flavonoids (Nogués et al. 1998). In *Salvia officinalis*, an increase in phenols was observed in half irrigated plants (Bettaieb et al. 2011). Here, a mid to late increase in total phenols and flavonoids occurred in plants subjected to 50% CC. According with Paulsen and Selmar (2016), imposed drought stress generally results in decrease of aerial biomass gain and the corresponding dry or fresh weight are strongly influencing the content of natural products in MAPs. Here, as indicated in Table 2, moderate drought conditions resulted in a significant increase of the total content of phenols in comparison to severe drought stressed plants and controls (16.44, 22.85 and 10.43 mg/plant in 100% CC, 50%CC and 0% CC, respectively). While, no differences were noted in flavonoid content between moderate drought

stress and control conditions (6.42, 6.47 and 2.73 mg/plantFW of flavonoids in 100% CC, 50%CC and 0% CC, respectively). These findings should be taken in consideration for future industrial purposes.

Drought induces oxidative stress in plants, in which reactive oxygen species (ROS) are produced (Munné-Bosch and Penuelas 2003). Plant resistance to ROS is associated with an increase in antioxidant activity to prevent stress damage (Bor et al. 2003). In this study, only plants under 50% CC showed a significant increase of antioxidant activity at the end of the experiment (Fig. 4C). Similarly, at the end of the experiment the total amount of antioxidant activity per plant was much superior in moderate drought stressed plants than the others (106.37, 125.98 and 20.86 mg/plantFW in 100% CC, 50% CC and 0% CC, respectively) (Table 2). These findings confirm the ability of this plant to encounter the water limitation (50% CC) activating stress defense signaling and pathways respect to severe drought stress (0% CC) where metabolic and physiological constraints are not able to allow plant survival.

Changes in emitted BVOCs

Different authors observed that any stress factor can potentially change the rate of volatile release and alter the bouquet of BVOCs (Dicke and Baldwin 2010; Holopainen and Gershenzon 2010; Niinemets et al. 2013). Their emission can vary drastically depending on the species, organ, developmental stage and environmental conditions (Holopainen and Gershenzon 2010). Abiotic stress can enhances the emission of BVOC rates and patterns by the alteration of the communication with other organisms and photochemical cycles (Loreto and Schnitzler 2010). In the present study, the volatiles emitted and identified from the analyzed twigs are reported in Supplementary Table 2. In total a number of 20, 32 and 38 compounds were recognized in plants treated with full irrigation, half irrigation and no irrigation, respectively, accounting for 92.1%, 96.6% and 95.4% of the total compositions, respectively. The volatile fractions were characterized mainly by sesquiterpene hydrocarbons (sh) although a mild reduction by increasing stress conditions was observed (57.5% < 42.8% < 42.7% in full irrigation, half irrigation and no irrigation, respectively). No irrigation affected the production of four of the six reported volatile molecule classes. In particular, an increase in oxygenated monoterpenes (om) (9.7%, 16.2% and 23.6% in full irrigation, half irrigation and no irrigation, respectively), was observed. Among the other categories not-terpene derivates (nt), oxygenated sesquiterpene (os) and apocarotenoids (ac) were observed only in few percentage. However, plants under 50% CC exhibited the highest content in monoterpene hydrocarbons (mh) (24.2%, 37.1% and 22.0% in full irrigation, half irrigation and no irrigation, respectively). All investigated headspaces showed different array of the main constituents. The chemical profile in fully irrigated plants was characterized by Germacrene D > β-Caryophyllene >

β-Pinene, in moderate stressed plants by β-Pinene > Camphor > β-Caryophyllene and in severe stressed plants by β-Caryophyllene > Camphor > β-Pinene. Among the various constituents, a very sharp decrease (4.4 times) was observed for the Germacrene D (from 22.0% to 5.0% in full irrigation and no irrigation, respectively). On the opposite the oxygenated monoterpene Camphor increased 2.5 times when plants were subjected to 0% CC.

The literature is ambiguous concerning BVOC emission in relation to water availability. Several recent reviews addressed the roles of BVOCs in enhancing the tolerance of plants to various general abiotic stressors (Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010; Possell and Loreto 2013; Rinnan et al. 2014). In particular, under drought Mediterranean climatic condition, the emission of oxygenated monoterpene is common (Loreto et al. 2014) and is thought to promote direct and indirect defense by modulating the signaling that biochemically activates defense pathways in response to stressful conditions (Loreto and Schnitzler 2010; Rinnan et al. 2014). Pistelli et al. (2013) reported an increase in oxygenated monoterpenes in *Salvia officinalis* plants under *in vitro* growing conditions. Moreover, there is evidence that the strength of the emission of BVOCs can be quantitatively related to the severity of abiotic stresses (Niinemets et al. 2013). As reported by Niinemets et al. (2004) the emission of oxygenated BVOCs depends on stomatal behavior. The stomatal closure in response to drought stress is therefore expected to drastically affect the emission of these compounds as showed in *Quercus ilex* L. (Bertin and Staudt 1996; Llusia and Peñuelas 1998; Loreto et al. 2001). Here, on the contrary we observed an increase in oxygenated monoterpenes under mild and severe drought stress associated with a not completely stomatal closure. Progressing soil water deficiency enhanced monoterpene emissions also in *Pinus halepensis* Mill. and *Cistus albidus* L., whereas did not affect it in *Rosmarinus officinalis* L. and *Quercus coccifera* L. (Ormeño et al. 2007). By contrast, overall emissions of sesquiterpenes were reduced by water deficiency in all the four species (Ormeño et al. 2007). Moreover, Hansen et al. (1997) and Ormeño et al. (2007) noticed that monoterpene emission in *R. officinalis* was not dependent by photosynthesis, but may originate from "*de novo*" synthesis in the photosynthetic tissues (Steinbrecher et al. 1999; Ormeño et al. 2009; Nogués et al. 2015). Taking together, all these results on BVOCs could represent important findings for studying the plant-environment interactions and can be used in breeding strategies to improve volatile compounds yield and quality.

Changes on essential oil

In a wide range of experiments was highlighted that plants under drought stressed showed an increase in secondary metabolites content (Selmar and Kleinwächter 2013). Here, a total of 78 constituents were detected in the investigated EOs of which 59, 41 and 62 in full, half and not irrigated plants, respectively (Supplementary Table 3). Drought stress conditions only slightly increased the total number of constituents identified in the EOs (94.0, 99.6 and 98.8% at full irrigation, half irrigation and no irrigation, respectively). Similar results were observed in *Satureja hortensis* L. (Baher et al. 2002), *Lippia berlandieri* Schauer (Dunford and Vazquez 2005), *Petroselinum crispum* (Mill.) (Petropoulos et al. 2008), *S. officinalis* (Bettaieb et al. 2009), and *Laurus nobilis* L. (Maatallah et al. 2016) plants treated with similar drought stressed conditions. Regarding the type of constituents, moderate drought stress increased the total amount of monoterpenes (both oxygenated monoterpenes and monoterpene hydrocarbons: 51.5, 77.1 and 54.9% and 1.4, 6.0 and 1.9% in full irrigation, half irrigation and no irrigation, respectively) and deeply reduced the oxygenated sesquiterpenes (29.2, 6.3 and 26.9% in full irrigation, half irrigation and no irrigation, respectively). Similarly, imposed moderate drought stress strongly increased (2 to 4 fold) the content of Camphor, a specific compound in the essential oil, as also reported in *S. officinalis* plants (Bettaieb et al. 2009; Nowak et al. 2010) and in *Picea abies* L. and *Pinus silvestris* L. (Turtola et al. 2003). Nowak et al. (2010) reported that moderate stressed condition resulted in a massive increase of concentration of monoterpenes compensating the reduction in biomass. In fact, the stimulation to produce high terpene content under drought stress could be due to the low allocation of carbon to the growth, suggesting a trade-off between growth and defense (Turtola et al. 2003).

The main constituent of EOs in all the studied plants was Camphor (an oxygenated monoterpene). This constituent strongly increased under 50% CC (36.5, 62.8, 38.7% in full irrigation, half irrigation and no irrigation, respectively). Our results showed a chemical profile composed by Camphor > Caryophyllene oxide > α-Cadinol in 100% CC; Camphor > β-Caryophyllene > Borneol in 50% CC; and by Camphor > Caryophyllene oxide > β-Caryophyllene in 0% CC. Abreu et al. (2008) reported interesting antioxidant properties of *S. sinaloensis* due to the presence of phenolic diterpenes in comparison with *S. officinalis* extracts. In the present study, moderate drought stress application induced an increase in oxygenated monoterpenes that are generally the most active components in many EOs (Ruberto and Baratta 2000; Imanshahidi and Hosseinzadeh 2006), such as in *S. cinnabarina* L. with hypotensive effect (Alfieri et al. 2007), *S. dolomitica* with anti-bacterial activity (Kamatou et al. 2007) and in *S. officinalis* with antifungal and anti-inflammatory activity on mammalian cells (Abu-Darwish et al. 2013). These results may contribute to a good utilization of *S. sinaloensis* EOs for plant breeding and pharmaceutical applications.

Conclusions

Drought is one of the abiotic stresses that most severely affects plant growth and development. Consequently, plants adjust their structure, metabolism and function to withstand it. Generally, it is characterized by reduction of water content and leaf water potential, turgor loss, closure of stomata and decrease in cell enlargement and growth. However, with respect to MAPs, the water shortage can lead to an enhancement of the content of PSMs, Our results showed that in *S. sinaloensis* drought affects morphological, ecophysiological and chemical traits depending on the stress severity. Plant growth parameters and chlorophyll were generally reduced but the photosynthetic apparatus demonstrated to tolerate moderate to severe drought conditions, without a complete stomatal closure. Interestingly, under moderate stressed conditions, monoterpene hydrocarbons increased in both BVOCs and Eos (e.g. camphene), while volatile oxygenated monoterpenes increased still with increasing stress severity (e.g. camphor). Thus, drought stress did not inhibit per se the biosynthesis of monoterpenes, even better the highest BVOCs and EOs contents were obtained in moderate drought stress condition. Furthermore, this stress condition induced a mid to late increase in phenols, flavonoids and antioxidant activity, suggesting that active molecule accumulation is associated with drought tolerance.

In conclusion, here an integrated approach, combining metabolomic and physiological studies, allowed us to get new insights in mechanisms and processes involved in *S. sinaloensis* adaptation to drought stress and PSMs production. Coupling this information, breeders and industries may optimize MAPs breeding and cultivation, in order to produce high quality plant materials adopting sustainable cultivation techniques.

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Tables

Table 1

Aerial and root dry mass accumulation and root:aerial (R:A) ratio of *Salvia sinaloensis* plants under full irrigation (100% container capacity, CC), half irrigation (50% CC), or no irrigation (0% CC). In brackets are reported the percentage variations referred to controls.

Water regime	Dry mass accumulation (gDW/plant)			R:A ratio
	Total	Aerial part	Root	
100% CC	3.57a (100%)	1.06a (100%)	2.51a (100%)	2.37b (100%)
50% CC	3.75a (105%)	0.88ab (83%)	2.87a (114%)	3.26a (137%)
0% CC	1.59b (44%)	0.77b (73%)	0.82b (33%)	1.06c (45%)
P	**	*	**	*

The statistical relevance of 'Between-Subjects Effects' tests (*=P<0.05, **=P<0.001, ns=not significant). Mean values showing the same letter are not statistically different at P≤0.05 according to the REGW-F test.

Table 2

Total amount (mg/plantFW) of phenols, flavonoids and antioxidant activity at the end of the experiment in *Salvia sinaloensis* plants under full irrigation (100% container capacity, CC), half irrigation (50% CC), or no irrigation (0% CC). In brackets are reported the percentage variations referred to controls.

Water regime	Total amount (mg/plantFW)		
	Total phenols	Flavonoids	Antioxidant activity
100% CC	16.44 b (100%)	6.42 a (100%)	106.37 b (100%)
50% CC	22.85 a (139%)	6.47 a (101%)	125.98 a (118%)
0% CC	10.43 c (63%)	2.73 b (43%)	20.86 c (20%)
P	**	*	*

The statistical relevance of 'Between-Subjects Effects' tests (*=P<0.05, **=P<0.001, ns=not significant). Mean values showing the same letter are not statistically different at P≤0.05 according to the REGW-F test.

Figure captions

Fig. 1 Dynamics of leaf water potential (LWP) of *Salvia sinaloensis* plants treated with full irrigation (100% container capacity, CC - black line), half irrigation (50% CC - dark grey line), or no irrigation (0% CC - light grey line). Means superscripted by the same letter do not differ significantly, according to REGW-F post-hoc test (NS = non significant).

Fig. 2 Gas exchange (internal CO_2 concentration, C_i - A; transpiration rate, E - B; stomatal conductance, g_s - C; net photosynthetic rate, A - D) and dynamic of the instantaneous water use efficiency (WUE, A/E - E) measured on *Salvia sinaloensis* plants treated with full irrigation (100% container capacity, 100% CC - black line), half irrigation (50% CC - dark grey line), or 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 (ns=non significant, *=$P<0.05$) was evaluated.

Fig. 3. Average values of growth index (G.I., cm^3) during the experiment. *S. sinaloensis* plants were treated with three irrigation regimes: full irrigation (100% container capacity, 100% CC, black line), half irrigation (50% CC, dark grey line), or no irrigation (0% CC, light grey line). Means superscripted by the same letter do not differ significantly, according to REGW-F test (NS = non significant; * $P<0.05$; ** $P<0.001$).

Fig. 4 Leaf total phenols (A) and flavonoids (B), and antioxidant activity (C) of *Salvia sinaloensis* plants under control condition (black line, 100% container capacity, CC), moderate drought stress (dark grey line, 50% CC), and severe drought stress (light grey, 0 % CC). Means superscripted by the same letter do not differ significantly, according to REGW-F test (NS = not significant; * $P<0.05$; ** $P<0.001$).

Ecophysiological and phytochemical responses of *Salvia sinaloensis* Fern. to drought stress

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Supplementary files

Supplementary Table 1. Chlorophyll (*a* + *b*), total carotenoid (Car) and their rate (Chl:Car) measured on *Salvia sinaloensis* plants under full irrigation (100% container capacity, CC), half irrigation (50% CC), and no irrigation (0% CC).

		Days										
		0	4	7	11	14	18	21	25	28	32	34
Chl *a* + *b* (mg/g FW)	100% CC	1.40	1.49	1.43	1.38	1.35	1.50a	1.32a	1.36	1.42	1.43	1.45
	50% CC	1.40	1.43	1.25	1.27	1.17	1.15b	1.15b	1.16	1.17	1.24	1.30
	0% CC	1.40	1.39	1.44	1.42	1.38	1.13b	1.07b	-	-	-	-
	P	ns	ns	ns	ns	ns	*	*	*	*	*	*
Car (mg/g FW)	100% CC	0.23	0.29	0.24	0.25	0.25	0.21	0.26	0.24	0.26	0.23	0.26
	50% CC	0.23	0.22	0.21	0.24	0.20	0.16	0.18	0.19	0.24	0.24	0.23
	0% CC	0.23	0.25	0.24	0.23	0.24	0.17	0.19	-	-	-	-
	P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Chl:Car	100% CC	6.20	5.21	5.92	5.51	5.43	7.07	5.17	5.63	5.41	6.12	5.61
	50% CC	6.20	6.54	5.86	5.24	5.97	7.35	6.45	6.25	4.97	5.15	5.76
	0% CC	6.20	5.45	5.96	6.16	5.89	6.48	5.63	-	-	-	-
	P	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

The statistical relevance of 'Between-Subjects Effects' tests (*=$P<0.05$, ns=not significant). Mean values showing the same letter are not statistically different at $P \leq 0.05$ according to the REGW-F test.

Supplementary Table 2. Chemical composition (%) of volatiles emitted from *Salvia sinaloensis* plants after full irrigation (100% CC), half irrigation (50% CC) or no irrigation (0% CC). All constituents are ordered on the basis of their linear retention index (L*RI*).

Category	**Constituents**	**l*RI***	**100% CC**	**50% CC**	**0% CC**
mh	α-Pinene	939	4.7	6.5	2.0
mh	Camphene	953	2.6	6.6	2.6
mh	β-Pinene	980	12.0	16.7	7.5
mh	Myrcene	991	0.0	0.5	1.9
mh	Limonene	1031	4.4	3.3	2.3
mh	(*Z*)-β-Ocimene	1040	0.0	1.9	0.5
mh	(*E*)-β-Ocimene	1050	0.0	0.9	4.6
mh	Terpinolene	1088	0.5	0.7	0.6
om	*Trans*-sabinene hydrate	1097	0.0	0.5	0.7
om	Linalool	1098	0.0	0.0	0.6
nt	1-octen-3-yl-acetate	1110	0.0	0.1	4.3
om	α-Campholenal	1125	0.0	0.0	0.4
om	Camphor	1143	8.3	12.9	20.2

om	Borneol	1165	1.4	2.2	0.7
om	α-Terpineol	1189	0.0	0.5	0.7
nt	(*N*)-decanale	1204	0.0	0.1	0.6
om	Isobornyl acetate	1285	0.0	0.1	0.7
sh	δ-Elemene	1339	1.4	0.6	0.2
sh	α-Copaene	1376	2.0	1.0	0.7
ac-13	(E)-α-damascenone	1380	0.7	0.3	1.0
sh	β-Elemene	1391	1.7	1.7	0.9
sh	Cyperene	1398	0.6	0.5	0.6
sh	β-Caryophyllene	1418	16.0	12.4	24.2
sh	β-Copaene	1429	1.1	1.1	0.3
sh	β-Gurjunene	1432	0.0	0.5	0.4
sh	α-Guaiene	1439	1.7	0.8	0.3
sh	α-Humulene	1454	3.9	3.5	6.5
sh	(*E*)-β-Farnesene	1458	1.3	2.7	1.0
sh	α-Himachalene	1476	0.0	0.4	0.1
sh	γ-Muurolene	1477	0.0	0.0	0.2
sh	Germacrene-D	1480	22.0	12.1	5.0
sh	α-Selinene	1485	1.5	2.5	0.8
sh	Bicyclogermacrene	1494	4.3	1.4	0.2
sh	Germacrene-A	1503	0.0	0.3	0.2
sh	7-epi-α-selinene	1517	0.0	1.3	0.7
sh	δ-Cadinene	1524	0.0	0.0	0.4
so	Caryophyllene oxide	1581	0.0	0.0	0.5
so	α-Cadinol	1653	0.0	0.0	0.3
	Total		**92.1**	**96.6**	**95.4**
	Non Terpene Derivates (nt, %)		**0.0**	**0.2**	**4.9**
	Monoterpene Hydrocarbons (mh, %)		**24.2**	**37.1**	**22.0**
	Oxygenated Monoterpene (om, %)		**9.7**	**16.2**	**23.6**
	Sesquiterpene Hydrocarbons (sh, %)		**57.5**	**42.8**	**42.7**
	Oxygenated Sesquiterpene (os, %)		**0.0**	**0.0**	**0.8**
	Apocarotenoids (ac, %)		**0.7**	**0.3**	**1.0**

*****All the constituents identified belong to non terpene derivates (nt), monoterpene hydrocarbons (mh), oxygenated monoterpene (om), sesquiterpene hydrocarbons (sh), oxygenated sesquiterpene (os), and apocarotenoids (ac).

Supplementary Table 3. Effect of full irrigation (100% CC), half irrigation (50% CC) or no irrigation (0% CC) on the essential oil constituents of *Salvia sinaloensis* plants. Constituents are ordered on the basis of their linear retention index (LRI).

Category *	**Constituents**	**l*RI***	**100% CC**	**50% CC**	**0% CC**
nt	Hexenal	800	0.2	1.0	0.0

nt	(*E*)-3-hexen-1-ol	851	0.2	0.5	0.0
mh	α-Pinene	939	0.0	0.5	0.1
mh	Camphene	953	0.6	3.6	0.5
mh	Sabinene	976	0.4	1.2	1.0
nt	3-Octanone	988	0.0	0.2	0.0
mh	Myrcene	991	0.0	0.3	0.1
nt	3-Octanol	993	0.0	0.0	0.1
mh	Limonene	1031	0.0	0.1	0.1
om	1,8-Cineole	1033	0.7	0.2	0.1
mh	γ-Terpinene	1062	0.2	0.2	0.0
om	*Cis*-sabinene hydrate	1068	0.0	0.0	0.4
nt	(*N*)-octanol	1070	0.0	0.0	0.0
om	*Trans*-linalol oxide (furanoid)	1074	0.2	0.2	0.2
om-no	Camphenilone	1083	0.2	0.3	0.2
mh	Terpinolene	1088	0.2	0.0	0.0
om	Linalool	1098	0.2	0.2	0.7
nt	1-octen-3-yl acetate	1110	0.5	1.8	1.8
om	α-Campholenal	1125	0.3	0.4	0.9
om	*Trans*-pinocarveol	1142	0.6	1.4	1.7
om	Camphor	1143	36.5	62.8	38.7
om	Isoborneol	1156	0.0	0.0	0.2
om	Pinocarvone	1162	0.5	0.8	1.1
om	Borneol	1165	7.5	4.7	5.1
om	4-Terpineol	1177	1.3	1.6	1.0
om	Cymen8-ol-para	1187	0.2	0.0	0.2
om	α-Terpineol	1189	0.5	0.7	0.6
om	Myrtenal	1193	1.4	1.5	2.1
om	Verbenone	1217	0.0	0.1	0.2
om	*Trans*-carveol	1217	0.0	0.0	0.2
om	Isobornyl formate	1233	0.6	0.2	0.3
om	Carvone	1242	0.0	0.0	0.2
om	Perilla aldehyde	1271	0.0	0.0	0.1
om	Isobornyl acetate	1285	1.0	1.0	0.9
nt	2-undecanone	1296	0.1	0.0	0.0
nt	(*N*)-tridecene	1292	0.1	0.0	0.0
om	α-Terpenyl acetate	1352	0.0	1.1	0.0
nt	Undecanol-N	1374	0.3	0.0	0.1
sh	β-Bourbonene	1384	0.3	0.3	0.0
ac-13	(*E*)-β-damascenone	1380	0.3	0.0	0.2
sh	Cyperene	1398	0.2	0.0	0.1
sh	α-Gurujene	1409	0.0	0.0	0.9

sh	β-Caryophyllene	1418	1.5	5.7	8.3
sh	β-Gurjunene	1432	0.5	0.0	0.0
sh	α-Guaiene	1439	0.5	0.0	0.0
sh	Aromadendrene	1439	0.3	0.0	0.9
sh	α-Humulene	1454	0.2	0.0	0.1
ac-12	Geranylacetone	1453	0.0	0.0	0.2
sh	(*E*)-β-farnesene	1458	0.0	0.0	0.2
sh	γ-Himachalene	1476	0.2	0.0	0.0
sh	Germacrene-D	1480	0.0	0.3	0.0
sh	β-Selinene	1485	0.8	0.0	0.6
sh	*Cis*-β-guaiene	1490	0.2	0.0	0.1
sh	Bicyclogermacrene	1494	0.4	0.0	0.0
sh	Cuparene	1502	1.2	0.3	0.2
sh	7-epi-α-selinene	1517	1.0	0.2	0.5
sh	δ-Cadinene	1524	1.3	0.1	0.3
sh	α-Calacorene	1542	0.3	0.0	0.0
sh	Selina-3,7(11) diene	1542	0.0	0.0	0.2
os	Elemol	1549	0.3	0.2	0.8
os	Longicamphenylone	1559	0.3	0.4	0.2
os	*Trans*-nerolidol	1564	0.4	0.0	0.2
os	Spathulenol	1576	1.9	0.5	1.6
os	Caryophyllene oxide	1581	9.8	3.0	10.4
os	*Cis*-β-elemenone	1594	0.4	0.1	0.2
os	Humulene oxide	1606	0.8	0.3	1.3
os	1,10-di-epi-cubenol	1614	1.5	0.3	1.0
os	α-Acorenol/g-Eudesmol	1630	0.6	0.0	0.7
os	β-Acorenol	1634	0.3	0.0	0.1
os	Epi-α-cadinol	1640	0.8	0.0	0.3
os	β-Eudesmol	1649	0.2	0.0	0.2
os	α-Cadinol	1653	9.1	1.4	6.2
os	14-hydroxy-9-epi-E-caryophyllene	1664	1.6	0.0	1.6
os	*Cis*-14-nor-muurolen 5-en 4-one	1682	0.9	0.1	1.4
os	(*E*,*E*)-Farnesol	1722	0.2	0.0	0.2
os	14-oxy-a-muurolene-	1764	0.2	0.0	0.2
os	(*Z*)-α-santalol acetate	1786	0.0	0.0	0.4
nt	(*N*)-octadecane	1800	1.5	0.0	0.1
	Total		**94.0**	**99.6**	**98.8**
	Non Terpene Derivates (nt,%)		**2.8**	**3.5**	**2.2**
	Monoterpene Hydrocarbons (mh, %)		**1.4**	**6.0**	**1.9**
	Oxygenated Monoterpene (om, %)		**51.5**	**77.1**	**54.9**
	Sesquiterpene Hydrocarbons (sh, %)		**8.8**	**6.8**	**12.4**

Oxygenated Sesquiterpene (os, %)	**29.2**	**6.3**	**26.9**
Apocarotenoids (ac,%)	**0.3**	**0.0**	**0.4**

*All the constituents belong to non terpene derivates (nt), monoterpene hydrocarbons (mh), oxygenated monoterpene (om), sesquiterpene hydrocarbons (sh), oxygenated sesquiterpene (os), and apocarotenoids (ac).