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Do volatile organic compound emissions of Tunisian cork oak populations originating from contrasting climatic conditions differ in their responses to summer drought?

Michael Staudt, Amel Ennajah, Florent Mouillot, and Richard Joffre

Abstract: The intrinsic variability of volatile organic compound emissions and photosynthetic parameters in response to two drying cycles was investigated on *Quercus suber* L. saplings originating from three Tunisian populations native to contrasting climates. Emissions mainly included monoterpenes plus traces of sesquiterpenes and methylsalicylate that steadily decreased during the experiment unrelated to treatments. Instead, monoterpene emissions increased by 30% during a first moderate drought and remained enhanced after rewatering, while CO₂-H₂O gas exchange was reduced by 30%. A second severe drought decreased monoterpene emissions to about two third of controls, strongly inhibited gas exchange and photochemical efficiency, and caused a partial loss of chlorophyll. Rewatering led to only partial recovery of emissions and photosynthetic parameters of stressed plants whose biomasses and leaf nitrogen and chlorophyll contents were lower than in control plants. There was no clear difference among populations in volatile organic compound emissions or photosynthetic parameters. However, the population inhabiting the most arid region had intrinsically smaller leaves with lower specific leaf masses and higher drought-related leaf losses than populations native to less arid climates. These results indicate an adaptive shift from stress-tolerating towards stress-avoiding ecotypes under arid climates involving mainly morphological adaptations without apparent differentiation in volatile organic compound production, probably because of its high phenological plasticity.

Résumé : La variabilité des émissions foliaires de composés organiques volatils et des paramètres photosynthétiques ont été étudiés au cours de deux cycles de dessèchement sur des plants de *Quercus suber* L. provenant de trois populations tunisiennes adaptées à des différentes conditions climatiques. Les composés organiques volatils émis étaient principalement des monoterpènes, plus, indépendamment du traitement, des traces de sesquiterpènes et méthyle-salicylate. Un premier dessèchement modéré réduisant les échanges gazeux en CO₂-H₂O de 30 %, a augmenté de 30 % les émissions de composés organiques volatils qui sont restées élevées après réhydratation. Un second dessèchement plus sévère a réduit les émissions d'environ 30 % accompagné d'une forte inhibition des échanges gazeux et de l'efficacité photochimique et d'une perte partielle de chlorophylle. Après réhydratation les émissions et les paramètres photosynthétiques ont partiellement récupéré. Les biomasses aériennes et souterraines finales ainsi que l'azote foliaire et la teneur en chlorophylle des plantes stressées étaient inférieurs à ceux des témoins. Les émissions de composés organiques volatils et les paramètres photosynthétiques n'étaient pas nettement différents entre populations. Cependant, la population provenant du site le plus aride présentait des feuilles plus petites de poids spécifique plus faible, et des pertes de feuilles plus importantes après le stress que les populations adaptées à des sites moins arides. Ces résultats pourraient indiquer l'évolution adaptative d'une stratégie de tolérance au stress vers une stratégie d'évitement du stress sous climat aride impliquant surtout des adaptations morphologiques sans différence apparente dans la production de composés organiques volatils, probablement à cause d'une grande plasticité phénologique.

Introduction

Volatile organic compounds (VOCs) are unsaturated complex carbon molecules that are produced and emitted by many plants (Kesselmeier and Staudt 1999). They may act as signalling compounds in trophic interactions, being cru-

cial for the plant's reproduction or defence against herbivores (Holopainen 2004). Because of their abundance and high reactivity, biogenic VOCs influence the oxidative capacity and the radiative properties of the troposphere. As a result, natural VOC emissions are important for climate and radiative forcing as well as air quality and visibility

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(Monson et al. 2007). To evaluate these effects, precise estimations of the total amount of VOC released into the atmosphere by the biosphere are needed. These so-called biogenic emission inventories predict VOC fluxes on regional and global scales on the basis of species-dependent emission potentials (defined as the emission rate measured at standard temperature and light conditions of 30 °C and 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density (PPFD)) and models that account for temporal variations in emissions driven by environmental factors and phenological processes (Guenther 1997). Both emission potentials and models have been found to be inaccurate and therefore are major limitations in the estimation of VOC fluxes. While in recent years, considerable progress has been made in understanding the taxonomic distribution of VOC-producing plants (e.g., see www.es.lanccs.ac.uk/cnhgroup/download.html) and in simulating the influences of some environmental factors on VOC emissions (Zimmer et al. 2000; Guenther et al. 2006; Arneth et al. 2007; Monson et al. 2007), the extent of genetic variability within a given species to produce VOCs is largely unknown (Staudt et al. 2001; Funk et al. 2005).

In temperate climates, the most important contribution of reactive VOC comes from broadleaf forest tree species such as oaks, which produce large amounts of isoprene or monoterpenes inside the chloroplasts of their photosynthetic tissues (Csiky and Seufert 1999). Because the biosynthesis of these compounds is energetically costly, an ecophysiological function of this apparent production of expensive waste has queried plant ecophysiologicalists for years (Sharkey et al. 2008). Results from many laboratory experiments provided evidence that the formation of these volatile isoprenoids helps the plants to cope with environmental stresses such as high temperatures, oxidants, or as a metabolic valve to dissipate excessive energy (Logan et al. 2000; Loreto et al. 2001; Sharkey et al. 2001; Owen and Peñuelas 2005; Magel et al. 2006; Behnke et al. 2007). Whatever the most relevant of these protective functions, all stresses related to them coincide in Mediterranean climates during the summer period when heat and high radiation co-occur with drought. Drought can increase leaf temperatures by decreasing the cooling effect via leaf transpiration and can effectively reduce the activity of metabolic sinks of photosynthetic products, which in turn will create an excess of energy and the subsequent formation of reactive oxygen species in the light-collecting photosynthetic apparatus (Chaves et al. 2003; Sharkey 2005; Sharkey et al. 2008). Therefore, one would expect that populations native to warmer and dryer sites would have a higher capacity to produce volatile isoprenoids than populations native to less stressful environments. On the other hand, maintaining a year-round high production rate of volatile isoprenoids would reduce the availability of energy and carbon in the periods of growth when large amounts of resources are required to complete the growing and reproduction cycles, especially in the more stressful sites. For that reason, the best strategy might be if plants enhance foliar isoprenoid production only during the period of stress when protection is needed and when growth is restrained as it is. Such a phenotypic plasticity in the formation of protective metabolites would increase plant fitness unless the associated sensory and regulatory machinery entails large extra costs (Valladares et al. 2007).

The goal of the present study was to investigate whether cork oak (*Quercus suber* L.) saplings stemming from three Tunisian populations representing a marked climate gradient differ intrinsically in their responses to repeated water deficits of different strength. Cork oak is an evergreen oak species whose VOC release from leaves may express remarkable intraspecific variation in both quality and quantity of emissions (Staudt et al. 2004). Cork oak forests are widely present in the coastal and pre-coastal areas of the western Mediterranean basin extending from southern France over the Iberian and Italian Peninsula to northern Africa (Lopez de Heredia et al. 2007). It is well adapted to withstand bushfires but to a lesser extent cold and snow cover, which are likely key environmental constraints in northern and mountainous-continental populations (Aranda et al. 2005). However, in the northwest African area, summer drought combined with heat increases rapidly from the coastal mountains towards the Sahara Desert and becomes a major limiting factor (Ghouil et al. 2003). In that region, cork oak populations are exposed to different selective pressures with respect to environmental stresses that may lead to local differentiations even in the absence of barriers to gene flow (Linhart and Grant 1996).

Materials and methods

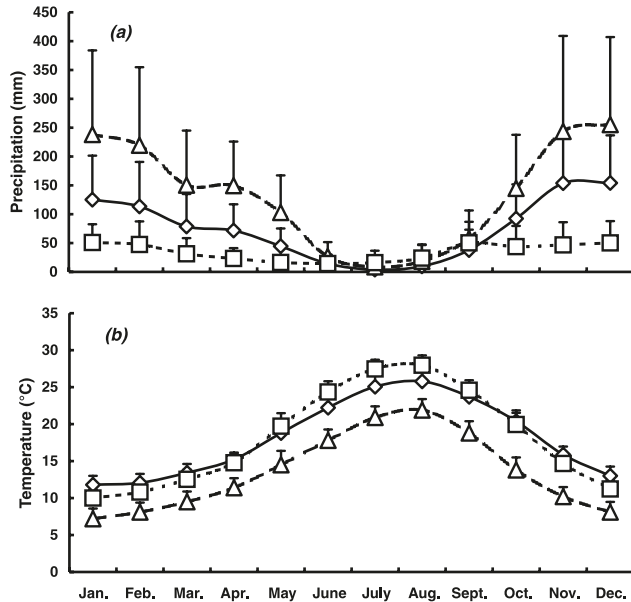
Plant material

In Tunisia, cork oak populations exist in the northwestern provinces starting from the Mediterranean coast extending southwards over the Kroumirie Mountains to the Mejerda Plain located about 50 km inland (Boudy 1955). We used cork oak saplings stemming from three natural populations representing a marked climate gradient (Gounot and Le Houerou 1967) with respect to summer stress conditions (Fig. 1): one of the northern coastal populations around Tabarka (ta) that are exposed to a subhumid climate with mild winters and moderate hot summers (36°58.21'N, 008°53.41'E, 50 m), one population on the northern slopes of the Kroumirie Mountains (Ain Draham (ad)) characterized by a rather humid climate with cold winters and modest warm summers (36°46.98'N, 008°43.79'E, 800 m), and one population situated in the southern hills and plains around Jendouba (je) growing in a semi-arid climate with moderate cool winters and hot dry summers (36°38.84'N, 008°39.56'E, 340 m). In these populations, acorns of different mother trees (dominating individuals between 94 and 165 years old) were collected at the end of November 2005. Acorns were germinated and grown outside in pots containing 50% sand and 50% peat. Plants were regularly fertilized and watered with demineralized water by means of an automatic drip irrigation system. In February 2007, saplings were transplanted into 11 L pots and transferred to a greenhouse compartment in June.

Experimental protocol

Leaf VOC emissions and other ecophysiological variables were measured in normal, well-watered conditions and during two consecutive drying cycles of different intensity. The whole experiment was completed in a 3-month period between July and October 2007. Daily temperature inside the greenhouse and the global radiation outside were recorded

Fig. 1. Annual course of (a) precipitation and (b) air temperature at the three stations Tabarka (diamonds), Ain Draham (triangles), and Jendouba (squares) in northern Tunisia representing subhumid, humid, and semi-arid climate conditions, respectively (Gounot and Le Houerou 1967). Data are monthly means + SD over a 28-year period (1975–2002) (source Direction Générale des Ressources en Eau, Tunis).



throughout the experimental period. Ten healthy individuals of similar size were selected from each population and randomly subdivided into five test plants for stress exposure and five control plants. Treatment plants were exposed to two consecutive drying cycles interrupted by a 1-week recovery. In the first drying cycle, test plants were exposed to a moderate water stress (target relative soil water content of 60%–70% of field capacity) and in the second one to a severe stress (target soil water content 20%–30% of field capacity). After each stress, plants were rewatered to field capacity to determine stress recovery. During each drying cycle, water stress was applied progressively over approximately 3 weeks. Progress of water stress was monitored daily by determining mass losses of pots. According to the measured mass losses, individual plants received a small amount of water to slow down soil desiccation and to even out differences among individuals.

Leaf VOC emissions, $\text{CO}_2\text{-H}_2\text{O}$ gas exchange, chlorophyll fluorescence parameters, chlorophyll content, and water potential were determined on treated and control plants during each phase of the experiment: before stress (PRE), first light water stress (S1), first recovery after light stress (R1), second severe water stress (S2), and second recovery (R2). Weather conditions were fairly good throughout the first part of the experiment (PRE to R1) but rather unstable during phases S2 and R2. Mean air temperature inside the greenhouse and outside global radiation were, respectively, 26.7 °C and 275 $\text{W}\cdot\text{m}^{-2}$ (PRE), 25.2 °C and 264 $\text{W}\cdot\text{m}^{-2}$ (S1), 26.1 °C and 233 $\text{W}\cdot\text{m}^{-2}$ (R1), 23.7 °C and 190 $\text{W}\cdot\text{m}^{-2}$ (S2), and 24.3 °C and 104 $\text{W}\cdot\text{m}^{-2}$ (R2).

At the end of the experiment, foliar nitrogen content and whole aboveground (intact leaves and stems) and belowground (main and fine roots) dry biomasses of saplings

were determined. Generally, in cork oak, leaf growth is usually limited to the period of spring and early summer (Mediavilla and Escudero 2003). By contrast, the radial growth of stems and main roots and the growth of fine roots may continue during the main vegetation period unless restricted by drought or other environmental constraints. In the present study, leaf growth was ended by July in all populations and no second flush occurred later during the experiment. During the second drought period, many leaves on stressed plants became senescent and were shed (usually older leaves of the lower parts), while stem organs always remained intact. Therefore, differences between control and treated plants in their final leaf biomasses can be attributed to the drought-related losses of leaves, whereas differences in their stem and main root biomasses can be attributed to reduced growth rates. Finally, differences in fine root biomasses between control and stressed plants could be attributed to both stress-related reduction in growth rates and decay of plant tissues.

Leaf water potential

Predawn water potentials (Ψ_{pd}) were measured with a standard Scholander-type pressure chamber. Measurements were made 1 h before sunrise on two individual leaves of each plant. The means of these two measurements were used for data evaluation.

Gas exchange and fluorescence measurements

Chlorophyll fluorescence parameters following the definitions of Maxwell and Johnson (2000) were determined with a PAM-2000 instrument (Walz, Germany). Maximum photochemical efficiency of photosystem II (F_v/F_m) was measured at dawn (after Ψ_{pd}) on overnight dark-adapted leaves using Walz leaf clips. Quantum yield of photosystem II (Φ_{PSII}) was measured during bright days around noon together with $\text{CO}_2\text{-H}_2\text{O}$ gas exchange using a Li-COR 6400 portable gas exchange system (LI-COR, Lincoln, Nebraska) equipped with a standard leaf chamber featuring an incorporated holder for the PAM-2000 fibre optic. Leaf temperature was set to 30 °C. Photosynthesis, transpiration, and stomatal conductance were calculated with the instrument software and corrected for leaf area if necessary. Leaf areas were determined with a Delta-T area meter (MK2) (Delta-T Devices, Ltd., UK).

Monoterpene emission measurements

VOC emissions were determined at standard temperature and light conditions (30 °C and 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD) using a dynamic, temperature-controlled enclosure system (flat PTFE chamber of approximately 150 mL volume) installed in a small laboratory close to the greenhouse. The chamber was continuously flushed with compressed air (Ingersoll Rand compressor model 49810187) at a constant rate of 0.4 $\text{L}\cdot\text{min}^{-1}$ (regulated by a Brooks mass flow controller), which was cleaned and dried in a clean air generator (AIRMOPURE, Chromatotec, France) and re-humidified to a relative humidity of about 40% by bypassing a variable portion of the air stream through a washing bottle. Chamber and plants were illuminated with a white light source (OSRAM 1000 W) filtered by a 5 cm water bath.

Monoterpenes were trapped by directing 1 L of air exiting the chamber through an adsorption tube (200 mg Tenax TA, 20–35 mesh; Chrompack) at a constant flow rate of 0.1 L·min⁻¹. The adsorption tubes were analysed by gas chromatography with a flame ionization detector (GC/FID) using a Chrompack CP9003 gas chromatograph equipped with a Chrompack TCT4002 thermodesorber (all Varian Inc.). Occasionally, parallel VOC samples were taken for analyses with gas chromatography coupled with mass spectrometry (Varian CP3800/Saturn2000 MS plus a Perkin-Elmer Turbomatrix thermodesorber) using the same analytical column and temperature program as in the GC/FID instrument (see Staudt and Lhoutellier (2007) for more details).

Peaks were identified by comparing their mass spectra and retention times with those from standards analysed under the same conditions. All gas chromatography systems were calibrated using authentic standards (Fluka Chemie, Buchs, Switzerland; Roth, Karlsruhe, Germany) diluted in methanol. Standard solution (0.5–3 µL) was injected on the head of the adsorption trap through a T-fitting equipped with a septum and purged with 300 mL of pure nitrogen at a flow rate of 50 mL·min⁻¹. Average precision as determined by repeated measurements of standards at realistic concentrations was within 5% (relative coefficient of variation of $n = 7$ repetitions).

The VOC emission rate was calculated as the difference between the VOC concentration in the chamber enclosing leaves and the concentration in the empty chamber multiplied by the inlet air flow and divided by the surface or dry mass of the leaves.

Foliar nitrogen and chlorophyll content

Nitrogen content of leaves was determined using a Thermo-Finishing EA1112 NC elementary analyser, and chlorophyll content was assessed by a Minolta SPAD 502 meter.

Statistical analyses

Two-way ANOVAs with Student–Newman–Keuls post hoc tests were conducted on the data of each phase for the factors treatment (stress versus control) and population (ta, ad, and je). Differences were considered as significant at a probability level of $P < 0.05$. If necessary, data were log-transformed to meet normality and homoscedasticity. All analyses were carried out using SigmaStat 3.0 (SPSS Inc., Chicago, Illinois).

Results

Evolution of water potential and photosynthetic parameters during repeated water stress

The Ψ_{pd} values of control plants were high and stable throughout the experiment (Table 1). In treated plants, Ψ_{pd} significantly decreased to -1.6 ± 0.2 MPa during S1 and to -3.8 ± 0.4 MPa during S2. After rewatering, Ψ_{pd} recovered almost to the PRE level especially following S1. Within the treated or control plants, Ψ_{pd} values were not different among the three populations during all stages of the experiment.

In control plants, no significant changes in any of the measured gas exchange and fluorescence parameters or in

chlorophyll content (Table 1) were observed throughout the experimental period, suggesting that these plants were never exposed to drought stress and that seasonal changes in climate conditions had no or only minor effects on the plant's carbon acquisition.

Gas exchange and fluorescence data of treated plants were very similar to those of the control plants before stress. Among the three populations, the plants from je showed the highest photosynthesis rates when expressed on a leaf dry mass basis. However, during S1, photosynthesis and stomatal conductance were significantly lower than in controls and this drop was significantly more pronounced in je than in the two other populations. In all populations, mean leaf internal CO₂ concentration (C_i) was decreased, although this decrease was only marginally significantly different from that in control plants (248 ± 12 versus 258 ± 15 µmol·mol⁻¹, $P = 0.099$; data not shown). The Φ_{PSII} data showed a trend very similar to that of photosynthesis but without significant differences among populations. The F_v/F_m values as well as foliar chlorophyll content remained unchanged in response to moderate water limitations. All of these data indicate that the lower photosynthesis rates during this light stress mainly resulted from a higher diffusion resistance of CO₂ to the carboxylation site.

After watering (R1), mean Φ_{PSII} of moderately stressed plants fully recovered to PRE levels, while photosynthesis and stomatal conductance remained somewhat lowered with respect to control plants. In this phase, leaf area based photosynthesis rates of je plants were significantly lower than those of ad plants.

Under S2, Φ_{PSII} , photosynthesis, and stomatal conductance were heavily inhibited and did not recover to PRE or control plant levels when plants were irrigated again (R2) regardless of the origin of the plants (Table 1). Unlike under light stress, photosynthesis rates of plants were on average more reduced than Φ_{PSII} . As a result, the ratio between Φ_{PSII} (or photosystem II electron transport derived from Φ_{PSII} and PPFD) and photosynthesis rate was significantly higher during and after S2 than in the previous phases (and also higher than in unstressed controls); even so, measurements were always made at comparable temperature and light conditions. The C_i values of heavily stressed plants were highly variable and on average not different from those of controls (253 ± 50 versus 254 ± 16 µmol·mol⁻¹). Furthermore, F_v/F_m and chlorophyll content significantly decreased under S2 and only barely recovered to PRE levels afterwards.

These results highlight that during the second drought phase, treated plants indeed experienced a severe stress with persistent, slowly reversible inhibition of photosynthesis including biochemical components and losses of chlorophyll and that an increased proportion of the remaining electron flow from photosystem II was utilized in alternative electron sinks such as photorespiration and Mehler peroxidase reactions.

VOC emissions

Foliar VOC emissions from all measured cork oak plants were dominated by five monoterpenes: α -pinene, β -pinene, sabinene, myrcene, and limonene. Other isoprenoid compounds were found in minor or trace levels, namely α -thujene, camphene, terpineol, p -cymene, (Z -

Table 1. Predawn leaf water potential (Ψ_{pd}), photosynthesis rates expressed on unit leaf area basis (PS_{area}) and unit leaf dry mass (PS_{dm}) basis, maximum photochemical efficiency and quantum yield (F_v/F_m and Φ_{PSII}) of photosystem II, and foliar chlorophyll content of stressed (S) and unstressed (C) cork oak saplings originating from three Tunisian populations native to different climate conditions (Ta-barka (ta), subhumid; Ain Draham (ad), humid; Jendouba (je), semiarid) during the different stages of the experiment.

Phase	Origin-treatment	Ψ_{pd} (MPa)	PS_{area} ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	PS_{dm} ($\text{nmol}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$)	Water conductance ($\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	F_v/F_m	Φ_{PSII}	Chlorophyll (arbitrary units)
PRE	ta-C	-0.52±0.03	10.2±1.8	63±9	174±34	0.83±0.03	0.38±0.03	29.9±1.7
	ad-C	-0.52±0.03	8.2±1.2	50±12	137±28	0.81±0.01	0.44±0.13	33.2±3.5
	je-C	-0.49±0.02	10.1±2.3	69±13	203±68	0.82±0.03	0.31±0.06	27.4±0.8
	ta-S	-0.51±0.02	7.5±2.1	51±16	136±47	0.83±0.01	0.31±0.11	28.4±4.9
	ad-S	-0.53±0.04	9.1±1.0	52±3	167±30	0.81±0.04	0.36±0.12	29.0±1.1
	je-S	-0.50±0.01	9.1±1.4	61±12	183±38	0.79±0.06	0.42±0.11	29.2±2.2
	ANOVA	ns	ns	O (je > ad)	ns	ns	ns	ns
S1	ta-C	-0.52±0.03	10.0±2.0	61±8	178±33	0.83±0.02	0.40±0.04	29.9±1.5
	ad-C	-0.52±0.03	10.3±1.1	62±6	219±37	0.83±0.02	0.31±0.06	33.6±3.3
	je-C	-0.49±0.02	7.7±1.6	53±8	143±40	0.81±0.02	0.44±0.11	29.1±3.7
	ta-S	-1.55±0.14	7.5±2.1	50±16	130±43	0.83±0.01	0.29±0.14	28.1±5.5
	ad-S	-1.56±0.19	9.2±0.5	53±2	162±33	0.82±0.01	0.24±0.06	29.2±4.9
	je-S	-1.62±0.13	5.1±1.3	34±10	79±15	0.80±0.06	0.20±0.11	29.4±4.2
	ANOVA	T (S < C)	T (S < C)	T (S < C)	T (S < C)	ns	T (S < C)	ns
R1	ta-C	-0.52±0.03	10.1±1.3	63±11	163±43	0.82±0.01	0.36±0.05	31.9±2.7
	ad-C	-0.52±0.03	10.3±1.1	62±6	219±37	0.82±0.02	0.30±0.06	33.5±3.3
	je-C	-0.49±0.02	8.4±1.5	58±8	152±41	0.81±0.03	0.32±0.07	28.5±3.5
	ta-S	-0.64±0.09	6.5±1.0	44±11	99±18	0.81±0.02	0.30±0.14	28.8±5.5
	ad-S	-0.76±0.07	8.7±1.2	50±9	153±40	0.83±0.03	0.39±0.10	30.1±3.6
	je-S	-0.69±0.09	7.6±1.9	50±14	155±87	0.81±0.04	0.36±0.08	29.4±4.2
	ANOVA	T (S < C)	T (S < C)	T (S < C)	T (S < C)	ns	ns	ns
S2	ta-C	-0.52±0.03	9.8±1.1	61±7	168±26	0.82±0.02	0.39±0.03	32.8±2.9
	ad-C	-0.52±0.03	9.6±1.6	58±8	188±52	0.80±0.03	0.32±0.06	34.0±3.8
	je-C	-0.49±0.02	8.1±1.5	55±9	143±40	0.81±0.02	0.43±0.11	28.8±4.2
	ta-S	-4.06±0.56	1.9±1.6	13±10	25±18	0.64±0.26	0.19±0.15	24.9±4.4
	ad-S	-3.61±0.25	0.7±0.5	4±2	19±5	0.67±0.21	0.15±0.10	26.7±4.8
	je-S	-3.68±0.30	2.3±0.7	16±6	31±15	0.69±0.09	0.13±0.06	26.8±2.7
	ANOVA	T (S < C)	T (S < C)	T (S < C)	T (S < C)	T (S < C)	T (S < C)	T (S < C)
R2	ta-C	-0.52±0.03	9.7±0.3	61±6	177±10	0.81±0.04	0.37±0.03	32.5±3.2
	ad-C	-0.52±0.03	10.0±1.4	60±6	202±47	0.81±0.09	0.31±0.06	35.0±3.8
	je-C	-0.49±0.02	9.3±1.8	64±8	185±54	0.82±0.03	0.35±0.09	29.3±2.9
	ta-S	-1.13±0.53	5.0±2.1	36±18	93±36	0.78±0.03	0.3±0.04	27.7±3.9
	ad-S	-0.88±0.27	3.3±1.0	19±6	73±20	0.81±0.03	0.23±0.05	28.2±4.1
	je-S	-0.87±0.26	4.3±2.5	29±21	121±101	0.76±0.09	0.23±0.10	28.1±2.3
	ANOVA	T (S < C)	T (S < C)	T (S < C)	T (S < C)	ns	T (S < C)	T (S < C)

Note: PRE, prestress; S1, light water stress; R1, recovery after light water stress; S2, severe water stress; R2, recovery after severe water stress. Treatment (T) and origin (O) effects analysed by ANOVA are indicated when significant at $P < 0.05$ (ns, not significant). Differences among groups are given in parentheses. There were no significant interactions between T and O. Values are means \pm SD ($n = 5$).

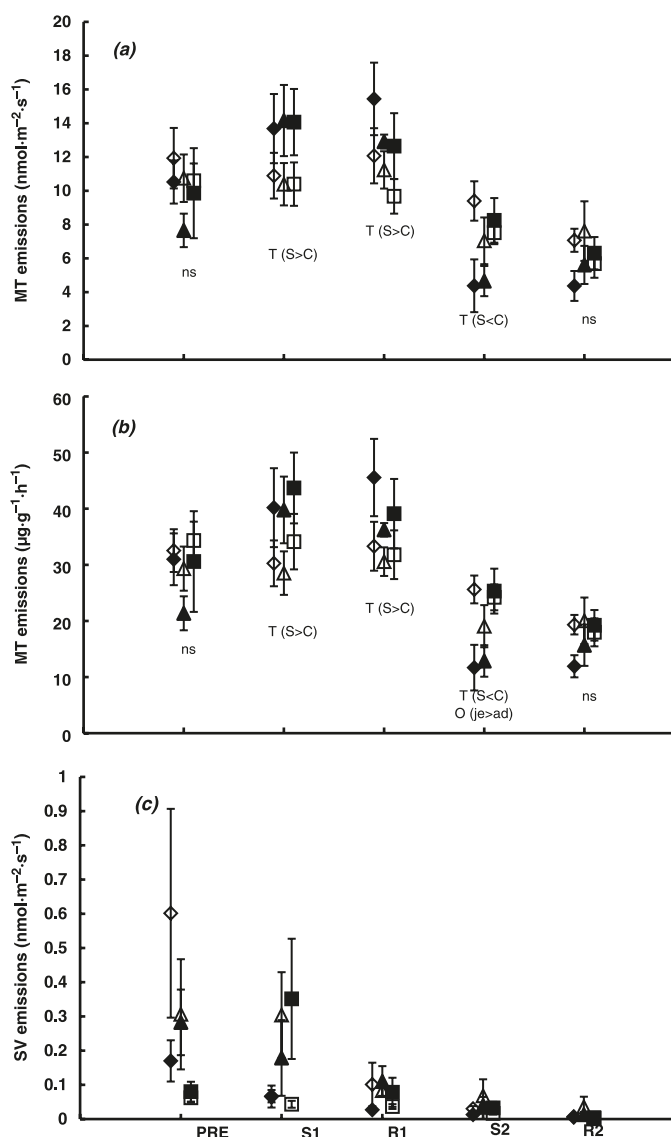
and (*E*)- β -ocimene, γ -terpinene, tricyclene, α -terpinene, β -phellandrene, (*Z*)- and (*E*)-linalool oxide, terpinolene, linalool, borneol, terpinen-4-ol, two unidentified monoterpenoids, the homoterpene (*E*)-4.8-dimethyl-1.3.7-nonatriene (DMNT), and a few sesquiterpenes, of which two were identified as β -caryophyllene and germacrene D. Occasionally, we observed some C6 green leaf volatiles, basically (*Z*)-3-hexenol and (*Z*)-3-hexenyl-acetate, and further, methylsalicylate, a derivative of the hormone salicylic acid.

Generally, the emission rates of individual VOC were positively correlated with each other (coefficients of linear correlations $R^2 > 0.3$) within compound classes (monoter-

penes, sesquiterpenes, and green leaf volatiles) but not across compound classes ($R^2 < 0.1$, data not shown). However, DMNT, methylsalicylate, and the monoterpene alcohols linalool and terpinen-4-ol scaled positively with the emissions of sesquiterpenes and much less with the emissions of other monoterpenes. Therefore, these compounds were pooled together with sesquiterpenes as a class of semi-volatiles.

Emissions of semivolatiles were highest in the first phase of the experiment and decreased rapidly during the following phases in all groups irrespective of the plants' treatment or origin (Fig. 2c). Green leaf volatiles occurred irregularly without relation to water stress or plant origin (data not

Fig. 2. Standard VOC emission rates (emission potentials) of stressed (S) (solid symbols) and unstressed (control (C)) (open symbols) cork oak saplings originating from three populations located along a climate gradient in northern Tunisia (diamonds, Tabarka (ta), subhumid; triangles, Ain Draham (ad), humid; squares, Jendouba (je), semi-arid) during the diverse phases of the experiment: (a) Monoterpene (MT) emissions per leaf area, (b) MT emissions per leaf dry mass, and (c) emissions of semivolatiles (SV, (sesquiterpenes, methylsalicylate, DMNT, linalool, and terpinen-4-ol)) per leaf area. All measurements were made at 30 °C and a light intensity of about 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD. Data are means \pm SE of $n = 5$ plant replicates. Annotations in Figs. 2a and 2b indicate significant ($P < 0.05$) effects of treatment (T) or plant origin (O) (ANOVA detected no significant interactions between T and O). Differences among groups are given in parentheses (ns, not significant). There were no significant T or O effects in SV emissions (Fig. 2c). PRE, prestress; S1, light water stress; R1, recovery after light water stress; S2, severe water stress; R2, recovery after severe water stress.



shown). Monoterpene emissions from control plants of all populations were stable during the first part of the experiment (until R1) and substantially decreased afterwards dur-

ing S2 and R2 (Figs. 2a and 2b). This decrease in the second part of the experiment coincided with a period of unstable weather conditions and an overall reduction in global radiation and air temperature (see Materials and methods) and therefore can be attributed to weather effects on the foliar capacity of unstressed control plants to produce monoterpenes. Monoterpene emission rates of treatment plants were similar to those of control plants at the beginning of the experiment before application of water stresses (PRE). After the application of a light water stress (S1), emissions were significantly increased by about 30% and remained high after recovery (R1).

During S2, emissions were strongly reduced with respect to the PRE level and were significantly lower than emissions of control plants. After R2, emissions from stressed plants remained low although not significantly different from those of the control plants.

In none of the phases were monoterpene emissions, expressed on a leaf area basis, significantly different among populations. However, on a leaf dry mass basis, emission rates of je plants were significantly higher than those of ad plants during S2.

Plant biomasses and leaf traits

Mean aboveground and belowground biomasses of control plants were similar in all populations (Fig. 3). Drought treatments significantly reduced leaf, stem, and root biomasses, whereas they had no effect on fine root biomasses (Fig. 3c) for which the differences between stressed and control plants were only marginally significant ($P = 0.063$) owing to the large variability among individuals. Premature leaf senescence and leaf shedding were observed during and after the S2 event. In particular, on je plants, foliage dry mass was almost 60% lower in stressed than in control plants. Overall, stress-related reductions in all organ biomasses were highest in the je population and smallest in the ad population.

Leaf size, specific leaf mass (SLM), and foliar nitrogen content of stressed and control saplings are shown in Table 2 in comparison with values obtained from adult field-grown trees of the same populations (A. Ennajah, unpublished results). Given that leaves of the saplings were fully developed before the experiment started, leaf size and SLM were unaffected by the drought treatments. On the other hand, across all sapling populations, foliar nitrogen content was significantly lower in treated than in control plants. In all leaf traits, there were significant differences among populations in either treated or control plants, the saplings from je showing significantly lower values than the leaves of the two northern populations, ta and ad.

Leaves sampled in situ on adult trees were on average much larger in size and had slightly higher nitrogen and lower SLM than the saplings used in the drought experiment. However, they exhibited similar differences among populations: as for the saplings, the lowest values were always found in the most southern, continental population of je.

Discussion

The effects of water limitations on isoprenoid emissions have been experimentally studied on several other plant species including the two other monoterpene-emitting evergreen

Fig. 3. Dry biomasses of control (C, open bars) and water-stressed (S, shaded bars) cork oak saplings stemming from three populations along a climate gradient in northern Tunisia (ta, Tabarka (subhumid); ad, Ain Drahem (humid); je, Jendouba (semiarid)). (a) Leaves, (b) stems, (c) fine roots, and (d) main roots. Error bars represent SDs of the mean ($n = 5$). Annotations indicate significant ($P < 0.05$) effects of treatment (T) or plant origin (O) or interactions of both ($O \times T$). Differences among groups are given in parentheses (ns, not significant).

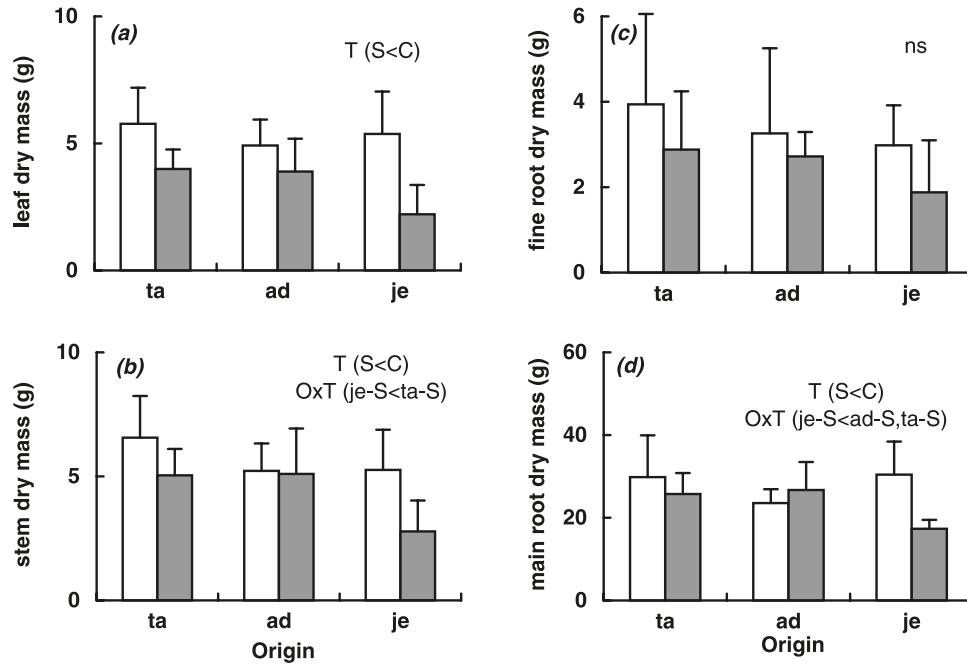


Table 2. Leaf size, specific leaf mass (SLM), and leaf nitrogen content of three Tunisian cork oak populations native to different climate conditions: Tabarka (ta), subhumid; Ain Drahem (ad) humid; Jendouba (je) semiarid.

Origin-treatment	Leaf size (cm ²)	SLM (g·m ⁻²)	Leaf nitrogen (%)
A			
ta-C	2.7±0.2	176±17	1.1±0.2
ta-S	2.6±0.2	172±7	1.0±0.1
ad-C	2.7±0.2	182±40	1.1±0.1
ad-S	2.5±0.3	166±18	0.9±0.1
je-C	1.5±0.1	153±11	1.0±0.2
je-S	1.4±0.2	156±17	0.8±0.2
ANOVA	O (je < ta, ad)	O (je < ta, ad)	T (S < C) O (je < ta, ad)
B			
ta	9.6±1.3	147±25	1.6±0.2
ad	15.0±4.3	150±10	1.8±0.2
je	7.2±1.2	129±12	1.5±0.1
ANOVA	je < ta < ad	je < ta, ad	je < ta < ad

Note: A, potted saplings exposed to water stress (S) or untreated controls (C); B, adult trees in natural populations. Treatment (T) and origin (O) effects analysed by ANOVA are indicated when significant at $P < 0.05$. Values are means ± SD of $n = 5$ (A) and $n = 10$ (B).

oaks *Quercus ilex* L. and *Quercus coccifera* L. (Bertin and Staudt 1996; Llusia and Penuelas 1998; Staudt et al. 2002; Blanch et al. 2007; Ormeno et al. 2007) as well as the isoprene-emitting deciduous oaks *Quercus virginiana* Mill., *Quercus robur* L., and *Quercus pubescens* Willd. (Brügge-mann and Schnitzler 2002; Pegoraro et al. 2004). We extended these studies by investigating the possible existence of intraspecific variability in VOC emissions and drought resistance as an adaptation to contrasting regional climate

conditions using another widespread evergreen oak species for which emissions responses to drought have not yet been described. Furthermore, we slowly applied water limitations during two consecutive drying cycles of different intensity, which represents an ecologically more realistic situation, especially for tree species developing extended root zones. To our knowledge, only two studies on isoprene-emitting plants have examined the effects of several drought cycles applying repeated water limitations of similar intensities

(Fang et al. 1996; Pegoraro et al. 2004). Finally, in this study, we focussed not only on the main emitted compounds (i.e., a few monoterpenes) but also on trace compounds including other compound classes such as sesquiterpenes for which very little information is available (Duhl et al. 2008).

Our study confirms that cork oak is a strong emitter of monoterpenes. In the three Tunisian populations, we found no low- or nonemitting individuals as has been observed in some other European populations (Csiky and Seufert 1999; Staudt et al. 2004). The monoterpene emission rates measured at standard conditions were high, similar to those found in previous studies (Staudt et al. 2004; Pio et al. 2005) and in the range of other monoterpene-producing oak species (Csiky and Seufert 1999; Kesselmeier and Staudt 1999). The emission profiles closely resemble that of *Q. ilex*, but the fraction of sabinene compared with pinenes in the emissions is generally higher in *Q. suber* than in *Q. ilex*. Other compounds, namely sesquiterpenes, DMNT, methylsalicylate, and C6 green leaf volatiles, were found more or less frequently in the emissions, which comprised, however, only a very small fraction of the total VOC release under standard conditions (on average 1.4%).

Monoterpene emissions from all Tunisian cork oak populations proved to be highly resistant to drought and were even enhanced under moderate water limitation. Thus, during S1, when Ψ_{pd} values reached about -1.5 MPa and carbon assimilation and photosystem II quantum efficiency were reduced by 20%–30%, standard emission rates of monoterpenes were increased by about 30% and remained high when plants were rewatered. Sesquiterpene emissions did not follow the same trend but tended to decrease throughout the experimental period (Fig. 2c) in both stressed and control plants. Similar to our results, Ormeno et al. (2007) observed in the VOC emissions from *Q. coccifera* a decrease in sesquiterpene and an increase in monoterpene emissions during increasing water deficit. However, while these authors attributed both changes to drought effects, our work clearly demonstrates that only monoterpene emissions from *Q. suber* were affected by water limitations, whereas the decrease in sesquiterpene emissions was unrelated to any water treatment and therefore must have been controlled by an unknown seasonal driver. Seasonal variations in sesquiterpene emissions might be associated with the atmospheric load of pathogen spores as observed by Hakola et al. (2006). Indeed, in our study, the emissions of sesquiterpenes were correlated with those of DMNT and methylsalicylate, VOCs that are typically formed by plants in response to biotic stresses (Staudt and Lhoutellier 2007). In particular, methylsalicylate and its precursor salicylate are known to be involved in plant stress responses where they may act as signalling compounds inducing defence reactions such as the activation of VOC biosynthesis (Fujita et al. 2006; Heil and Ton 2008). Thus, in our study, methylsalicylate might have been implicated in the regulation of sesquiterpene emissions but not in the regulation of drought-related changes in monoterpene emissions.

The mechanism by which moderate drought amplifies the production of volatile isoprenoids is unknown. One possibility is that the reduced transpiration rate of stressed plants increases the overall leaf temperature regime (Pegoraro et al. 2007). Temperature effectively modulates emission rates

not only in the short term but also in longer terms (Staudt et al. 2003), probably by up- and down-regulating key enzymes in the plastid isoprenoid pathway (Mayrhofer et al. 2005), thus causing oscillations in the emission potentials with weather conditions (Boissard et al. 2008), as could be observed in the present work on control trees during the second part of the experiment (Figs. 2a and 2b). Because these oscillations are slowly reversible (Staudt et al. 2003) and because in stressed trees, transpiration rates did not fully recover to PRE levels after moderate drought, emissions of treated plants remained high during R1. The emission enhancement could also be related to a decrease in C_i values during drought, when photosynthesis is essentially reduced by diffusive resistances, i.e., by stomatal closure. The biosynthesis of volatile isoprenoids was shown to be sensitive to C_i through changes in the cytosolic CO_2 -fixing enzyme phosphoenolpyruvate carboxylase, which competes with isoprenoid biosynthesis for phosphoenolpyruvate (Rosenstiel et al. 2003). Isoprene emissions generally increase with decreasing C_i (Possell et al. 2005) and this is what we observed during moderate stress when C_i levels in stressed plants were lowered compared with the PRE level and those of control plants. However, under S2, the emission potential of stressed plants was reduced, while mean C_i was not different with respect to control plants, suggesting that monoterpene biosynthesis in stressed plants was ultimately controlled by drivers other than C_i or leaf temperature, probably by a reduction in the amount and activity of enzymes regulating the chloroplast isoprenoid pathway (Fortunati et al. 2008) and (or) by the lack of primary substrates coming from photosynthetic processes (Brügmann and Schnitzler 2002), which were strongly inhibited during this phase of the experiment (Table 2).

However, the major goal of our work was to reveal potential intrinsic variability of isoprenoid emissions response to drought among cork oak populations native to contrasting climate conditions. Cork oak populations have existed for several thousands of years in northern Tunisia, which may have been one of the species refuges during ice ages (Ben Tiba and Reille 1982). In that area, geographic differentiations in adaptive traits may have evolved in response to contrasting local growth conditions as has been reported elsewhere (e.g., Diaz-Fernandez et al. 2004). Indeed, on the basis of allozyme polymorphism, Toumi and Lumaret (1998) observed a high genetic differentiation among populations located in the same regions. Given that monoterpene formation in plants may rapidly diverge and confer protection against stresses associated with summer drought (Hyatt and Croteau 2005; Owen and Peñuelas 2005), we hypothesized that leaves of populations native to hot and more arid sites are more resistant to drought and exhibit higher and (or) compositionally different VOC emissions than populations native to cooler and more humid regions. Our data suggest that VOC emissions were overall similar in quantity and quality in all populations and therefore do not support this hypothesis. Nevertheless, during S2, the highest emission rates were indeed observed in the southern je population, but this difference was only significant when emission rates were expressed per leaf mass because leaves of the je population had significantly lower SLM than leaves of the northern populations. Leaves of je plants were also smaller

in size and showed comparable high dry mass based photosynthesis rates, at least before stress. Furthermore, je plants showed the strongest reduction in photosynthesis under moderate drought and the strongest rate of leaf abscission during severe drought, suggesting that leaves of je plants were more sensitive to water limitations than those of the northern populations. Indeed, SLM is generally related to the sclerophylly of leaves (thicker leaves with more thick cuticles) and scales positively with leaf longevity (Reich et al. 1997). Although the light environment rather than water availability is likely the major determinant of SLM during leaf development (Aranda et al. 2007), leaves with high SLM are reported to better endure drought than low-SLM leaves (Westoby et al. 2002). However, plants with low SLM are expected to occupy more favourable sites than those with high SLM (Orians and Solbrig 1977), which disagrees with our observations. One may argue that the differentiation in leaf traits among cork oak populations observed in this study does not represent that occurring under natural conditions but would rather reflect limitations in the plants' plasticity to respond to optimal growth conditions because all saplings had developed their leaves in the absence of water limitations. Yet, leaf samples taken in situ from adult trees provided similar differences in leaf traits among the three populations (Table 2). Therefore, we hypothesize that low SLM and small-sized leaves that are more sensitive to water limitations and that are shed under severe drought are intrinsic traits reflecting an adaptation to arid conditions. Leaf shedding is the most effective way to reduce water losses by transpiration, thus preventing plants from lethal stresses during severe water deficits (Chaves et al. 2003 and references therein). Such extreme drought events are more frequent in the southern continental plains of je than in the northern coastal regions of ta and ad (Fig. 1). Given that in the je region, winter and spring temperatures are rather mild with short and irregular rainy periods, small fast-growing leaves with high gas exchange rates enable plants to maximize carbon gain before the onset of severe drought. Small leaves also require proportionally less investment in structural support than large leaves (Lusk and Warton 2007) and therefore are less costly and can more easily be replaced. On the other hand, in the regions with shorter drought periods and less risk of extreme stress events, larger leaves of robust structure that are able to persist and to maintain carbon assimilation under moderate water limitations are more advantageous, especially in the cooler mountainous regions.

To conclude, our results may indicate the existence of an adaptive shift from stress-tolerating ecotypes under sub-humid climates towards stress-avoiding ecotypes under semi-arid climates involving mainly morphological adaptations at the leaf and whole-plant level without apparent differentiation in the production of VOCs. Additional studies on more populations are necessary to corroborate this hypothesis. Our data further suggest that the application of one common emission potential for all populations in regional emission inventories is justified and that the protective function of foliar VOC production is less important in potential selection processes for drought resistance. However, as mentioned above, emission potentials are responsive to the prevailing temperature and light conditions, which will covary with the intensity of drought in most sites

(cf. Fig. 1). Thus, under field conditions, a given cork oak genotype is expected to have a higher average VOC production capacity when growing in warmer, arid sites than when growing in cooler sites. This high plasticity in VOC production may explain why no genetic differentiation under local growth conditions has evolved. Whether Tunisian cork oak populations indeed exhibit different emission potentials under natural conditions and whether genetic variability in VOC emission is absent within and among other cork oak provenances remain to be established.

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