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Research paper

The diversification of terpene emissions in Mediterranean oaks: lessons from a study of *Quercus suber*, *Quercus canariensis* and its hybrid *Quercus afares*

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Interspecific gene flow is common in oaks. In the Mediterranean, this process produced geographical differentiations and new species, which may have contributed to the diversification of the production of volatile terpenes in the oak species of this region. The endemic North African deciduous oak *Quercus afares* (Pomel) is considered to be a stabilized hybrid between the evergreen *Quercus suber* (L.) and the deciduous *Quercus canariensis* (Willd.), presumably being monoterpene and isoprene emitters, respectively. In a common garden experiment, we examined the terpene emission capacities, terpene synthase (TPS) activities and nuclear genetic markers in 52 trees of these three oak species. All but one of the *Q. suber* and *Q. canariensis* trees were found to be genetically pure, whereas most *Q. afares* trees possessed a mixed genotype with a predominance of *Q. suber* alleles. Analysis of the foliar terpene emissions and TPS activities revealed that all the *Q. canariensis* trees strongly produced isoprene while all the *Q. suber* trees were strong monoterpene producers. *Quercus afares* trees produced monoterpenes as well but at more variable and significantly lower rates, and with a monoterpene pattern different than that observed in *Q. suber*. Among 17 individuals tested, one *Q. afares* tree emitted only an insignificant amount of terpenes. No mixed isoprene/monoterpene emitter was detected. Our results suggest that the capacity and pattern of volatile terpene production in Algerian *Q. afares* populations have strongly diverged from those of its parental species and became quantitatively and qualitatively reduced, including the complete suppression of isoprene production.

Keywords: Algerian oak, allozyme polymorphism, chemo-taxonomy, chemotype, cork oak, isoprenoid, isoprene synthase, speciation.

Introduction

The genus *Quercus* is very diversified and widespread in the Northern hemisphere where it represents one of the most important phytogenic sources of highly reactive volatile organic compounds (Kesselmeier and Staudt 1999). Oak leaves and perhaps other green tissues constitutively produce considerable amounts of volatile terpenes (isoprenoids) in a light-dependent process from recently fixed photosynthates (e.g., Loreto et al. 1996). Biogenic terpenes emitted from vegetation are the major source of reduced carbon released into the atmosphere and are important precursors to the photochemical production of ozone and secondary organic aerosols (e.g., Peñuelas and Staudt 2010). To date, all oak species studied for volatile organic compound emissions were found to constitutively emit the hemiterpene isoprene (C5), with the exception of a few Mediterranean species, among which some monoterpene (C10) emitters and some non-emitters have been identified (Csiky and Seufert 1999, Harley et al. 1999, Loreto 2002). The mechanisms and driving forces underlying this apparently unique diversification in European oaks are still unknown. In

fact, the extent of intraspecific variability in volatile terpene production in oaks has been rarely studied (e.g., Staudt et al. 2001, Loreto et al. 2009) and the biological and ecological roles of this production are not completely understood. Several hypotheses on the function of chloroplastic isoprene formation have been put forward and independently tested (for recent overviews see Loreto and Schnitzler 2010, Velikova et al. 2011, Way et al. 2011). Isoprene may confer resistance to high temperature and oxidative stress and may maintain metabolic homeostasis between subcellular compartments. Theoretically, the diversification of terpene emissions in European oaks could result from the natural selection of chemotypes under varying growth conditions, because all volatile terpenes formed in chloroplasts may not have the same production costs and protection abilities (Niinemets 2004, Copolovici et al. 2005). However, a clear relationship between ecotypes and chemotypes has not yet been demonstrated for oaks (Staudt et al. 2008).

The variability of terpene emissions within and between oak populations has only been extensively investigated in the monoterpene-emitting oak *Quercus ilex* L. (Staudt et al. 2001, 2004) and *Quercus suber* (L.) (Staudt et al. 2004, 2008, Loreto et al. 2009). For both species, inherent differences in the emission profiles of individual trees could be detected. The abundance of these chemotypes varied among populations, perhaps reflecting the palaeogeographic fragmentation of the range of species in the Mediterranean area and selective adaptation of ecotypes to new habitats (Loreto et al. 2009). Other potential sources contributing to the diversification of isoprene and monoterpene production in oaks are the selection and breeding associated with human activities and the naturally occurring interbreeding between sympatric oak species (Staudt et al. 2004). Hybridization and subsequent genetic introgression by one parental species are common phenomena in European oak populations, thus making unambiguous species discrimination difficult (Piredda et al. 2011). In some cases, hybridization resulted in stable geographical differentiations or even new species (Bellarosa et al. 1996). Such populations may have peculiar terpene emission profiles if the parental species differed in their terpene production capacities. For example, early-generation hybrids (*Q.* × *turneri*) between the monoterpene emitter *Q. ilex* and the isoprene emitter *Q. robur* have been shown to emit both terpene classes (Schnitzler et al. 2004).

Here we focus on populations of *Quercus afares* (Pomel), *Quercus suber* (L.) and *Quercus canariensis* (Willd.). Genetic studies by Mir et al. (2006) conducted on sympatric oak populations in Northern Africa provided evidence that the deciduous oak *Q. afares* results from an ancient stabilized hybridization between the evergreen *Q. suber* and the deciduous *Q. canariensis,* presumably being monoterpene and isoprene emitters, respectively (*Csiky and Seufert 1999, Staudt et al. 2004*). *Quercus afares* is endemic to the coastal mountainous regions of northern Algeria and Tunisia. Its distribution extends from 200 m to 1200–1600 m of elevation. Contact zones with *Q. suber* (mixed stands) are most common in the lowlands while contact zones with *Q. canariensis* are more frequent at mid-altitudes, particularly above 700 m (see Mir et al. 2006 and references therein). *Quercus afares* also constitutes monospecific stands, especially on fire-degraded soils above 1200 m.

In the present work, we aimed to analyse the degree of variation in the terpene emission patterns of populations of these three oak species in a common garden experiment. More specifically, we wanted to know whether the terpene production characteristics in Mediterranean oaks are rather invariable traits that are conserved over many generations and could be used as chemotaxonomical markers to identify mixed genotypes resulting from interspecific gene flow or to classify oak species within the genus *Quercus*, or whether these traits are unstable and evolve rapidly, perhaps as an adaptive response caused by shifts in ecological requirements. To address these questions, we studied the foliar terpene production pattern together with the genetic markers (allozyme variation) of 52 adult trees originating from northeastern Algeria. Terpene emissions and CO_2/H_2O gas exchanges were quantitatively measured under environmentally controlled conditions and were complemented by the analysis of the activities of isoprene and monoterpene synthases (mono-TPSs). Compared with emission data, the terpene synthase (TPS) activity might be more efficient in detecting inherent quantitative differences between individuals/populations because the actual terpene emission rate of leaves is more strongly modulated by environmental factors than the in vitro TPS activity, which reflects the leaf's intrinsic capacity for isoprene and/or monoterpene biosynthesis (Lehning et al. 1999, Schnitzler et al. 2004). To date, the TPS activities in oaks have been mainly studied to examine their temporal variability and less for elucidating intraspecific variability (Lehning et al. 2001, Fischbach et al. 2002, Lavoir et al. 2009).

Materials and methods

Plant material and sampling protocol

Quercus suber, *Q. canariensis* and *Q. afares* were grown from acorns collected from native populations in eastern Algeria in the massif of Akfadou (36.48°N, 4.30°E) (Acherar and Rambal 1992), and they were cultivated since 1986 in a common garden experiment close to our institute in Montpellier (43°36′43″N, 3°52′38″E), France. The garden consists of a rectangular 6 × 15 m plot extending in east–west direction with an average tree density of ~1.2 m^{-2}. The canopy reached a height ~3.5 m. The soil in the garden is a loamy clay soil.

All measurements were made using terminal light-exposed leaves (current-year leaves) of the upper tree crowns, which

were accessed using a scaffold tower, during August and the beginning of September under sunny and hot weather conditions. Average air temperature during the measurement period (between 9 a.m. and 4 p.m.) was 27.4 °C. On 17–18 individual trees of each population, terpene emissions were measured twice on the same leaves in two successive measurements: (i) in the field on intact branches using a portable photosynthesis system (GFS-3000 Walz, Effeltrich, Germany) and (ii) on freshly cut branches in a nearby laboratory. We applied two different cuvette systems, because commercial photosynthesis devices do not allow accurate analysis of low emission rates (see Niinemets et al. 2011 for an overview on methodological aspects). Additional leaves were collected to determine nuclear genetic markers and in vitro activities of the isoprene and mono-TPSs. Individuals were assayed randomly by alternating the species every day to avoid confounding of potential weather and daytime effects with species effects. Furthermore, at the end of the campaign terpene emission and enzyme activity measurements were repeated on three individuals of each population to assess the temporal and intra-tree variability of foliar terpene biosynthesis and emission. All trees were watered regularly during the experimental period to prevent them from experiencing water stress.

Terpene sampling and analysis

All terpene emission measurements were performed after an acclimation period of the enclosed leaves of at least 30 min to the light and temperature measuring conditions of ~1000 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and 35 °C. This temperature was chosen because it was close to the actual leaf temperature of sun-exposed leaves during the hours of measurement and could be achieved and maintained by the air conditioning of the commercial gas exchange system under all circumstances. For emission measurement, a healthy leaf was clamped in the standard leaf chamber (8 cm^2 window) and flushed with ambient air at a rate of 0.4 l min^{-1}. An ozone scrubber device consisting of eight layers of MnO_2-coated copper nets was placed in the inlet air of the system to avoid the decomposition of ozone-sensitive terpenes. After acclimation and stabilization of photosynthetic gas exchange, two air samples, one for isoprene and one for monoterpenes, and other semi-volatiles were taken from the chamber air via a PTFE T-fitting connected to the chamber outlet line: (i) a gas-tight glass syringe wrapped with aluminium foil was gently filled with ~100 ml of air and immediately brought to the laboratory where it was connected to an online gas chromatograph (GC) for analysis of isoprene; and (ii) 2 l of outlet air were sucked through a glass cartridge filled with 200 mg Tenax TA (20–35 mesh, Agilent, Geneva, Switzerland) at a flow rate of 0.1 l min^{-1}. The cartridge was analysed directly on the day of sampling or at the latest, the day afterwards, after being stored at 4 °C in darkness.

After terpene sampling in the field, the whole branch was cut under water and transferred to the laboratory for the second terpene emission measurement. This was performed with a custom-made dynamic temperature and light-controlled enclosure system consisting of a flat rectangular chamber of ~ 105 ml volume (Bracho-Nuñez et al. 2011). The chamber was continuously flushed with compressed air (Ingersoll Rand compressor Mod. 49810187, Baurès Montpellier, France) at a constant rate of 0.5 l min^{-1}. Before entering the chamber, the air was cleaned and dried in a clean air generator (AIRMOPURE, Chromatotec, Bordeaux, France) and re-humidified to achieve relative humidity of 30–40% in the chamber inlet. The air and leaf temperature inside the chamber were measured by two thermocouples and PPFD outside of the chamber by a LiCOR Quantum sensor (PAR-SB 190, LiCOR Inc., Lincoln, NE, USA). Data were recorded on a Campbell 21x data logger. The chamber was illuminated with a white light source (OSRAM 1000 W) filtered by a 5-cm water bath. Measurements of CO_2/H_2O gas exchange were made by directing a part of the air entering and exiting the chamber through a LiCOR 6000 infrared gas analyser (LiCOR Inc., Lincoln, NE, USA) that was run in estimated reference mode. Monoterpenes were trapped on cartridges as in the field and analysed offline by GC. Isoprene emission was analysed online by coupling the chamber outlet air to an online GC system. After the emission measurements, the leaves were harvested for the determination of the projected leaf area (Delta-T Area Meter MK2, Delta-T Devices Ltd, Cambridge, UK), and the dry mass after drying for at least 48 h at 60 °C. Between two emission measurements, the chambers and sampling lines were flushed with ambient air for at least 30 min to remove terpene residuals.

Isoprene was analysed by an AirmoVOC C2-C6 online GC (Chromatotec, Bordeaux, France) with a flame ionization detector (FID). The instrument continuously drew air from the syringe or chamber air via a 1/8 inch PTFE tubing at a flow of 12 ml min^{-1}. For analysis, the air was directed for 5 min into the internal multi-phase trap, which was maintained at –10 °C and subsequently flash-heated for 2 min to release the trapped isoprene into a fused silica PLOT Al_2O_3/KCl column. The temperature programme of the oven was: 1 min at 40 °C, 15 °C increase per min up to 180 °C, 20 min at 180 °C. The online GC was calibrated with an isoprene gas standard in nitrogen (Deuste Steininger GmbH, Mühlhausen, Germany).

Monoterpenes and other semi-volatiles trapped in the cartridge were analysed by a Chrompack CP9003 GC-FID equipped with a Chrompack TCT4002 thermo-desorber (all Agilent Technologies, Geneva, Switzerland). Before desorption, the tubes were pre-flushed with pure nitrogen (flow 40 ml min^{-1}) for 1 min at room temperature to remove excessive humidity. The terpenes were separated on a Chrompack Sil 8CB low bleed capillary column (30 m × 0.25 mm) using the following temperature programme: 3 min at 40 °C,

3 °C min^{-1} to 100 °C, 2.7 °C min^{-1} to 140 °C, 2.4 °C min^{-1} to 180 °C, 6 °C min^{-1} to 250 °C. Peaks were identified and quantified by comparison with pure standards dissolved in MeOH that were analysed under the same conditions. In addition, GC analyses coupled with mass spectrometry were performed to confirm compound identifications (for details see Bracho-Nuñez et al. 2011).

Analysis of isoprene synthase and mono-TPS activities

All preparation of protein extracts from individual trees was started from two shock-frozen leaves sampled adjacent to the leaves where gas exchange analysis was performed. Samples were stored until use at −80 °C. Transfer of samples from Montpellier to Germany was performed on dry ice. Protein extractions and foliar isoprene synthase (ISPS) and mono-TPS activities were performed according to established standard protocols for oaks described in Schnitzler et al. (2004).

Genetic markers

Proteins were extracted from leaves in a Tris-HCl buffer (pH 7.6) and were stored at −80 °C until analysis (Toumi and Lumaret 2001). Horizontal starch-gel electrophoresis and staining were performed as described in Mir et al. (2006) for five enzyme systems corresponding to five polymorphic loci, three diagnostic and two semidiagnostic (a single common allele) loci between *Q. canariensis* and *Q. suber*. These are as follows: phospho-glucose isomerases (EC 5.3.1.9., locus *PGI-1*), alcohol dehydrogenases (EC 1.1.1.1, locus *ADH-1*), isocitrate dehydrogenases (EC 1.1.1.42, *IDH-1*), leucine amino-peptidases (EC 3.4.11.1, *LAP1*) and acid phosphatases (EC 3.1.3.2, *AcPh-1*).

Data treatments and calculations

Isoprene and monoterpene emission rates were calculated as the difference between the air concentration in the chamber enclosing a shoot and the concentration measured in the empty chamber multiplied by airflow and divided by the projected leaf area (ng m^{-2} s^{-1}) or leaf dry mass (μg g^{-1} h^{-1}). Empty chamber measurements were made once a day and the mean of all empty chamber measurements was used for background corrections.

The basal emission rates of the tree species emission rates were assessed by normalizing the emission rates to a leaf temperature of 30 °C assuming a Q_{10} of 2.66 for both isoprene and monoterpene emissions (Niinemets et al. 2011). Net CO_2 assimilation and transpiration rates and other gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981).

Paired *t*-tests were used to compare terpene emission and gas exchange data measured in the field with those measured in the laboratory. One-way analyses of variance (ANOVAs) were performed to evaluate data for differences among the three oak species (SigmaStat 2.0 Jandel Scientific Software, San Jose, CA, USA). If the data failed assumptions of normality and homogeneity of variance, the non-parametric Kruskal–Wallis test was applied. Differences were considered significant at $P < 0.05$.

A model-based clustering method implemented in the software STRUCTURE version 2.2 (Pritchard et al. 2000) was used to identify the 52 sampled individuals with *Q. canariensis* or *Q. suber* ancestry and to estimate the part of their genome attributable to either parental species based on their genotype score at the five polymorphic loci (multi-locus genotypes). Data from all loci were used to calculate the posterior probabilities of membership by assuming two groups of individuals ($K = 2$) corresponding to the two parental species. Assuming admixture and correlated allele frequencies, 2×10^4 burn-in periods and 3×10^4 Markov chain Monte Carlo simulations were performed. A probability value ≥0.95 belonging to either parental species was assumed to indicate a non-mixed (pure-bred) genotype and a value <0.95 to indicate a mixed genotype.

Results

Genetic markers

In the clustering analysis, no mixed genotype was observed in the *Q. suber* population, in which all individuals were grouped in the same cluster. The same situation was observed for *Q. canariensis*, where all individuals were grouped in the second cluster, with the exception of tree number 3, in which 7% of its genome was attributed to *Q. suber* (Figure 1). As expected in a hybrid species, 13 out of the 17 *Q. afares*

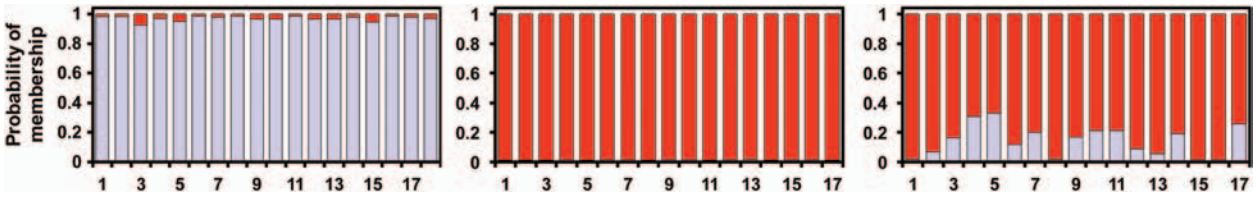

Figure 1. Ancestry estimates based on posterior probabilities ($K = 2$) of belonging to *Q. canariensis* (light blue) and to *Q. suber* (red) from the Bayesian analysis of allozyme variation at five diagnostic loci in 52 individuals identified morphologically as *Q. canariensis* (left), *Q. suber* (middle) and *Q. afares* (right).

individuals displayed a mixed genotype, with a probability ranging from 67 to 94% of belonging to *Q. suber*. The four other *Q. afares* individuals had a 95–98% probability to belong to this species. These individuals possessed allele characteristics of *Q. suber* at the three diagnostic loci between the parental species and alleles common to both parents at the two semidiagnostic loci.

Terpene emissions and CO_2/H_2O gas exchange

Individuals of *Q. canariensis* morphotype almost exclusively emitted isoprene (> 99%), whereas individuals of *Q. suber* and *Q. afares* morphotypes almost exclusively emitted monoterpenes (> 99%), in which α-pinene, sabinene, β-pinene, myrcene and limonene were the main compounds emitted. (*Z*)- and (*E*)-β-ocimene were occasionally observed in the emission profiles of each oak species, and traces of α-thujene, camphene, α- and γ-terpinene and terpinolene were only present in the emission profile of *Q. suber*.

Measurements in the field on intact branches and measurements in the lab on cut branches yielded similar terpene emission rates and patterns. Trace monoterpene emissions were more scattered and overall lower in the field than in the laboratory due to the higher background noise and lower detection limits associated with the commercial photosynthesis system. However, average emission rates of cut *Q. canariensis* branches were lower than intact *Q. canariensis* branches (2291 ± 810 (SD) vs. 3601 ± 1406 ng m^{-2} s^{-1}, $P = 0.004$), while oppositely, mean emission rates of cut *Q. suber* and *Q. afares* branches were higher than those of intact branches (*Q. suber*: 3509 ± 1950 vs. 2472 ± 976 ng m^{-2} s^{-1}, $P = 0.054$; *Q. afares*: 2105 ± 1203 vs. 1168 ± 804 ng m^{-2} s^{-1}, $P = 0.005$). Net CO_2 assimilation and transpiration rates showed no difference between field and laboratory measurements in all cases ($P > 0.05$). Mean values (±SD) across all three species of net CO_2 assimilation and transpiration rates were, respectively, 5.6 ± 3.5 μmol m^{-2} s^{-1} and 1.7 ± 1.1 mmol m^{-2} s^{-1} in the field and 4.3 ± 2.8 μmol m^{-2} s^{-1} and 2.0 ± 1.1 mmol m^{-2} s^{-1} in the laboratory. For further data evaluation, we used the means of both field and laboratory measurements.

Mean actual emission rates based on a leaf area or derived basal emission rates were significantly lower in *Q. afares* than in the two putative parental species that emitted isoprene and monoterpenes at almost equal rates (Table 1). On a dry mass basis, terpene emission rates were highest for *Q. canariensis*, because its leaves had significantly lower leaf masses per area than the leaves of *Q. suber* and *Q. afares*. Mean net CO_2 assimilation, transpiration rates and derived CO_2/H_2O gas exchange parameters, such as stomatal conductance and water use efficiency (data not shown), were highly variable in each population and not significantly different between the three species. The percentage loss of assimilated carbon by volatile terpene production ranged between 1 and 20% and was significantly lower in *Q. afares* leaves than in *Q. canariensis* leaves on average (Table 1).

On a relative scale, the lowest between-tree variability in the total emission of terpenes was observed in *Q. canariensis* (coefficient of variation: *Q. canariensis:* ~27%, *Q. suber:* ~39%, *Q. afares:* ~51%), whose emission pattern also showed the lowest variability (Figure 2, upper graphs). In fact, all *Q. canariensis* individuals emitted large amounts of isoprene. Traces of monoterpenes (<30 ng m^{-2} s^{-1}), mostly limonene, were observed in eight out of 17 trees. *Quercus suber* also showed relatively low variation in terpene release; all the individuals emitted monoterpenes in high amounts, principally pinenes and sabinene, except for four trees that also emitted large amounts of limonene. Very low isoprene emission rates (19 ± 5 ng m^{-2} s^{-1}) were recorded in most *Q. suber* measurements, though not in all. Traces of isoprene were also detected in the emissions of all *Q. afares* trees (28 ± 9 ng m^{-2} s^{-1}) without having an apparent correlation with the presence of *Q. canariensis* alleles in their genetic fingerprints. In contrast to *Q. suber*, the emission profile of *Q. afares* trees was dominated by limonene and had pinenes and sabinene only as by-emissions. However, four individuals showed an intermediate terpene pattern, as observed in the *Q. suber* population. Furthermore, one *Q. afares* individual (no. 7) with a low net CO_2 assimilation rate (1.1 μmol m^{-2} s^{-1}) emitted only traces of ocimenes. Terpene emission rates and net CO_2 assimilation rates were weakly

Table 1. Standard terpene emission rates expressed on a leaf area (E_S area) and dry mass (E_S mass) basis, net CO_2 assimilation (A_N), relative C-losses by terpene emissions and leaf masses per area (LMA) of the three oak species. Statistical results from ANOVA and Kruskal–Wallis tests (C-loss) are shown. DF residuals: 49; ns: not significant.

	Q. canariensis (ca; $n = 18$)	*Q. suber* (su; $n = 17$)	*Q. afares* (af; $n = 17$)	*P* value	Pairwise comparison
E_S area[1] (ng m^{-2} s^{-1})	1832 ± 500	1854 ± 707	1022 ± 523	<0.001	af < ca, su
E_S mass[1] (μg g^{-1} h^{-1})	55.9 ± 13.7	43.1 ± 17.0	25.8 ± 12.5	<0.001	af < su < ca
A_N[2] (μmol m^{-2} s^{-1})	4.4 ± 2.4	5.2 ± 2.8	5.4 ± 2.9	0.517	ns
C-loss[2] (%)	9.0 ± 7.2	6.8 ± 4.3	3.8 ± 2.5	0.016	af < ca
LMA (g m^{-2})	119 ± 21	158 ± 28	142 ± 22	<0.001	ca < su, af

[1]Normalized to temperature of 30 °C.
[2]Under assay conditions, i.e., 35 °C and saturating light.

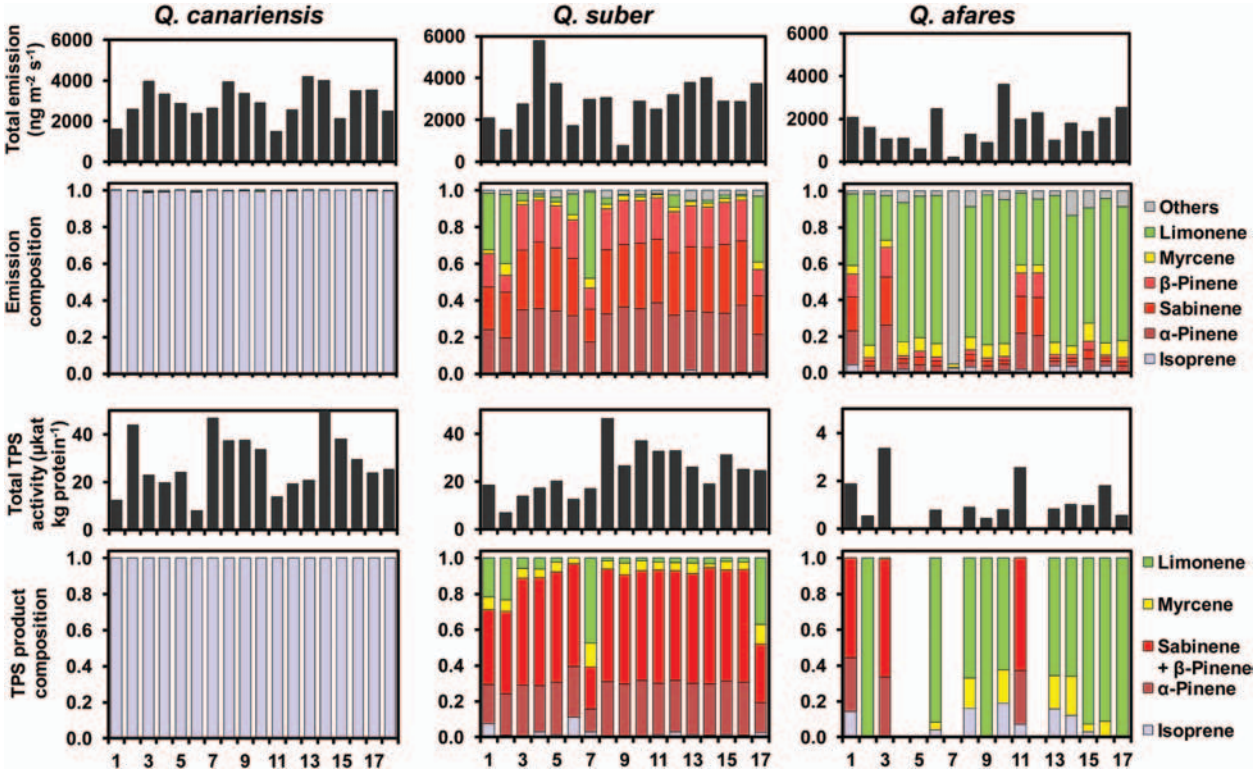

Figure 2. Foliar terpene (isoprene and monoterpene) emissions (upper graphs) and TPS activities (lower graphs) of 52 individual *Q. canariensis* (left), *Q. suber* (middle) and *Q. afares* (right) trees growing in a common garden experiment. Black columns show the sum of terpene emissions and TPS activities and coloured columns show the relative contributions of isoprene and single monoterpenes. Note that in the enzyme activity assays sabinene and β-pinene could not be separated from each other.

correlated in *Q. afares* ($R^2 = 0.20$, $P = 0.07$). No correlation was found for both parameters in the two other species (*Q. suber*: $R^2 = 0.08$, $P = 0.25$; *Q. canariensis*: $R^2 = 0.02$, $P = 0.60$).

Repeated measurements performed on some individuals revealed that isoprene and monoterpene emission rates more or less varied between different leaves of a given tree, whereas the relative emission patterns were almost stable (Figure 3). No significant emissions of isoprene or monoterpenes were found in the replicate measurement of *Q. afares* no. 7, despite a fairly high photosynthetic activity (2.9 µmol m^{-2} s^{-1}) that led us to suggest that all leaves of this individual had a very low capacity to produce volatile terpenes.

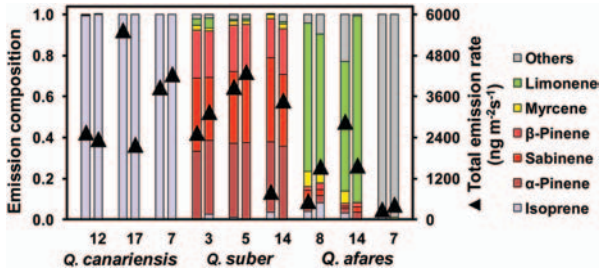

Figure 3. Emission profiles (columns, left-hand *y*-axis) and total amount (triangles, right-hand *y*-axis) of two replicate measurements made on nine trees (three per species).

Isoprene and mono-TPS activities

Measurements of ISPS and mono-TPS synthase activities (Figure 2, lower graphs) confirmed the intraspecific and interspecific variability of foliar terpene production observed in the emission data. However, some discrepancies with the emission data became obvious. Mono-TPS synthase activity could never be detected in *Q. canariensis* leaves, even in those individuals with trace emissions of monoterpenes. By contrast, low ISPS activity was detected in all *Q. suber* and in many *Q. afares* individuals. Similar to isoprene emission rates, average catalytic rates of ISPS were about two orders of magnitude lower in *Q. suber* and *Q. afares* than in *Q. canariensis* leaves. Surprisingly, in all *Q. afares* samples, mono-TPS activity was very low as well, whereas in *Q. suber*, mono-TPS activity was in a range similar to that of ISPS in *Q. canariensis* leaves. On average, total TPS activities of *Q. canariensis* and *Q. suber* were not significantly different and were both significantly higher than the total TPS activity of *Q. afares* (Kruskal–Wallis test, $P < 0.001$). No TPS activity at all could be detected in four *Q. afares* individuals, among which was the apparent non-emitter (no. 7). Nevertheless, distinct differences in the enzymatic monoterpene formation pattern could be observed among *Q. afares* individuals and among *Q. suber* individuals expressing different

TPS activities, which approximately corresponded to the observed differences in the emission pattern. For example, consistent with the emission pattern, the *Q. afares* individuals 1, 3 and 11 expressed TPS activity for pinenes and sabinene, while most other individuals possessed an enzyme activity for limonene formation. Interestingly, the total TPS activity of the three *Q. afares* individuals producing pinenes and sabinene was on average higher than that of the limonene-producing individuals (2.6 ± 0.7 ($n = 3$) vs. 0.9 ± 0.4 μkat kg protein^{-1} ($n = 10$)). The results from replicate TPS activity assays performed on a few trees suggested that the activity pattern is quite stable and inherent for all leaves of a given tree (data not shown).

Discussion

We determined allozyme markers, terpene emissions and TPS activities of three sympatric oak species in North Africa; the endemic species, *Q. afares*, has likely been derived from the other two (*Q. canariensis, Q. suber*). *Quercus afares* has never been studied for foliar terpene production and its classification within the genus *Quercus* has been unclear (Mir et al. 2006). European oaks are known to constitutively produce different amounts and classes of terpenes and it has been suggested that this diversification of foliar terpene production in European oaks reflects their taxonomic relations and biogeographic origins (Csiky and Seufert 1999, Harley et al. 1999, Loreto 2002, Loreto et al. 2009). European oaks encompass three groups (Figure 4; Manos et al. 1999). First, the group of white oaks (*Quercus* s.s. or *Lepidobalanus*), of which all species, including

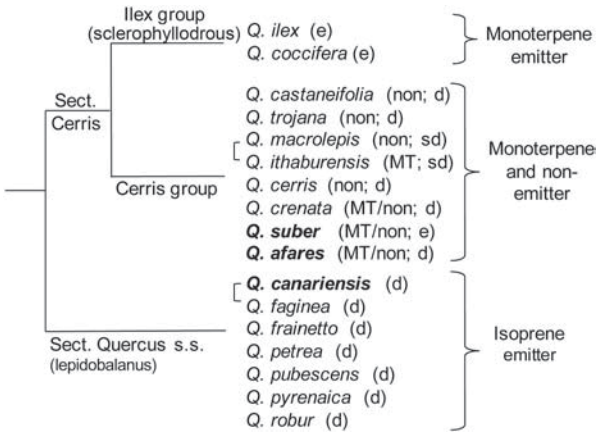

Figure 4. Simplified scheme showing the classification of European and North African oaks (adapted from Manos et al. 1999) that have been screened for terpene emissions according to Steinbrecher et al. (1997), Csiky and Seufert (1999), Loreto (2002), Loreto et al. (2009) and this study (bold species names). MT: monoterpene emitter; non: non-emitter; MT/non: species for which both monoterpene-emitting and non-emitting individuals have been reported; e: evergreen; d: deciduous; sd: semideciduous. Brackets heading pairs of species names denote two very closely related species that are sometimes considered to be single species.

Q. canariensis, are reported to emit isoprene. *Quercus canariensis* is a close relative of *Q. faginea* and both have been studied only once on a few saplings by Csiky and Seufert (1999), who described them as weak and strong isoprene emitters, respectively (E_S of 11 and 111 μg g^{-1} h^{-1}). Our results confirm that *Q. canariensis* emits mainly isoprene with standard emission rates falling in the range of those reported for the same species and other isoprene-emitting oak species (Csiky and Seufert 1999, Kesselmeier and Staudt 1999). The second group is the *Ilex* or *Sclerophyllodrous* group that consists of the two circum-Mediterranean evergreen oaks *Q. ilex* and *Q. coccifera*. Both species have been reported to emit large amounts of monoterpenes. The third group, which is called *Cerris* and to which *Q. suber* belongs, includes monoterpene-emitting species (e.g., *Q. ithaburiensis*), non-emitting species (e.g., *Q. cerris*) and possibly in Asia also isoprene-emitting species (Loreto 2002). *Quercus suber* has been studied several times for its terpene emissions and was mostly described as a strong monoterpene emitter (Staudt et al. 2004, Pio et al. 2005, 2008, Loreto et al. 2009), which is further corroborated by the results of the present study. However, *Q. suber* was initially considered to be a non-emitter because non-emitting individuals have been observed within some *Q. suber* populations in Italy and France (Steinbrecher et al. 1997, Delfine et al. 2000, Staudt et al. 2004; Staudt and Hilbig, unpublished results). *Quercus crenata*, a natural hybrid of *Q. suber* × *Q. cerris* was also described as both a non-emitter and monoterpene emitter (Loreto 2002, Loreto et al. 2009). Furthermore, Csiky and Seufert (1999) outlined that the apparent non-emitting species *Q. macrolepis* and the monoterpene-emitting *Q. ithaburiensis* are two very closely related species that are often considered to be a single species. These details collectively suggest that the classification in monoterpene and non-emitting oak species within the *Cerris* group is not always distinct and that both high interspecific and intraspecific variability of the foliar terpene emission capacity is typical in this group. In the present study, *Q. afares* trees by far expressed the strongest intraspecific variability by having monoterpene emission capacities ranging over one of magnitude between 200 and 3600 ng m^{-2} s^{-1} (6–89 μg g^{-1} h^{-1}). Thus, the emission traits we found for *Q. afares* assign this species to the *Cerris* group, which corroborates previous classifications based on morphological traits and genetic markers (Mir et al. 2006 and references therein). They also demonstrate that monoterpene emission is not a trait specific to evergreen oaks in the Mediterranean (see, e.g., Loreto 2002) but can also occur in deciduous oaks, as already indicated by the study of Csiky and Seufert (1999) who observed monoterpene emissions from the semi-deciduous oak *Q. ithaburensis*.

The field survey by Mir et al. (2006) provided clear evidence that *Q. afares* originates from ancient hybridizations between *Q. suber* and *Q. canariensis*, with *Q. suber* being the maternal

species. Our genetic analysis confirmed that the *Q. afares* genome shares alleles from both species and has a predominance of *Q. suber* alleles. Given that both parental species were strong isoprene and monoterpene emitters, respectively, we hypothesized that *Q. afares* would be a strong terpene emitter as well and that some individuals would display mixed emission patterns, as reported for early-generation hybrids of *Q. ilex* × *Q. canariensis* (Staudt et al. 2004). Furthermore, we expected that the monoterpene emission pattern of *Q. afares* would be similar to its parent *Q. suber.* To our surprise, we observed no individual with mixed isoprene/monoterpene emissions or mixed ISPS/mono-TPS activities. Moreover, we found that the mean emission capacity and TPS activity of *Q. afares* were significantly lower with respect to the parental species and that the monoterpene pattern of *Q. afares* was dominated by limonene while that of *Q. suber* was constituted predominantly by a mix of pinenes and sabinene. We also noted a discrepancy between the mono-TPS activity of *Q. afares* and its emission capacity, the former being extremely low and sometimes undetectable even though most *Q. afares* trees emitted monoterpenes at relevant rates.

Taken together, our results led us to suggest that the capacity and pattern of volatile terpene production in Algerian *Q. afares* populations have strongly diverged from those of their parental species and became quantitatively and qualitatively reduced, including the complete suppression of isoprene production. What could be the mechanisms behind this apparent diversification? Volatile terpenes exist in numerous isomers that are formed by single or multi-product enzymes and encoded by strongly related genes (Christianson 2008, Chen et al. 2011). Comparative and functional genomics studies have shown that the product pattern of TPS genes can evolve rapidly in plants by gene duplication, allelic variation and changes in the transcription regulation (e.g., Köllner et al. 2004, Huang et al. 2010). Inbreeding can efficiently reduce the number of single terpenes and the total amount of terpene produced as demonstrated by Delphia et al. (2009) for the volatile production by horsenettle (*Solanum carolinense*). We believe that the impoverished and quantitatively variable terpene production in *Q. afares* results from bottlenecks and subsequent genetic drift acting at the early stages of the species establishment and leads to a restricted gene pool. Mir et al. (2006) concluded that *Q. afares* has differentiated to a new stabilized species for a long time and that limited backcrosses of hybrids with the parental species happened early, rarely and exclusively with *Q. suber. Quercus afares* possibly evolved from a relatively few initial hybridizations followed by recurrent mating events in rather small populations (Mir et al. 2006), whereby a high amount of non-functional alleles and TPS transcripts with a fast turnover could have arisen. Apparently, isoprene biosynthesis became most rapidly and strongly suppressed in the accessions of *Q. canariensis* × *Q. suber* hybrids. Further, the fact that mono-TPS activities in *Q. afares* leaves were extremely low despite moderate emission rates suggests that mono-TPS enzymes in this species are particularly unstable and/or that *Q. afares* leaves contain protein-destroying metabolites (e.g., tannins) that could not be neutralized by buffer systems, which are commonly successful for intact isolation of TPS activity from tree tissues (Fischbach et al. 2001, Schnitzler et al. 2004).

Natural selection could also have contributed to the differentiation and suppression of volatile terpene production in *Q. canariensis* × *Q. suber* accessions. The constitutive formation of isoprene and monoterpenes in chloroplasts can confer thermotolerance and antioxidative protection to the foliar photosynthetic system (for overview see Loreto and Schnitzler 2010), but due to their volatility, this production entails a considerable loss of biochemical energy and amounts of reduced carbon provided by photosynthetic processes (Table 1). We observed no clear differences among the photosynthetic performances of the three oak populations. However, the correlation between terpene emission and photosynthesis exclusively observed within the *Q. afares* population suggests that these processes were most interdependent in this species and that low terpene-emitting *Q. afares* individuals were less competitive under hot summer conditions. Compared with its parental species, *Q. afares* populations are more frequently at higher altitudes in North Africa (Mir et al. 2006). A shift in the species distribution towards cooler and less arid climates might explain why low emitters have survived and even been favoured over strong emitters in *Q. afares* populations. In addition, the loss of protection by volatile terpenes in *Q. afares* might have been functionally replaced or compensated by changes in metabolomic homeostasis (Behnke et al. 2009).

In conclusion, our results suggest that foliar terpene emissions can differentiate rapidly in oak populations and that the capacity to produce both isoprenes and monoterpenes is not preserved in populations ascending from hybrids between isoprene and monoterpene emitters. In the case of the North African endemic oak *Q. afares*, we believe that specific founding effects (ancient localized hybridizations followed by few backcrosses and a high initial level of inbreeding) together with shifts in species range have favoured the differentiation and impoverishment of terpene production in this species. More studies are needed to elucidate the mechanisms and ecological drivers behind quantitative and qualitative differentiation of terpene emissions in oaks, especially within the highly diversified *Cerris* group.

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Conflict of interest

None declared.

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