Drought stress modifies early effective resistance and induced chemical defences of
Aleppo pine against a chewing insect herbivore

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Supplementary material:

Figure S1. Aleppo pine natural distribution range, origin of the pine populations
included in this study and location of the Centro de Investigación y Tecnología
Agroalimentaria (CITA, Zaragoza, Spain) where the experiment was set-up. Mean
annual temperature (T) and annual precipitation (P) is shown in the companion table.
Figure S2. Details of the experimental set-up showing (a) an Aleppo pine seedling confined inside the acrylic transparent cylinders fitted to the pot and covered by a gauze; (b) the pine weevil, *Hylobius abietis*, our model insect used for the herbivory treatment; (c) the damage caused by the bark beetle on the stem; and (d) the calibrated template used to measure the debarked area along the stem.

Table S1. Summary of the chemical species identified by GC-FID in the hexane extracts. Name, concentration, relative abundance of each compound in relation to their chemical group, proportion of presence in the 90 composite stem samples analysed, retention time and Kovat Index are shown.

Table S2. Summary of the mixed models for the direct effects of drought stress (D), herbivory (HERB) and population (POP) and the interactive effect of $D \times HERB$ on the concentration of the 17 selected terpenes extracted from the composite stem samples of one year-old Aleppo pine.
Abstract

During their long lifespan, pines must cope with simultaneous abiotic and biotic stresses such as drought and herbivory. Mediterranean pines are isohydric species that rapidly close their stomata in response to drought reducing carbon fixation. In such situation, the synthesis of chemical defences could be impaired. Here, we tested the hypothesis that drought stress may constrain the capability of Mediterranean pines to defend against herbivory and to induce chemical defences. For this purpose, we subjected three contrasting populations of Aleppo pine (*Pinus halepensis* Mill.) to three levels of drought stress, thereafter exposing the seedlings to the herbivore *Hylobius abietis* L. A suite of ecophysiological and defensive traits was measured to explore the interaction between both stresses.

Drought significantly affected the $^{13}$C signature and reduced starch and fatty acids concentration. Damage caused by the insect was affected by drought stress, being 75% higher at the moderate stress level but returning under severe stress to similar values as control seedlings.

Pine seedlings responded to herbivory by decreasing the concentration of total polyphenolics and condensed tannins, increasing the concentration of total diterpenes, and modifying the profile of major terpenes. Induced responses to herbivory were, as expected, altered by drought. Inducibility of polyphenolics decreased as drought stress increased while for diterpenes it was higher at moderate stress. Moreover, a significant drought × herbivory interaction was found on the multivariate terpene profile. Results should be considered for predicting responses of pine forests to the forecasted increase of abiotic and biotic risks associated to global change.

**Key Words:** carbon economy, chemical defences, drought stress, *Hylobius abietis*, pest resistance, *Pinus halepensis*. 
Introduction

Pines are large and long-lived organisms that must cope with a mixture of biotic and abiotic stressors along their lifespan (Hampe and Petit, 2005). These events are temporally and spatially variable and may cause conflicting selective pressures. Although the array of biotic and abiotic pressures is present along every life stage of the pine ontogeny, early seedling stages are particularly susceptible to them (Frei et al., 2018; Thanos, 2000). In particular, early drought events and the pressure exerted by insect herbivores stand out as major threats in temperate forest ecosystems (Anderegg et al., 2015). Each of these threats has a deep impact on survival and population persistence by itself (Klos et al., 2009; Myers and Sarfraz, 2017; Stephens and Westoby, 2015), but in combination they may have a greater effect on seedling performance and survival. Limited resistance to the combined action of such abiotic and biotic factors (drought and herbivory) could have strong consequences in the Mediterranean basin and in other areas with Mediterranean climate around the globe, where models forecast rising temperatures, reduced rainfall and increased probability of insect outbreaks as the result of global change (Granda et al., 2013; Hódar et al., 2003; Hódar and Zamora, 2004).

Mediterranean pines are paradigmatic isohydric species that rapidly close their stomata in response to water deficit (Baquedano and Castillo, 2006; Klein et al., 2013; Tardieu and Simonneau, 1998). Regulating stomatal conductance under drought conditions prevents massive losses of water and maintains near-constant leaf water potentials, increasing water-use efficiency (Attia et al., 2015). As a side effect, this strategy increases stomatal and mesophyll resistance to carbon dioxide diffusion and reduces photosynthesis, which ultimately limits the available carbon required for the synthesis of structural and non-structural organic compounds if drought stress is long-
lasting (Fitter and Hay, 2012; Flexas et al., 2004). Due to these resource-derived conflicts, carbon allocation to plant secondary metabolites providing resistance against pests and pathogens may be constrained, leading to reduced defensive capabilities and impairing effective resistance against herbivores (Gaylord et al., 2013; Klutsch et al., 2017). Besides the potential medium- and long-term consequences of a limited carbon budget, more direct, short-term and interactive effects of drought stress on herbivore resistance could derive from the lack of carbon resources for boosting induced chemical defences in response to herbivore damage (Suárez-Vidal et al., 2017). Similarly, many other molecules (transcription factors, proteins, hormones and other messengers) involved in herbivore-damage recognition and defensive signalling pathways are potentially subjected to crosstalk and interference with those involved in tolerance to drought stress (Fujita et al., 2006; Nguyen et al., 2016).

Here we hypothesize that, as a result of acclimation to a short event of drought stress during early development, (i) the resistance of a model isohydric Mediterranean pine tree to a chewing insect herbivore could be functionally impaired; (ii) drought stress may constrain the plant’s ability to produce induced chemical defences in response to damage; and (iii) effects of drought stress on resistance would differ among populations adapted to diverse water availability regimes. To test these hypotheses, we subjected Aleppo pine (Pinus halepensis Mill.) seedlings from three populations with contrasting climates and differing in drought tolerance to three levels of drought stress. Aleppo pine is a fast-growing, first-colonizer forest conifer native to the Mediterranean basin. It is considered a drought-avoiding and water-saving species with high water-use efficiency and fast stomatal closure under drought stress (Borghetti et al., 1998). There is strong evidence of local intraespecific adaptation to drought stress, with populations from drier areas showing more conservative water-use strategies than their counterparts.
from wetter environments, resulting in a strong genetic differentiation among populations in drought tolerance traits (Voltas et al., 2008; Klein et al., 2013).

After four months of differential watering treatments, seedlings were exposed to real herbivory during four days by the pine weevil *Hylobius abietis* L. (Coleoptera, Curculionidae). *Hylobius abietis* is considered one of the most important forest pests in Europe, feeding on the bark and phloem of young pine seedlings and causing large mortalities in pine plantations and forest regeneration (Day et al., 2004). Feeding by this insect has been shown to elicit induced chemical defences in several pine species (López-Goldar et al., 2016; Lundborg et al., 2016; Moreira et al., 2013; Suárez-Vidal et al., 2017; Zas et al., 2014) but no previous work has explored the interaction between pine weevils and Aleppo pine. Weevil damage (as an inverse proxy of plant resistance), carbohydrates and fatty acids concentration (indicators of carbon economy), and chemical defences (in control and herbivore-exposed plants) were measured to analyze how drought stress interfere with mounting effective induced defences at the early stages of development of this Mediterranean pine species.

**Material and methods**

**Plant material and Experimental design**

Seeds from three Spanish Aleppo pine populations of contrasting climate and different ecophysiological and growth performance (Voltas et al. 2008) were sown and grown under semi-natural conditions. The populations originated from Benamaurel (south-eastern Spain) (high intrinsic water-use efficiency [WUEi] and low growth rate), Benicàssim (eastern Spain) (intermediate WUEi and high growth rate) and Cabanelles (north-eastern Spain) (low WUEi and high growth rate) (Fig S1). One year after sowing, seedlings were subjected to three levels of drought stress (control, moderate and severe;
see next section) following a factorial design with four blocks, with drought stress (3 levels) and population (3 levels) as the main factors, and 10 replicates per treatment combination and block (i.e. experimental unit), leading to a total of 360 seedlings (3 treatments × 3 populations × 4 blocks × 10 replicates). One block was harvested for determining plant water potential, carbon isotope composition ($\delta^{13}C$) in recently fixed sugars and the concentration of non-structural carbohydrates as proxies of the effects of drought stress on water-use efficiency and carbon economy. The remaining plant material (3 blocks = 270 plants) was exposed to real herbivory by the pine weevil *Hylobius abietis* during four days (n = 135) or kept as control (n = 135). All seedlings were then harvested and sampled for measuring insect damage and the concentration of chemical defences (total polyphenolics, condensed tannins, mono-, sesqui- and diterpenes) in control (constitutive) and herbivore-exposed (induced) plants.

Growing conditions and drought stress treatments

Seeds were provided by CIFOR-INIA (Madrid, Spain) and sown in 2 L pots filled with a mixture 1:1 (v:v) of peat (Humin substrate N3, NEUHAUS, Belgium) and river sand enriched with NPK (14:16:18) 1.3 kg m$^{-3}$. The substrate mix was covered with a 1 cm layer of sand on which seeds were gently buried. Plants were grown in a shade-house (70% shade) under natural light and temperature conditions at CITA (Centro de Investigación y Tecnología Agroalimentaria, Zaragoza, Spain, Fig S1), and, during the first growing season, they were gently watered every week up to field capacity. Air temperature and relative moisture during this phase averaged 23.9 ± 0.1 ºC and 63.7 ± 23.9 % respectively (mean ± S. E.). When seedlings were one-year-old (July 2016), they were subjected to three drought stress treatments by periodically adjusting the watering for maintaining different amounts of water in the substrate: 70-100%, 45-
60% and 15-40% (in terms of weight basis) of field capacity in the control, moderate and severe drought stress treatments, respectively. One and a half month after the start of the treatments, plants in the drought treatments began to show alarming wilt symptoms (loss of needle turgid, needle desiccation and yellowing). To avoid seedling mortality, plants of these treatments were re-watered and maintained at around 50-70% of field capacity for another 45 days. Thereafter, during the last month prior to the insect bioassays and sampling, plants were submitted again to the three drought stress treatments, slightly adjusting the stress levels to avoid further wilting and mortality: 80-90%, 35-50% and 25-35% of field capacity in the control, moderate and severe drought stress treatments, respectively.

**Herbivory treatment**

Adult pine weevils were captured during early summer 2016 in a recently clear-felled mixed pine forest (Pontevedra, Spain, 42°19’34’’N, 8°26’18’’W) using Norlander traps baited with ethanol and turpentine as in Moreira et al. (2008). Then, they were separated by sex, maintained in moist culture chambers at 10 °C and fed weekly with fresh pine twigs as in Suárez-Vidal et al. (2017). For the bioassay, plants were confined inside acrylic transparent cylinders fitted to the pot and covered by a gauze as in López-Goldar et al. (2016) (Fig S2a). One randomly selected male and female, previously starved for 24 h, were weighted and confined inside the cylinders of half of the plants, randomly allotted (Fig S2b). Another half of the plants remained non-inoculated and worked as herbivory controls. After 4 days of weevil feeding, the cylinders were removed and the weevils recovered.

**Plant sampling and measurements**
Weevil feeding damage in the weevil-exposed pines (Fig S2c) was immediately measured as the debarked area along the stem with a calibrated template (López-Goldar et al., 2016) (Fig S2d). Thereafter, the plants were harvested by cutting the shoot just above the root collar. Needles stem and roots were gently separated and immediately deep frozen in liquid nitrogen and preserved at -80ºC. A total of 270 plants were harvested at this moment.

Chemical analyses

As a proxy of the impact of the drought stress treatments applied to the pine seedlings prior to the insect bioassay, we analysed $\delta^{13}$C, water potential and non-structural carbohydrates in one of the four blocks of the experimental design. $\delta^{13}$C was analysed in the soluble sugar fraction of the needles, following Offermann et al. (2011). Briefly, 1.5 ml of deionised water was added to 75 mg of grinded freeze-dried needles and shacked for 45 minutes at 4ºC, then subjected to a short hot extraction in a water bath (10 min at 100ºC) and cooled to 4ºC and centrifuged (10 min at 14000 rpm at 4 ºC; Eppendorf Centrifuge 5810 RE, Germany). About 0.5 ml of polivinylpolypyrrolidone (Across Organics ref # 227545000) was added to remove phenolic compounds, shacked and centrifuged as above. Then, 35 µl of the supernatant was pipetted into tin cups (ref # 176.9809.26, LÜDISWISS, Switzerland) and oven dried till solvent evaporation (4 hours at 60ºC). The water-soluble organic carbon residue, mostly representative of recently fixed sugars (Offermann et al., 2011), was analyzed for stable carbon isotopes by an elemental analyzer EA1108 (Carlo Erba Instruments, USA) coupled to a GC-IRMS (MAT 253) with an interface Conflo III (ThermoFinnigan, USA) at the Research Support Service (SAI, www.sai.udc.es) of University of Coruña (A Coruña, Spain).
Five individual plants per population × drought stress combination were used for this analysis.

Plant water status was also measured prior to the insect bioassay, by monitoring midday water potential with a Scholander Chamber (PMS instruments, Maximum Operating Pressure 100 bar, USA) in the apical section of the stems. Four plants per population and drought stress treatment were destructively harvested for this determination.

The concentration of non-structural carbohydrates in seedlings was also determined before the insect bioassay following Buysse and Merckx (1993). Briefly, a subsample (ca. 50 mg) of freeze-dried coarse root tissues was extracted with aqueous ethanol (80%, v/v), incubated at 60 °C during 30 min and centrifuged in a bench centrifuge (Eppendorf Centrifuge 5810R, Germany) at 3180 rpm for 10 minutes. The concentration of soluble sugars in the supernatant was determined colorimetrically at 490 nm by the phenol-sulphuric method of Dubois et al. (1956) modified by Buysse and Merckx (1993) in a microplate reader (Spectra MR Dynex Technologies, USA) using glucose as standard (SIGMA, ref # G8270-100g). The pellet after the above extraction was re-suspended in 4 ml of sodium acetate buffer in a hot bath (100 °C for 60 minutes), and digested enzymatically with amyloglucosidase (0.5% in sodium acetate buffer; SIGMA, ref # 10115-5g-F) to release glucose as described in Palacio et al. (2007). The concentration of soluble sugars in the solution resulting from starch breakdown was also determined colorimetrically. Five seedlings per population × drought stress combination were used for this analysis.

Analyses of the concentration of plant secondary metabolites as a proxy of investment in plant defence were performed in both control (constitutive defences) and herbivore-exposed plants (induced defences) immediately after the insect bioassay. To
achieve enough plant material for the multiple analyses and to maintain a contained sample size, composite samples were prepared by pooling three seedlings per population × drought stress × herbivory treatment combination across blocks.

The concentration of total phenolics and condensed tannins in the stem was determined in these composite samples as in Moreira et al. (2009) with slight modifications. Briefly, 20 mg of finely grounded freeze-dried samples were extracted with 1 ml of aqueous methanol (1:1, v:v; HPLC grade, HiperSolv Chromanorm) in 1.1 ml reaction tubes (VWR, Microtiler ref # T100-25). The tubes were vortexed, sonicated during 15 min, centrifuged at 3500 rpm during 20 minutes (Eppendorf Centrifuge 5804, Germany) and the supernatant saved. A diluted aliquot was allowed to react with Folin reagent (ref # 1.09001.0500, MERCK, Germany) and sodium carbonate (ref # 131648.1210, PANREAC, Germany) for 2.5 h and absorbance measured at 740 nm in a microplate reader (680 Microplate Reader, Biorad, USA). Concentration of total phenolics was estimated using tannic acid (PANREAC ref # 141065) as standard and expressed as tannic acid equivalents.

Condensed tannins were analysed in the same methanolic extract (Moreira et al., 2009). The methanolic extract was mixed with buthanol (VWR AnalR NORMAPUR, ref # 20810.323) and ferric ammonium sulphate solution (VWR Prolabo, ref # 24254.293) allowing reacting for 50 minutes, cooling them fast with ice. Absorbance was read at 550 nm in a microplate reader and concentration of condensed tannins estimated using quebracho extract as standard (Schinopsis balansae Engl; Droguería Moderna, Pontevedra, Spain) and expressed as quebracho tannins equivalent.

Concentration of mono-, sesqui- and diterpenes was determined in the stem of deeply frozen samples as in López-Goldar et al. (2018) with modifications. Briefly, 300 mg of fresh composite subsamples of pine stem tissues were extracted in 4 ml glass
extraction vials with 1000 μl of GC-grade hexane (VWR, Chromanorm, ref # 83992.320) using dodecane (MERCK, ref # 1.09658.0005) and pentadecane (Sigma, ref # 7610) at 100 μg ml⁻¹ as internal standards. After vortexing, sonication (20 min) and overnight extraction in darkness, the supernatant was transferred into 1.5 ml GC vials. A 150 μl aliquot of this hexane extract was used directly for the analysis of mono and sesquiterpenes. Resin acids and fatty acids in the extract were methylated before GC analysis. A second 150 μl aliquot of the hexane extract was dried under N₂, rediluted in HPLC-gradient grade methanol (VWR ref # 20864.320, HiPerSolv CHROMANORM) with heptadecanoic acid (Sigma-Aldrich ref # H3500) as internal standard, and methylated by adding tetramethylammonium hydroxide (Sigma-Aldrich ref # 334901; 1:10 in methanol, v:v). Separation and quantification was performed using a GC-FID Clarus 500 (Perkin Elmer, MA, USA) equipped with an Elite-5 capillary column (30 m, ID 0.25 mm, film thickness 0.25 μm, Perkin Elmer, MA, USA) coupled to a FID and using the Total Chrom Navigator Clarus 500 v6.3.2 software (Perkin Elmer, MA, USA). The FID temperature was set at 300ºC. A volume of 1 μl of each sample was injected in splitless mode, using hydrogen as the carrier gas. Instrument calibration and checking was done with the internal standards. For mono- and sesquiterpenes in the hexane extract, the oven temperature was set up at 40ºC for 2 min, followed by a first temperature rise of 4ºC × min⁻¹ up to 200ºC, then by a second temperature ramp of 10ºC × min⁻¹ up to 250ºC and maintained at this temperature for 5 minutes. For resin acids and fatty acids in the methylated methanolic extract, the oven was set at 152ºC for 2 min, followed by a temperature ramp of 3ºC × min⁻¹ up to 260ºC and maintained at this temperature 5 min.

Peak identification in the GC-FID was performed by comparing the retention times and Kovat Index, calculated upon commercial alkane series (Alkane Standard C8-
C20 Fluka ref # 04070, 40µg ml⁻¹), with the retention times and Kovat Index of the
compounds identified in previous studies by GC-MS (López-Goldar et al. 2018; Suárez-
Vidal et al., in preparation). The minimum detectable peak area was 1000 areas unit for
mono- and sesquiterpenes, and 5000 for diterpenes. A total of 28 monoterpenes, 22
sesquiterpenes and 7 diterpenes were found (Table S1). Only those known-compounds
with a relative concentration in their respective group (mono-, sesqui- or diterpenes)
greater than 1% were retained for the statistical analyses, resulting in a total of 17
terpenes.

Statistical analyses

For the analyses of the effects of drought stress on pine performance before the
insect bioassay (δ¹³C, water potential and non-structural carbohydrates concentration),

drought stress, population and their interaction were considered as fixed factors. The
variability in the resistance to the pine weevil across populations and drought stress
treatments was analysed using the damage caused by the insect (debarked area
expressed in absolute terms) as the dependent variable. In this case, the biomass of the
two weevils and the diameter of the plants were incorporated as covariates to the
aforementioned statistical model and blocks were included as a random factor.

For the analysis of the concentration of plant chemical defences, the effects of
drought stress, population and herbivore induction (and the interactions among each
other) were considered as fixed factors in the model. Block was missed in the models
because composite samples across blocks were used for these analyses. All analyses
were carried out fitting mixed models by REML using the Proc Mixed procedure of the
SAS System (Litell and Milliken, 2006).
If deemed necessary, normality was achieved by log or square root transformations of the dependent variable, and residual heterogeneity models across drought stress treatments were used when significant deviations were found. For the analysis of terpenes, and due to the high number of compounds found, we adjusted the p-values by the False Discovery Rate (FDR) for $p \leq 0.05$ with a threshold of $\alpha = 0.05$ in order to avoid false positives (Benjamini and Hochberg, 1995).

To study the multivariate effect of the experimental factors on the terpene profile, we summarize the information by multivariate analyses. A PCA was performed on the 17 most abundant compounds to reduce the information into two principal components, which were subjected to analysis using the same mixed model as for plant chemical defences. Besides, a MANOVA was carried out with the GLM procedure of SAS System using drought stress, herbivory and population as fixed factors.

Results

Experimental drought stress reduced plant water potential and increased $\delta^{13}C$

Drought stress decreased plant water potential proportionally to the intensity of the drought stress applied (Fig 1a). Drought stress also significantly affected the carbon isotopic signature of recently fixed carbon (Fig 1b). Control seedlings showed significantly higher discrimination against the heavy carbon isotope (i.e. lower $\delta^{13}C$) than those grown under moderate or severe drought stress, indicating a strong effect of water limitation on stomata closure and reduced carbon dioxide fixation. Neither differences among populations nor population × drought stress interaction were observed for water potential and $\delta^{13}C$ (Fig 1).

Drought stress consistently reduced carbon reserves
Drought stress significantly affected reserves storage, with seedlings growing under control and moderate drought stress showing a 30% greater starch concentration than those plants growing under severe drought stress conditions (Fig 2a). The concentration of fatty acids was also consistently affected by drought stress, with control plants showing 20% greater concentration than plants subjected to moderate and severe drought stress (Fig 2b). No significant effect of drought stress was observed for soluble sugars ($F_{2,78} = 0.9, \ p = 0.431$). Moreover, we found no direct or interactive effects of population on carbon storage traits (Fig 2).

Drought stress affected pine resistance to the pine weevil

Drought stress significantly affected the effective resistance to the pine weevil. This effect was not proportional to the watering regime, as the damage caused by the insect was 75% greater in moderate drought stress than in control and severe drought stress conditions (Fig 3). The debarked area did not differ among populations and the effect of drought on the damage was consistent across populations, with no interaction between population and drought stress (Fig 3).

Interactive effect of drought stress and herbivory on the allocation to chemical defences

Aleppo pine seedlings strongly responded to four days of weevil feeding, decreasing the concentration of total polyphenolics and condensed tannins, and increasing the concentration of total diterpenes (Table 1; Fig 4). For polyphenolics, the response to herbivory differed among water stress treatments (Table 1), with lower responses as drought stress increased (Fig 4a). Moreover, total polyphenolics and condensed tannins differed among populations (Table 1), being Benamaurel the population with the lowest concentration.
The concentration of total mono- and sesquiterpenes were not affected by direct and interactive effects among drought stress and herbivory (Table 1). For individual major mono- and sesquiterpenes, only α-pinene was affected by drought stress (higher concentrations under severe drought stress), while β-pinene was the only one that significantly responded to herbivory (higher concentration after herbivore damage) (Table S2). Concentration of total sesquiterpenes (Table 1) and most individual mono- and sesquiterpenes (Table S2) differed among populations, with Benamaurel and Benicàssim showing higher concentrations than Cabanelles (Table 1). In the case of diterpenes, herbivory significantly increased the concentration of total diterpenes (Table 1), and the specific concentration of dehydroabietic and abietic acid (Table S2). The response to herbivory interacted with the drought stress treatments for the total diterpenes concentration (Table 1; Fig 4c) and, specifically, for isopimaric and abietic acid (Table S2), with greater inducibility at moderate water stress.

The Principal Component Analysis summarized well the relative concentration of the 17 most abundant terpenes. The first principal component (PC1) explained 31.3% of the total variance, being positively related to β-pinene and the sesquiterpenes α-amorphene, δ-cadinene, β-gurjunene and α-copaene and negatively related to the monoterpenes δ-3-carene and α-terpinolene and the sesquiterpenes β-caryophyllene and α-humulene (Fig. 5a). PC2 explained 15.9% of the total variance and was related to a few key monoterpenes (positively with δ-3-carene and α-terpinolene and negatively with α-pinene) (Fig 5 a). PC1 discriminated well between control and herbivory-exposed plants (Fig 5b), meanwhile PC2 did it for terpenes that responded to drought stress, but segregation across these factors was not clear (Fig 5b).

The MANOVA analysis confirmed the interactive effect between drought and herbivory on the profile of terpenes. Besides the significant effects observed for each
individual factor, a significant interaction between drought stress and herbivory was also found (Table 2).

Discussion

This study provides evidences that short events of drought stress may compromise the resistance of Aleppo pine seedlings against chewing insects such as the pine weevil (*Hylobius abietis*). We showed that drought stress not only affected the effective resistance of the young pines to weevil herbivory, but also altered some of the patterns of induction of chemical defences in response to insect damage.

*Drought affected plant water status and C storage of young pines*

As expected, the drought stress imposed in our experiment reduced the water potential of the young pines. The water potential measured at severe drought stress was close to the value resulting in 99% stomatal closure (-2.8 MPa) reported for Aleppo pine saplings of same age (Borghetti et al., 1998; Klein et al., 2011; Michelozzi et al., 2011). The high sensitivity of stomata closure to water limitation in this species (Klein et al., 2011) was also reflected on the differences in $^{13}$C isotopic signature of the recently fixed carbon among treatments (Ferrio et al., 2003; Klein et al., 2005; Moreno-Gutiérrez et al., 2012). Under drought stress, isohydric species close their stomata and diffusion of carbon dioxide across stomata is impaired. Then plants fix the remaining carbon dioxide located in the subestomatic chamber, enriched in $^{13}$C relative to air CO$_2$ (Farquhar et al., 1982; Flexas et al., 2007), which is translated into a higher $^{13}$C signature under water stress conditions, which is assumed to reflect higher intrinsic water-use efficiency (Ferrio et al., 2003; Moreno-Gutiérrez et al., 2012). Soluble carbohydrates were depleted under drought stress in agreement with a hypothetical C limitation imposed by
stomata closure. This is a typical response of many woody species to water shortage (Hartmann et al., 2013; Mitchell et al., 2013; Klein et al., 2014), and is, likely, a consequence of the allocation of carbon reserves to basal physiological processes occurring during periods of negative carbon balance. The concentration of fatty acids also decreased under drought stress. An increase in lipolytic activities and a decrease in fatty acids has been reported for water stressed *A. thaliana* (e.g. Gigon et al., 2004), and a down-regulation of transcripts related with fatty acid biosynthesis in drought stressed Aleppo pines (Fox et al., 2018).

Moderate, but not severe drought, affected pine resistance against weevil herbivory

By measuring the debarked area as an inverse proxy of resistance, we were able to find clear evidences that water shortage had a strong effect on resistance, where plants under moderate drought were 1.5-times more damaged than those in the well-watered control treatment. However, we found that plants exposed to intense drought stress were less damaged than those in intermediate levels of stress. Our results are clear and stronger as we accounted as covariate the potential effect of plant stem diameter and weevil weight. Plant defense theory predicts that plant resistance to herbivory is expected to be plastic to resource availability (Cipollini et al., 2014; Endara and Coley, 2010, Gianoli and Salgado-Luarte, 2017). For example, the Growth Defense Balance Hypothesis (GDBH; Herms and Mattson, 1992; Stamp, 2003) predicts that moderate resource limitation may favor the allocation of carbon resources to defenses rather than to growth, but that severe growth-limiting conditions may reverse this pattern. For example, Gutbrodt et al. (2012) reported that apple plants at the moderate drought stress were more resistant to *Spodoptera littoralis* damage than those in the control and severe treatments. Our results show, however, the opposite pattern, with well-watered control
and intense drought stressed plants showing low damage and moderate stress showing
the highest susceptibility. The comparison of control and moderate stress agree with our
hypothesis; however, the behavior of the weevil in the severe drought treatment
disagrees with a progressive increase in susceptibility to drought stress due to a lack of
carbon resources. We could speculate here that the reduced weevil damage observed in
severe drought treatment may be related to a reduced palatability because of too low
water content in those plants, more than to greater allocation to chemical defences. We
found a pattern of slight but significant increasing concentrations of phenolics and
tannins, and also diterpenes, with drought stress. But the observed pattern is not likely
explaining the big difference in damage between moderate and severe treatments.
Because responses of biological interactions to resource availability are far to be lineal,
our results evidence that exploring the full range of abiotic gradients is advisable for
completely retrieving the outcome of plant-insect interactions.

Most studies about damage caused by different pest and pathogens in conifer
species agree that severe drought stress would affect negatively plant defensive
allocation, because as drought stress increases less photosynthates are available to
synthesize chemical defenses, and more damage is expected (Anderegg et al., 2015;
Devkota et al., 2018; Gao et al., 2017; Klutsch et al., 2017). Thus, terpene concentration
may be reduced when water availability is too low (Bertin and Staudt, 1996; Llusià and
Peñuelas, 1998; Klutsch et al., 2017). However, our results do not provide strong
evidences that water shortage is depleting allocation to carbon based defenses directly
due to carbon starvation imposed by stomata closure. We found greater constitutive
concentration of phenolics and tannins with increasing drought, greater concentration of
diterpenes in the severe drought plants, and a different terpene profile in drought
stressed plants (significant effect in PC1 and MANOVA). All together, these secondary
metabolites could provide certain increased level of resistance (see for instance Moreira et al., 2009; Sampedro et al., 2011; López-Goldar et al., 2018). However, all those changes will unlikely explain the differences observed in debarked area, as the differences in constitutive concentration of chemical defences among drought stress treatments were not that large. We could speculate that, besides chemical defences, other alternative traits would contribute to resistance (Carmona et al. 2011). For instance, plant nutrient content, structural defences as lignification or density of resin canals, and other plant features such as water content could directly drive insect feeding behavior and thus debarked area (Fedderwitz et al., 2015; Fedderwitz et al., 2016). All together these facts suggest that the effect of drought stress on biotic resistance to chewing insects may be species-specific and highly dependent on the specific experimental conditions, including the intensity and duration of both stresses, the ontogeny of the plants, etc. (Ramegowda and Senthil-Kuma, 2015), being difficult to generalize. Further research should deepen on the physiological mechanisms that may explain the strong observed effect of water stress on herbivory damage, exploring a wider range of water deficit.

Water stress constrained the inducibility of chemical defences

Aleppo pine seedlings responded to the biotic stress imposed by weevil damage with both quantitative and qualitative changes in the concentration of chemical compounds. The most relevant quantitative responses were the decrease in the concentration of phenolics and tannins, and the increase of that of total diterpenes (specifically, abietic and dehydroabietic acids) and the monoterpene β-pinene. Although no previous studies have analyzed the interaction between pine weevils and Aleppo pine to date, this pattern of defensive induction is consistent with those observed in other
pine species such as *P. pinaster* (López-Goldar et al., 2016; Suárez-Vidal et al., 2017; Zas et al., 2014), *P. radiata* (Moreira et al., 2013; Zas et al., 2011) or *P. sylvestris* (Kovalchuk et al., 2015; Lundborg et al., 2016). One goal of our experimental design was to test whether drought stress could constrain the pine defensive responses to herbivory. It is known that inducibility of chemical defences is context-dependent and that it may be constrained by nutrient availability (Sampedro et al., 2011; Moreira et al., 2015), but this point have been not tested yet for drought stress. Up to our knowledge, this is the only work studying constitutive and induced chemical defensive allocation in pine seedlings under drought and herbivory stress. The tool for testing this hypothesis is the analysis of the drought x herbivory interaction on the concentration of chemical defences. If significant, it would evidence a negative or positive effect of drought in the ability for mounting an induced defensive response to herbivory. Interestingly, as a main result of our work, we found a significant interference (negative interaction) of the drought stress for the concentration of total polyphenolics, total diterpenes and singular diterpenes such as isopimaric and abietic acid. We also found interactive effects of drought x herbivory on the multivariate space defined by terpenoid chemical defences. Across all these compounds of different chemical nature, the interference is consistent showing a constrained plasticity, that is, a reduction in the pine ability to change the concentration of chemical defences after herbivore attack. For phenolics, which decreased in concentration after the attack, it meant a smaller decrease. A decrease in concentration of phenolics and a rise in that of terpenoids in pine trees in response to this insect have been reported in previous studies (Moreira et al., 2013). In the case of diterpenes, responses to weevil damage were only significant under moderate water-limiting conditions. This could be explained by the fact that weevil damage was notably greater under this treatment, and that induced responses are known to be proportional to
the intensity of the biotic damage (López-Goldar et al. 2016). In fact, quantifying the
effect of experimental treatments on the responsiveness of plants to herbivory is
challenging as it is difficult to separate direct effects of the treatments from those
mediated by modifications of the weevil feeding patterns (Suárez-Vidal et al. 2017). In
any case, multivariate analysis also revealed qualitative changes in the profile of major
terpenes in response to herbivory across the different watering treatments, suggesting
that drought stress actively modulated the physiological machinery involved in the
production of induced chemical defenses. Overall, these results suggest that drought
may modify the ability of pine seedlings to defend against biotic threats, either by
alterations of the basal constitutive physiological state or by altering the ability of pine
seedlings to elicit induced defences. As induced defences may be crucial to effectively
defend against biotic threats (Sampedro et al., 2011), these alterations may have
unsettling consequences for simultaneously cope with long-lasting drought and
herbivory stresses, two rising disturbances associated to global change (Bansal et al.,
2013, Granda et al., 2013; Hódar et al., 2003; Hódar and Zamora, 2004). The lack in the
inducibility of diterpenes in the severe drought treatment could help to explain, in part,
the pattern observed for damage, although we cannot affirm that it was the cause.
Despite diterpenes are known to be strong deterrents of weevil damage (López-Goldar
et al. 2018), it seems difficult to assign a major role of this type of compounds in
explaining differences in debarked area, as the differences in diterpenes concentration
among watering treatments were not that large. However, the interference that we report
here deserves further research, irrespective of whether the interference of our drought
stress treatments was or not the functional cause of the observed differences in
resistance. Further research specifically designed to explore the origin of carbon sources
used for the synthesis of (constitutive and induced) defensive compounds (Guérard et
al., 2007) and the interactions between hormonal signaling (Nguyen et al., 2016) will help to determine to what extent the observed interactive patterns between drought and herbivory are mediated by carbon starvation or tradeoffs among different signaling pathways.

Lack of variation in plasticity across provenances

Strong population differentiation in several life-history related traits and their plastic responses has been reported for Aleppo pine, including growth (Voltas et al., 2018), reproductive effort and early development (Santos del Blanco et al., 2010), and water-use efficiency (Voltas et al., 2008). We found differences among populations in the concentration of chemical compounds, with populations from the driest areas (Benamaurel and Benicàssim) showing lower concentrations of total polyphenolics and condensed tannins and higher of sesquiterpenes. However, none of these patterns of variation contributed to explain the variation in resistance across water stress treatments. The populations that we selected for our experimental design have been reported as extreme behavior within the distribution range of the species in the Iberian Peninsula (Benamaurel from south-eastern Spain with high intrinsic water-use efficiency [WUEi] and low growth rate; Benicàssim from eastern Spain with an intermediate WUEi and high growth rate; and Cabanelles from north-eastern Spain with a low WUEi and high growth rate) (Voltas et al. 2008). According to life history theory, we could expect that differentiation in water-use efficiency could covariate with carbon fixation, carbon storage and defensive investment, and subsequently in the plasticity of the response against herbivory, our third hypothesis. Such variation in plasticity would appear as a significant drought x herbivory x population interaction. Although we found differentiation among populations in some traits, we did not find evidences supporting
our hypothesis of variation in plasticity across populations. Recently, a comprehensive study has reported variation in resistance against *H. abietis* among populations in *Pinus pinaster* (López-Goldar et al. 2018). Those authors found differences in constitutive defences but not in their inducibility among populations (López-Goldar et al. 2018). The pattern of variation in resistance and plant secondary metabolites in *P. halepensis* deserves further research, perhaps examining a larger number of populations. Similarly, particular differentiation among populations not accounted for in other defensive traits, physiological processes or interactions among hormonal signaling pathways, responsible for herbivory and drought stress responses, could be behind the observed effect of water stress on herbivory resistance.

Conclusions

Our results confirmed the hypothesis that drought stress modified the defensive ability of Aleppo pine against the pine weevil *Hylobius abietis*. Although drought stress affected some basal constitutive chemical defences (e.g. diterpenes), the observed changes in the concentration of chemical defences may be not enough to explain the large observed effect of water stress on the effective resistance against the pine weevil. Further research is needed to determine the physiological mechanisms behind the non-linear effect of water stress on weevil damage. We also found that water stress interfered in induced chemical responses to insect damage, both on their profile and concentration. Despite the three analysed populations are known to differ in drought tolerance and other traits, we found that resistance to herbivory damage under drought stress was fairly consistent across populations. Overall, these results are relevant for understanding the effects of global change in forest ecosystems of the Mediterranean.
basin, where both drought stress and biotic pressure caused by pest and pathogens are forecasted to increase in the near future.

**Author contributions**

Zas R., Sampedro L. and Voltas J. designed the experiment, provided the reagents and lab infrastructures, performed the sampling, helped with the interpretation of the results and improved the different versions of the manuscript. Notivol, E. provided the infrastructure, carried out drought stress treatments and also contributed to the interpretation of the results and the improvement of the manuscript. Serrano, L. helped with drought stress treatments design, the measurements of water potential and contributed to the interpretation of the results and the improvement of the manuscript. Suárez-Vidal E. carried out the herbivory treatments (together with Sampedro L. and Zas R.), measurement of weevil damage, seedlings sampling, all chemical analysis (unless non structural carbohydrates analyzed in Voltas J. lab and δ13C at the Research Support Service of University of Coruña), most of the statistical analyses (with the help of Zas R.), produced the results, wrote the first draft with Zas R. and Sampedro L. and the improvement of subsequent versions of the manuscript.

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technical assistance in the experimental setup and with the chemical analysis at MBG and UdL, and María Lema from SAI-UTIA at the University of Coruña for the isotopic analysis. This research was supported by the grants AGL2012-40151-C03-01, AGL2015-68274-C03-02-R and AGL2015-68274-C03-03-R, founded by MINECO/FEDER. E.S-V. received financial support from the FPU grant program (Ministerio de Educación, Cultura y Deporte Gobierno de España).

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Figure 1. Effect of drought stress (control = white; moderate = grey; severe = dark bars) applied during four months to one-year-old Aleppo seedlings on midday plant water potential (a) and the carbon isotope composition ($\delta^{13}$C) on recently fixed sugars (b). Mean ± SE, N = 15. Different letters below the bars denote significant differences at $p \leq 0.05$ among drought stress treatments. The tables within the panels summarize the effect of the factors in the experimental design.
Figure 2. Effect of four months of drought stress (control = white; moderate = grey; severe = dark bars) on the concentration of starch from 90 composite samples from coarse roots (a) and fatty acids from 90 composite samples from the stem (b) in terms of mg g⁻¹ dry weight in one-year-old Aleppo pine seedlings. Mean ± SE, N = 30. Letters above the bars denote the significant differences at $p \leq 0.05$ among drought stress treatments. The tables within the panels summarize the effect of the factors in the experimental design.

**Starch (mg g⁻¹)**

<table>
<thead>
<tr>
<th>Effect</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drought (D)</td>
<td>*</td>
</tr>
<tr>
<td>Population (POP)</td>
<td>n.s</td>
</tr>
<tr>
<td>POP × D</td>
<td>n.s</td>
</tr>
</tbody>
</table>

**Fatty acids (mg g⁻¹)**

<table>
<thead>
<tr>
<th>Effect</th>
<th>$P &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drought (D)</td>
<td>***</td>
</tr>
<tr>
<td>Population (POP)</td>
<td>n.s</td>
</tr>
<tr>
<td>POP × D</td>
<td>n.s</td>
</tr>
</tbody>
</table>
Figure 3. Effect of drought stress (control = white; moderate = grey; severe = dark bar) applied during four months to one-year-old Aleppo pine seedlings on the damage caused by the pine weevil after four days of exposure to real herbivory. Mean ± S.E are shown, N = 45. Letters above the bars denote the significant differences at $p \leq 0.05$ among drought stress treatments. The table within the panel summarize the effect of the factors in the experimental design.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
Effect & $P > F$ \\
\hline
Drought (D) & * \\
Population (POP) & n.s. \\
POP $\times$ D & n.s. \\
\hline
\end{tabular}
\end{table}
Figure 4. Effect of the interactive effects between drought stress and herbivory on the concentration of chemical defences in the bark of one-year-old Aleppo pine seedlings. Concentration of total polyphenolics (a), condensed tannins (b) and total diterpenes (c) in the three drought stress treatments (control, moderate and severe) in control (bars without insect) and herbivory treatments (bars with insect). Drought was applied during four months, then plants were exposed (or not, controls) to 4 days of herbivory by the pine weevil. Mean ± E.S., N = 15. Different letters above the bars denote the significant differences at $p \leq 0.05$ among drought stress treatments and herbivory interaction.
Figure 5. Summary of the Principal Component Analysis performed on the relative concentration of the 17 most abundant terpenes in the 90 composite stem samples of one-year-old Aleppo pine seedlings of three populations from contrasting climates. (a) Explained variance, eigenvalues and component loadings for the two main PC axes and each vector in represents the correlation coefficient between each compound and the corresponding principal component. (b) Population means of both PCs for the control seedlings (open symbols) and those seedlings exposed to herbivory (dark symbols) across the three drought stress treatments (control = dots; moderate = squares; severe = triangle). The table within the panel summarize the effect of the factors in the experimental design.
Table 1. Summary of the mixed models for the direct effects of drought stress (D), herbivory (HERB) and population (POP) and the interactive effect of D × HERB on the concentration of chemical defences in one year-old Aleppo pine (measured in 90 composite stem samples), namely total polyphenolics, condensed tannins, mono-, sesqui- and diterpenes. F ratios and associated probability levels (p > F) for the main effects (drought stress treatments, herbivory, population) and D×HERB interaction are shown. p- values of significant effects (p ≤ 0.05) are highlighted in bold font. Interactions of drought stress and herbivory with population were discarded to simplify the table because they were not significant.

<table>
<thead>
<tr>
<th></th>
<th>Drought (D)</th>
<th>Herbivory (HERB)</th>
<th>Population (POP)</th>
<th>D × HERB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F&lt;sub&gt;2,70&lt;/sub&gt;</td>
<td>p&gt;F</td>
<td>F&lt;sub&gt;1,70&lt;/sub&gt;</td>
<td>p&gt;F</td>
</tr>
<tr>
<td>Polyphenolics</td>
<td>4.3</td>
<td>0.018</td>
<td>48.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tannins</td>
<td>8.8</td>
<td>&lt;0.001</td>
<td>24.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monoterpenes</td>
<td>2.0</td>
<td>0.154</td>
<td>0.2</td>
<td>0.688</td>
</tr>
<tr>
<td>Sesquiterpenes</td>
<td>1.1</td>
<td>0.346</td>
<td>1.8</td>
<td>0.179</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>0.0</td>
<td>0.983</td>
<td>5.1</td>
<td>0.027</td>
</tr>
</tbody>
</table>
Table 2. Summary of MANOVA analysis for the direct and interactive effects of drought stress (D), herbivory (HERB) and population (POP) on the variation of absolute concentration for the 17 selected terpenes in the 90 composite stem samples of one year-old Aleppo pine. Wilk’s Lambda value ($\lambda$), freedom degrees, $F$ ratios and associated probability levels ($p > F$) for the main effects (drought stress, herbivory and population) and their interactions are shown. $p$-values of significant effects ($p \leq 0.05$) are highlighted in bold font. Interactions of drought stress and herbivory with population were discarded to simplify the table because they were not significant.

<table>
<thead>
<tr>
<th>Effect</th>
<th>$\lambda$</th>
<th>DF</th>
<th>$F$</th>
<th>$p &gt; F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drought (D)</td>
<td>0.419</td>
<td>34,96</td>
<td>1.6</td>
<td><strong>0.049</strong></td>
</tr>
<tr>
<td>Herbivory (HERB)</td>
<td>0.498</td>
<td>17,48</td>
<td>2.9</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>Population (POP)</td>
<td>0.216</td>
<td>34,96</td>
<td>3.3</td>
<td>&lt;<strong>0.001</strong></td>
</tr>
<tr>
<td>D $\times$ HERB</td>
<td>0.353</td>
<td>34,96</td>
<td>1.9</td>
<td><strong>0.007</strong></td>
</tr>
</tbody>
</table>
Drought stress modifies early effective resistance and induced chemical defences of Aleppo pine against a chewing insect herbivore

Estefanía Suárez-Vidal, Luis Sampedro, Jordi Voltas, Luis Serrano, Eduardo Notivol, Rafael Zas.

Figure S1. Distribution map of Aleppo pine modified (http://www.euforgen.org/species/pinus-halepensis/). Aleppo pine natural distribution range (light green), origin of the pine populations (red dots) included in the study and location of the Centro de Investigación y Tecnología Agroalimentaria (CITA, Zaragoza, Spain, blue dot) where the experiment was set-up. Mean annual temperature (T) and annual precipitation (P) is shown in the companion table. Benamaurel is the population from the driest area, potentially the most tolerant against drought stress; Cabanelles is the one from the wettest area and Benicàssim from intermediate values.
Figure S2. Details of the experimental set-up showing (a) an Aleppo pine seedling confined inside the acrylic transparent cylinders fitted to the pot and covered by a gauze; (b) the pine weevil, Hylobius abietis, our model insect used for the herbivory treatment on one of the experimental Aleppo pine seedlings; (c) the damage caused by the bark beetle on the stem (debarked area); and (d) the calibrated template used to measure the debarked area along the stem.
Table S1. Summary of the chemical species identified by GC-FID in the hexane extracts. Name, concentration, relative abundance of each compound in relation to their chemical group, proportion of presence in the 90 composite stem samples analysed, retention time and Kovat Index are shown. Compounds in bold font are those selected as the 17 terpenes with a relative concentration higher than 1%.

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Overall mean concentration (mg g⁻¹) ± S.E</th>
<th>Relative abundance (%)</th>
<th>Presence in the samples (%)</th>
<th>Retention time (min) ± S.E</th>
<th>Kovat Index calculated ± S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoterpenes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1</td>
<td>tryciclene</td>
<td>0.0045 ± 0.0002</td>
<td>0.098</td>
<td>97.78</td>
<td>9.1 ± 0.61</td>
<td>919.4 ± 0.93</td>
</tr>
<tr>
<td>MT2</td>
<td>α-thujene</td>
<td>0.0040 ± 0.0002</td>
<td>0.086</td>
<td>94.44</td>
<td>9.3 ± 0.63</td>
<td>924.9 ± 0.88</td>
</tr>
<tr>
<td>MT3</td>
<td>α-pinene</td>
<td>2.3398 ± 0.0562</td>
<td>51.403</td>
<td>100.00</td>
<td>9.5 ± 0.61</td>
<td>932.8 ± 1.05</td>
</tr>
<tr>
<td>MT4</td>
<td>camphene</td>
<td>0.0221 ± 0.0008</td>
<td>0.480</td>
<td>100.00</td>
<td>10.0 ± 0.64</td>
<td>945.4 ± 1.02</td>
</tr>
<tr>
<td>MT5</td>
<td>sabinene</td>
<td>0.0340 ± 0.0009</td>
<td>0.749</td>
<td>100.00</td>
<td>10.8 ± 0.66</td>
<td>971.0 ± 0.94</td>
</tr>
<tr>
<td>MT6</td>
<td>β-pinene</td>
<td>0.2880 ± 0.0172</td>
<td>6.252</td>
<td>100.00</td>
<td>10.9 ± 0.66</td>
<td>973.8 ± 0.89</td>
</tr>
<tr>
<td>MT7</td>
<td>myrcene</td>
<td>0.3235 ± 0.0132</td>
<td>7.115</td>
<td>100.00</td>
<td>11.5 ± 0.66</td>
<td>989.8 ± 0.89</td>
</tr>
<tr>
<td>MT8</td>
<td>α-phellandrene</td>
<td>0.0036 ± 0.0001</td>
<td>0.077</td>
<td>91.11</td>
<td>11.9 ± 0.74</td>
<td>1002.2 ± 1.01</td>
</tr>
<tr>
<td>MT9</td>
<td>δ-3-carene</td>
<td>1.2819 ± 0.0333</td>
<td>28.207</td>
<td>100.00</td>
<td>12.1 ± 0.67</td>
<td>1009.4 ± 1.08</td>
</tr>
<tr>
<td>MT10</td>
<td>α-terpinene</td>
<td>0.0068 ± 0.0002</td>
<td>0.148</td>
<td>96.67</td>
<td>12.3 ± 0.72</td>
<td>1014.4 ± 1.02</td>
</tr>
<tr>
<td>MT11</td>
<td>p-cymene</td>
<td>0.0020 ± 0.0002</td>
<td>0.044</td>
<td>72.22</td>
<td>12.6 ± 0.83</td>
<td>1022.0 ± 0.99</td>
</tr>
<tr>
<td>MT12</td>
<td>limonene</td>
<td>0.0722 ± 0.0019</td>
<td>1.589</td>
<td>100.00</td>
<td>12.7 ± 0.72</td>
<td>1026.5 ± 1.07</td>
</tr>
<tr>
<td>MT13</td>
<td>β-phellandrene</td>
<td>0.0013 ± 0.0005</td>
<td>0.032</td>
<td>7.78</td>
<td>12.8 ± 3.15</td>
<td>1028.6 ± 0.79</td>
</tr>
<tr>
<td>MT14</td>
<td>cis-b-oicimene</td>
<td>0.0018 ± 0.0001</td>
<td>0.039</td>
<td>72.22</td>
<td>13.1 ± 0.88</td>
<td>1036.6 ± 0.95</td>
</tr>
<tr>
<td>MT15</td>
<td>trans-β-oicimene</td>
<td>0.0001 ± 0.0000</td>
<td>0.001</td>
<td>4.44</td>
<td>13.4 ± 4.70</td>
<td>1046.3 ± 0.96</td>
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<tr>
<td>MT16</td>
<td>γ-terpinene</td>
<td>0.0146 ± 0.0004</td>
<td>0.322</td>
<td>100.00</td>
<td>13.8 ± 0.73</td>
<td>1057.0 ± 1.02</td>
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<tr>
<td>MT17</td>
<td>unknown MT1</td>
<td>0.0018 ± 0.0003</td>
<td>0.041</td>
<td>35.56</td>
<td>14.3 ± 1.33</td>
<td>1070.8 ± 1.35</td>
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<tr>
<td>MT18</td>
<td>unknown MT2</td>
<td>0.0044 ± 0.0002</td>
<td>0.096</td>
<td>80.00</td>
<td>14.8 ± 0.92</td>
<td>1084.2 ± 1.03</td>
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<tr>
<td>MT19</td>
<td>α-terpinolene</td>
<td>0.1270 ± 0.0032</td>
<td>2.810</td>
<td>100.00</td>
<td>14.8 ± 0.74</td>
<td>1086.5 ± 1.08</td>
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<tr>
<td>MT20</td>
<td>linalool</td>
<td>0.0038 ± 0.0007</td>
<td>0.086</td>
<td>47.78</td>
<td>15.3 ± 0.98</td>
<td>1098.3 ± 0.98</td>
</tr>
<tr>
<td>MT21</td>
<td>solusterol</td>
<td>0.0001 ± 0.0000</td>
<td>0.003</td>
<td>7.78</td>
<td>15.5 ± 1.81</td>
<td>1104.3 ± 0.76</td>
</tr>
<tr>
<td>MT22</td>
<td>unknown MT3</td>
<td>0.0003 ± 0.0000</td>
<td>0.006</td>
<td>12.22</td>
<td>16.6 ± 2.36</td>
<td>1137.3 ± 1.19</td>
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<td>0.012</td>
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<td>1158.2 ± 1.15</td>
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<td>54.44</td>
<td>17.9 ± 1.00</td>
<td>1175.9 ± 0.96</td>
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<td>α-terpineol</td>
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<td>1189.7 ± 0.99</td>
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<td>19.2 ± 8.31</td>
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<th>Code</th>
<th>Name</th>
<th>Overall mean concentration (mg g⁻¹) ± S.E</th>
<th>Relative abundance (%)</th>
<th>Presence in the samples (%)</th>
<th>Retention time (min) ± S.E</th>
<th>Kovat Index calculated ± S.E</th>
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<th>0.0016 ± 0.0001</th>
<th>0.260</th>
<th>77.78</th>
<th>23.6 ± 0.98</th>
<th>1349.9 ± 1.11</th>
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<td>100.00</td>
<td>24.5 ± 0.82</td>
<td>1375.8 ± 0.99</td>
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<tr>
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<td>24.9 ± 0.93</td>
<td>1391.5 ± 1.05</td>
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<tr>
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<td>27.5 ± 0.85</td>
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<td>28.7 ± 0.97</td>
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<td>31.0 ± 1.01</td>
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<td>32.4 ± 1.17</td>
<td>1650.3 ± 1.58</td>
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<td>55.56</td>
<td>32.6 ± 1.76</td>
<td>1660.8 ± 2.36</td>
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<th>Diterpenes</th>
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<td>4.5770 ± 0.0948</td>
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<td>20.0 ± 1.63</td>
<td>2325.6 ± 1.38</td>
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<td>2333.7 ± 1.77</td>
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Table S2. Summary of the mixed models for the direct effects of drought stress (D), herbivory (HERB) and population (POP) and the interactive effect of D × HERB on the absolute concentration of the 17 selected terpenes extracted from the composite stem samples of one year-old Aleppo pine. F ratios with the correspondent DF and associated probability levels ($p > F$) for the main effects (drought stress treatments, herbivory, population) and D × HERB interaction. P values of significant effects ($p \leq 0.05$) are highlighted in bold font after the adjustment by FDR ($\alpha = 0.05$). Interactions of drought stress and herbivory with population were discarded to simplify the table because they were not significant.

<table>
<thead>
<tr>
<th>Name</th>
<th>Drought (D)</th>
<th>Herbivory (HERB)</th>
<th>Population (POP)</th>
<th>D × HERB</th>
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<td>$F_{2,70}$</td>
<td>$p&gt;F$</td>
<td>$F_{1,70}$</td>
<td>$p&gt;F$</td>
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<tr>
<td>α-pinene</td>
<td>3.9</td>
<td>0.025</td>
<td>0.4</td>
<td>0.534</td>
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<tr>
<td>β-pinene</td>
<td>1.8</td>
<td>0.168</td>
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<td>&lt;0.001</td>
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<td>myrcene</td>
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<td>0.189</td>
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<td>0.263</td>
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<td>β-gurjunene</td>
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<td>0.886</td>
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<td>0.048</td>
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