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Plant coexistence alters terpene emission and content of Mediterranean species

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Abstract

There is evidence that secondary metabolism may modulate plant interactions and is modified by different biotic stress agents, such as herbivores or pathogens. However, it is poorly understood whether secondary metabolism is altered during competition among plants. The intraspecific and interspecific coexistence of some Mediterranean potted seedlings, namely *Rosmarinus officinalis*, *Pinus halepensis*, *Cistus albidus* and *Quercus coccifera* was investigated through their terpene accumulation within leaves (except for *Q. coccifera*, a non-storing species) and terpene emissions (for all species). Competition had both positive and negative effects for both terpene emissions and content, depending on the species a seedling coexisted with. For *R. officinalis*, terpene concentrations (1.8-cineole and camphor) and terpene emissions (camphene, camphor and overall monoterpenes) were lower when the neighbour species was *P. halepensis*. For *C. albidus*, no changes were observed in its content, while the overall sesquiterpene emissions (70% of total emissions) were reduced in all competition conditions, except in intraspecific competition. In the case of *P. halepensis*, the highest terpene content occurred when it grew with *C. albidus*, and in intraspecific competition, while its emissions were reduced under these conditions. Only emissions of *Q. coccifera* showed no significant changes in the different competition treatments.

Keywords: *Rosmarinus officinalis*; *Pinus halepensis*; *Cistus albidus*; *Quercus coccifera*; Labiaceae; Pinaceae; Cistaceae; Fagaceae; Rosemary; Aleppo pine; Rockrose; Kermes oak; Plant secondary metabolism; Terpenes; Monoterpenes; Sesquiterpenes

1. Introduction

Plants produce a wide diversity of terpenes, with over 15,000 known compounds (Langenheim, 1994). Many Mediterranean species produce and release large amounts and a wide variety of mixtures of highly volatile terpenes. Some species, such as *Quercus ilex* L. (Staudt et al., 2001) and *Quercus coccifera* L. (Llusià and Peñuelas, 2000), release terpenes directly after being synthesized. Others, such as *Rosmarinus officinalis* L. (Hansen et al., 1997), *Cistus albidus* L. and *Pinus halepensis* Mill. (Llusià and Peñuelas, 2000), store these biogenic volatile organic compounds (BVOC) prior to release.

When a species possesses terpene reservoirs, such as glandular trichomes or resin ducts, the terpenes emitted do not necessarily depend either on the amount or on the composition of stored terpenes (Peñuelas and Llusià, 1997; Schindler et al., 1998; Seufert et al., 1995). Thus, studying terpene content within leaves will not necessarily lead to a better knowledge of terpene emission response. When the lack of relationship between emitted and stored compounds has been observed, this is commonly accepted to be due to the fact that the emission rate of a compound not only depends on its concentration inside reservoirs but also on its volatility, which is temperature dependent (Lerdau et al., 1997). However, some studies (e.g. Gershenzon et al., 2000) have shown that some highly volatile compounds may mainly contribute to terpenes sequestered in reservoirs rather than to emitted compounds, while less volatile compounds may contribute

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more to terpene emissions from leaves. Under these circumstances, these authors proposed that cell membranes might be more permeable to some compounds, thus limiting or favouring some terpene emissions.

Terpenes are known to ensure plant survival in the ecosystem since they favour plant defence against biotic (herbivores, pathogens) and abiotic stress factors (Gouinguene and Turlings, 2002; Mumm et al., 2004). Also, they may modulate interactions between competing plants (Gniazdowska and Bogatek, 2005). However, little is known about the way these carbon-based secondary metabolites are altered when plants compete with regard to any environmental factor. Competition between plants is a major ecological factor, which has often been reported in the Mediterranean area (Sardans et al., 2004). When plants compete for soil resources, growth, photosynthesis and leaf nitrogen content are reduced (Midoko-Iponga et al., 2005; Wang et al., 2005; Donaldson et al., 2006), these parameters affecting in turn plant terpene emissions (Staudt et al., 2003; Niinemets et al., 2002; Lerdau et al., 1995). Despite these findings which suggest that competition could play an important role in terpene storage and emission of plants, no study has been carried out to test whether competition has an impact on terpenes stored within leaves. Moreover, only one study has described the way terpenes released by plants are modified under different competition conditions (Peñuelas and Llusia, 1998).

An accurate insight into the ecological factors explaining leaf terpene variability is of particular interest from two points of view. Firstly, increases in terpene concentration within leaves may promote leaf flammability and thus fire risk, if terpenes possess low boiling points (Owens et al., 1998; Kaloustian et al., 2002). This is of special interest in the Mediterranean area since fire is highly recurrent and climate leads many species, such as *R. officinalis* or *P. halepensis*, to store high terpene concentrations (Trabaud, 1976; Llusià and Peñuelas, 2000). Secondly, biogenic terpene emissions play a role in the formation of troposphere secondary pollutants such as ozone (Atkinson and Arey, 2003) or aerosols (Hoffmann et al., 1996). This is relevant in the Mediterranean area since some of these pollutants, mainly O_3, outstrip several times per year the European tolerated limits (Millan et al., 1996) and many Mediterranean species show high terpene emission rates (e.g. *Quercus ilex* L., *Pinus pinea* L., Sabilllon and Cremades; *P. halepensis*, Simon et al., 2005). For these reasons, many attempts have been made to construct terpene emission prediction models. The accuracy of these models depends partly on the number and pertinence of factors that are considered in the model. So far, only some abiotic factors (light and temperature) have been taken into account in these models, while biotic factors are not accounted for at all.

Thus, in this study we ought to examine whether emitted and stored terpenes (monoterpenes and sesquiterpenes) varied according to the type of competition (intraspecific and interspecific competition).

2. Results and discussion

2.1. Terpene content and emission dependency on competition

Because no nutrients were supplied during the 2 years during which the seedlings grew together, competition for substrate nutrients is the main factor responsible for changes in leaf terpene emissions and content shown in this study.

R. officinalis mainly accumulates (Figs. 1 and 2) and emits (Figs. 3 and 4) monoterpenes (Table 2). Firstly, terpene content of this species is significantly altered in competition (MANOVA, $p < 0.05$; Table 3). When each terpene compound stored in leaves is analyzed separately, camphene concentration is significantly reduced when the neighbour species is *P. halepensis* (RP) (ANOVA, $p < 0.05$,

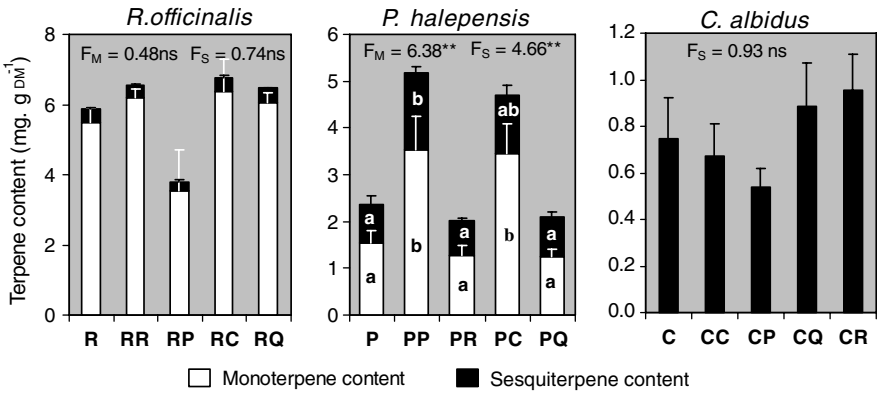

Fig. 1. Mean monoterpene and sesquiterpene concentrations within leaves of *R. officinalis*, *P. halepensis*, *C. albidus* and *Q. coccifera* alone (R, P, C, Q, respectively), in intraspecific (RR, PP, CC, QQ) and interspecific competition conditions. The effect of competition on the overall monoterpene and sesquiterpene content is tested through one way ANOVA, followed by a Tukey pots hoc test. Significant differences ($p < 0.05$) are denoted by different letters (a < b). F_M and F_S are the ANOVA values of the overall monoterpene and sesquiterpene content respectively. *: $0.01 < p < 0.05$. **: $0.001 < p < 0.01$. ns: not significant. $n = 6$. Bars indicate the s.e.

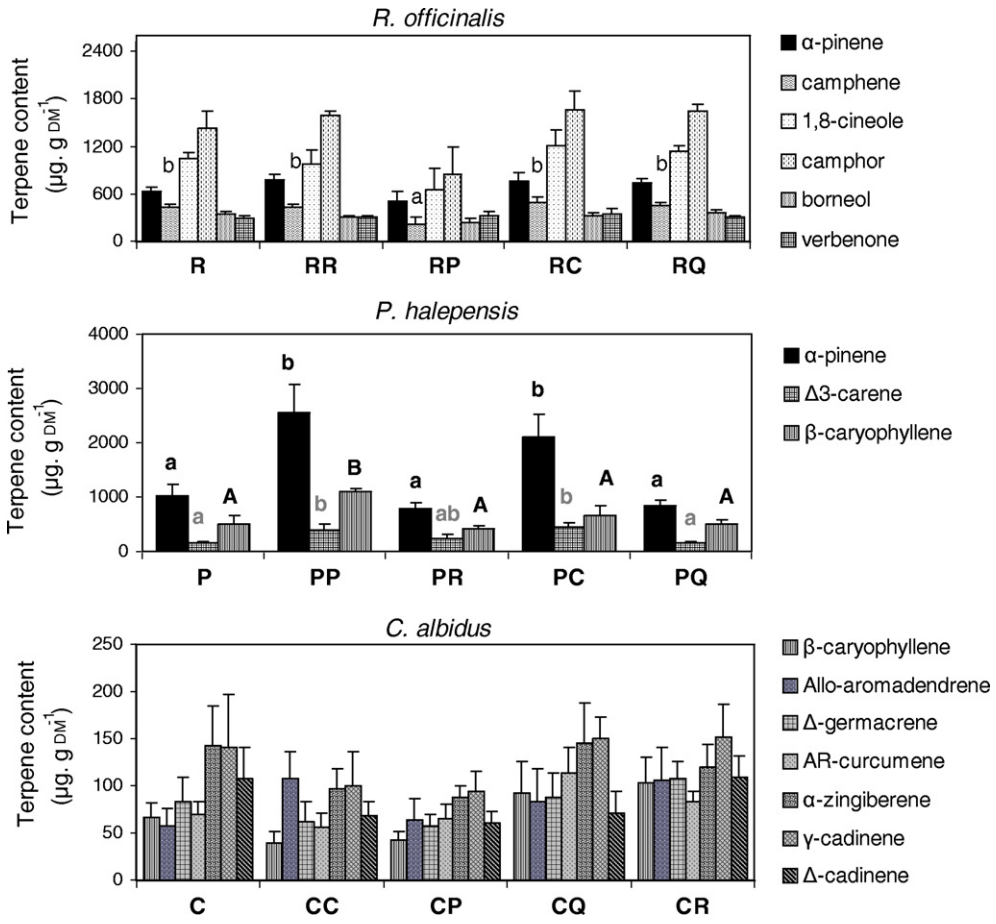

Fig. 2. Mean concentrations of major terpenes in leaves of *R. officinalis*, *P. halepensis*, *C. albidus* and *Q. coccifera* alone (R, P, C, Q, respectively), in intraspecific (RR, PP, CC, QQ) and interspecific competition. Tukey test performed for each major compound, shows differences between competition treatments. Significant differences ($p < 0.05$) are denoted by different letters (a < b). When more than one major compound is altered by competition different letter styles are used. $n = 6$. Bars indicate the s.e.

Tukey test; Fig. 2). Also, the concentrations of the overall monoterpenes (Fig. 1) and camphor (Fig. 2), tended to show lower concentrations in RP (ANOVA, $p = 0.06$, Tukey test). Secondly, contrary to terpene content, terpene emission factors (E_S) of *R. officinalis* are not significantly altered by competition (MANOVA, $p > 0.05$; Table 3). However, when each E_S type is analyzed separately (ANOVA), terpenes show similar changes to those reported for stored terpenes. Thus, E_S of both camphor and 1.8-cineole is also significantly lower when *R. officinalis* grows with *P. halepensis* (RP) (ANOVA, $p < 0.05$, Tukey test; Fig. 4). In addition, E_S of 1.8-cineole is also significantly reduced when *R. officinalis* grows with *C albidus* (RC) (ANOVA, $p < 0.05$, Tukey test; Fig. 4). Thus, stored and emitted terpenes of *R. officinalis* show similar alterations under the different competition treatments.

Monoterpene concentrations are slightly higher than sesquiterpene concentrations in needles of *P. halepensis* (Figs. 1 and 2, Table 2), while E_{SM} outstrips E_{SS} clearly (Figs. 3 and 4, Table 2). Firstly, competition significantly alters leaf terpene accumulation of this species (MANOVA, $p < 0.05$; Table 3). The overall monoterpene concentration increases when (i) *P. halepensis* is in intraspecific competition (PP), (ii) *P. halepensis* grows with *C. albidus* (PC) (ANOVA, $p < 0.05$, Tukey test; Fig. 1). The highest overall sesquiterpene concentration appears when *P. halepensis* is in intraspecific competition (PP) (ANOVA, $p < 0.05$, Tukey test; Fig. 1). Concentration of each major monoterpene shows similar changes to those observed for the overall monoterpene content, that is, they are favoured in PP and PC (ANOVA, $p < 0.05$, Tukey test; Fig. 2). Secondly, E_S of *P. halepensis* are also significantly affected by competition (MANOVA, $p < 0.05$; Table 3). However, intraspecific (PP) and most interspecific competition conditions (PC, PR) have a negative effect on E_{SM}, E_{SS} (Fig. 3) and E_S of most important major compounds released by *P. halepensis* (α-pinene and Δ^3-carene) (Fig. 4, ANOVA, $p < 0.05$, Tukey test). Therefore stored and emitted terpenes of *P. halepensis* show broadly opposite responses to competition. This opposite response of terpene emission factor and terpene content suggests that when *P. halepensis* releases terpenes from specialized structures, terpene pool filling does not occur in parallel, resulting in lower monoterpene concentrations and higher monoterpene emissions (e.g. in *P. halepensis* potted alone). Results reported by Peñuelas and Llusia (1998) for *P. halepensis* differ from

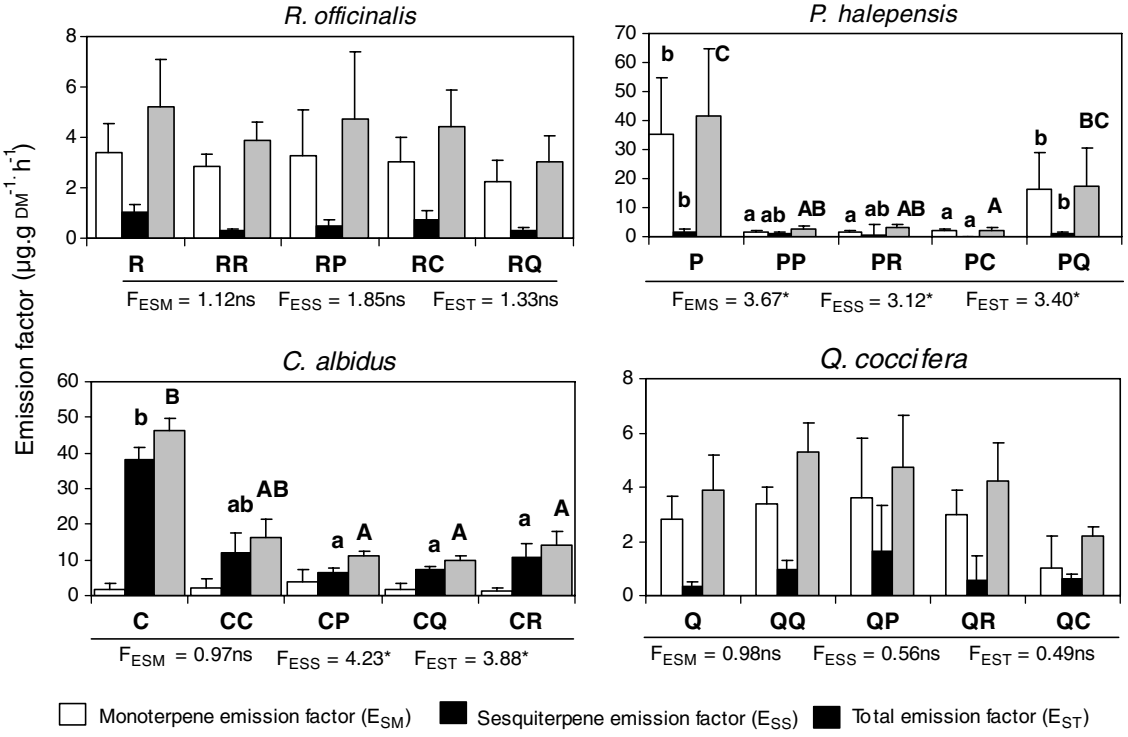

Fig. 3. Mean monoterpene, sesquiterpene and total emission factor (E_{SM}, E_{SS}, E_{ST}, respectively) from leaves of *R. officinalis*, *P. halepensis*, *C. albidus* and *Q. coccifera* alone (R, P, C, Q, respectively), in intraspecific (RR, PP, CC, QQ) and interspecific competition conditions. The effect of competition on these emissions is tested through one way ANOVA, followed by a Tukey post hoc test. Significant differences ($p < 0.05$) are denoted by different letters (a < b < c). F_{ESM}, F_{ESS} and F_{EST} are the ANOVA values of E_{SM}, E_{SS} and E_{ST}, respectively. ns: not significant. ∗: $0.01 < p < 0.05$. Black, grey and black capital letters are used for E_{SM}, E_{SS} and E_{ST}, respectively. $n = 6$. Bars indicate the s.e.

those provided in this study. Those authors reported that (i) monoterpene emissions of *P. halepensis* were favoured in intraspecific competition (PP), while in this study its emissions were limited in PP; (ii) monoterpene emissions of *P. halepensis* were disfavoured in interspecific competition with *Q. ilex*, whereas this study showed that when *P. halepensis* was in competition with *Q. coccifera*, emissions of *P. halepensis* were favoured. These apparently contradictory results might be explained by the fact that Peñuelas and Llusia (1998) added nutrients before the experiment took place while in this study no nutrients were added. However, some other factors such as the time during which plants compete for substrate resources (1 year in Peñuelas and Llusia, 1998), temperature, light, phenological state of plants or herbivory could also partly explain the differences in the results reported in the two studies.

For *C. albidus*, only sesquiterpenes were found in its leaves (Figs. 1 and 2, Table 2), whereas both sesquiterpenes and monoterpenes contribute to its leaf emissions (Figs. 3 and 4, Table 2), as reported by Llusià and Peñuelas (2000). Regarding the competition effect, firstly, terpene storage of *C. albidus* is similar under the different competition treatments (MANOVA, $p > 0.05$; Table 3). Similarly, when considering each terpene content type individually, the results show that neither the overall sesquiterpene concentration (Fig. 1), nor the concentration of any major compound (Fig. 2) are significantly affected by competition (ANOVA, $p > 0.05$). Secondly, similarly to terpene content, E_S of *C. albidus* are not significantly altered by competition (MANOVA, $p > 0.05$; Table 3). However, in contrast to stored terpenes, when each E_S is analyzed individually, some significant differences are observed (ANOVA, $p < 0.05$). E_{SS} and E_S of AR-curcumene are reduced under all interspecific competition treatments (Tukey test, Figs. 3 and 4). Moreover, E_S of β-caryophyllene, Δ-germacrene and AR-curcumene are mainly limited when *C. albidus* is in competition with *P. halepensis* (CP) (Tukey test; Fig. 4) (most emissions from *P. halepensis* were also strongly reduced when growing together with *C. albidus*, see Fig. 3). Thus, for this species, competition has a different effect on sequestered and emitted terpenes. It is interesting to note that this species stores 10-fold lower concentrations than *R. officinalis* and *P. halepensis* (Fig. 1). Because high terpene content contributes to an increased risk of plant flammability (Nuñez-Regueira et al., 2002), *C. albidus*, *R. officinalis* and *P. halepensis* probably contribute differently to fire propagation in natural vegetation stands.

Finally, E_{SM} and E_{SS} of *Q. coccifera* are similar. E_{SM} is only slightly higher than E_{SS} (Fig. 3). Qualitatively, however, monoterpenes are the main released compounds (Table 2). E_S of this species are not significantly altered by competition (MANOVA, $p > 0.05$; Table 3). Furthermore, when each E_S is analyzed separately, no significant differences are observed (ANOVA, $p > 0.05$; Figs. 3 and 4).

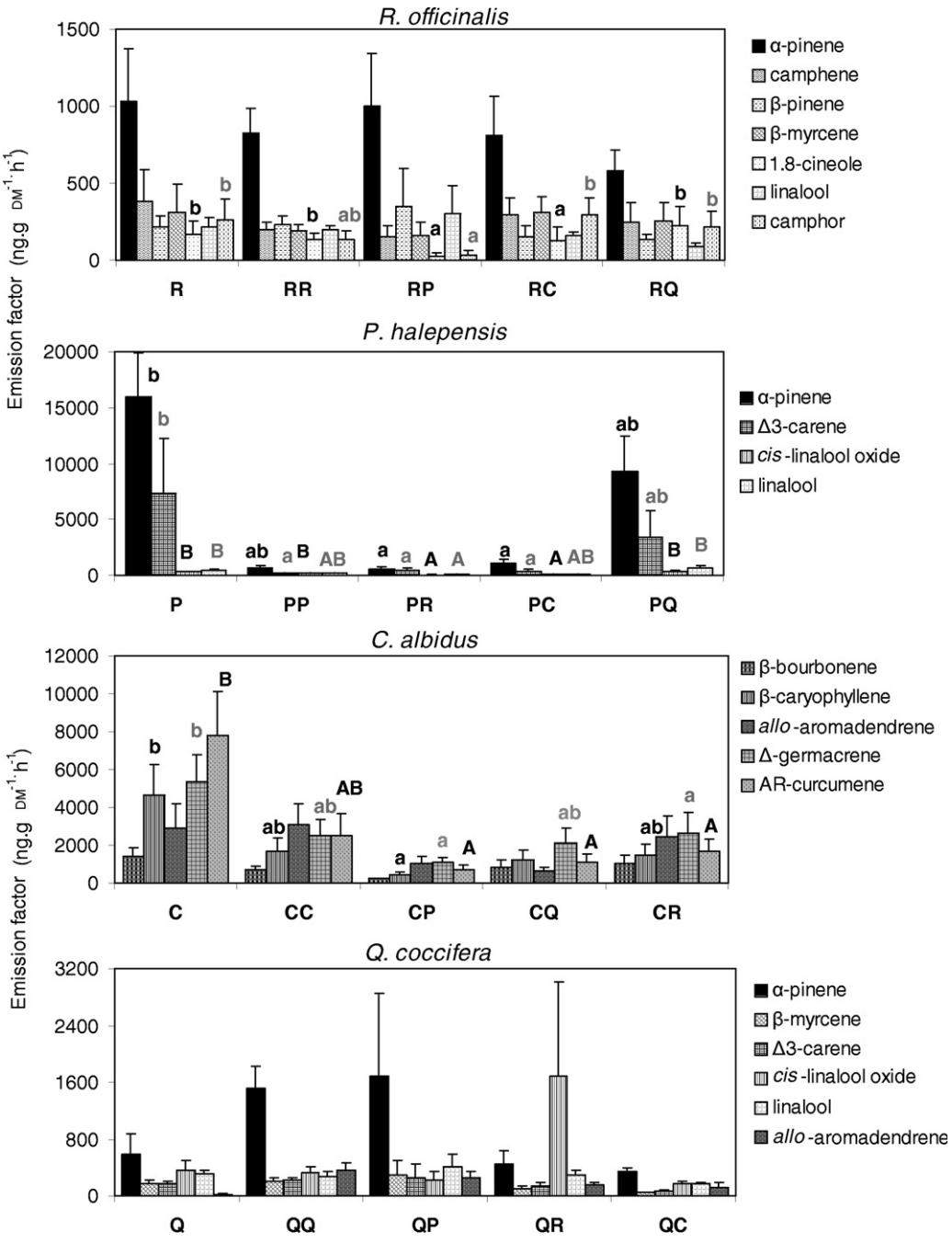

Fig. 4. Mean emission factor of major terpenes from leaves of *R. officinalis*, *P. halepensis*, *C. albidus* and *Q. coccifera* alone (R, P, C, Q, respectively), in intraspecific (RR, PP, CC, QQ) and interspecific competition conditions. Tukey test performed for each major compound, shows differences between competition treatments. Significant differences ($p < 0.05$) are denoted by different letters (a < b). When more than one major compound is affected by competition different letter styles are used. $n = 6$. Bars indicate the s.e.

To summarize, it was shown that (i) some terpene emissions and content from all species were affected by competition except for emissions of *Q. coccifera* and content of *C. albidus*; (ii) when terpene emissions of a species were reduced by competition, emissions of the neighbour species were also often reduced. Thus, E_{SM}, E_{SS} and E_{ST} of *P. halepensis* were reduced in PC and equally, E_{SS} and E_{ST} of *C. albidus* were reduced in CP. Similarly, some major compounds of *R. officinalis* were reduced in RP, and some major compounds of *P. halepensis* were also limited in PR; (iii) some emitted (emission factor) and stored terpenes of *R. officinalis* responded in a similar way to competition, while in the case of *P. halepensis* and *C. albidus* there were no similarities between changes observed in emitted and stored terpenes. Regarding this last point, other studies have also shown that stored and emitted terpenes respond differently to the same environmental conditions. Llusià and Peñuelas (1998) showed that monoterpene emissions

of several Mediterranean species were inhibited under water stress, while their terpene content was simultaneously promoted by this abiotic factor. Moreover, Peñuelas and Llusià (1997) reported a clear seasonal cycle trend for terpene emissions from *R. officinalis*, while no so clear seasonality was observed for its terpene content.

Since terpenes are considered as signal compounds between plants (Gniazdowska and Bogatek, 2005), the effect of competition on terpene emissions could reflect the fact that the same species develop a different signalling mechanism according to the species it grows with. However, the aim of this study was not to solve the controversy as to whether a species develops different signalling mechanisms depending on its neighbour species, but to examine whether terpene emissions from plants were affected by competition.

Because the substrate used in this study came from a relatively poor garrigue soil, results obtained with regard to terpene emissions could be useful to estimate the emission factor of a species according to the plant composition of natural garrigue sites. For example, in the light of the results obtained, it might be expected that emissions of *P. halepensis* in garrigue natural ecosystems will be greater when this species grows together with *Q. coccifera* (Fig. 3).

Moreover, since terpenes within leaves have been found to increase leaf flammability, when the terpene compound is liquid at ambient temperature (Owens et al., 1998), a better knowledge of factors having an impact on terpene content might lead to a better understanding of natural factors that determine the flammability of Mediterranean vegetation. In this context, considering the terpene content response of species examined in the present study could be of great interest, since all are widespread in the Mediterranean Basin.

2.2. Relationship between emitted and stored terpenes

Quantitatively, neither the overall emissions (field emissions), nor the emission of any major compounds, are significantly correlated with their concentration in leaf tissues ($-0.003 < r < 0.36$, $p > 0.05$). Only the overall sesquiterpene emissions (oxygenated sesquiterpenes not considered) and emissions of camphor from *R. officinalis* are linearly correlated with their respective concentrations in specialized compartments of leaves ($p < 0.05$; $r = 0.40$ and 0.50, respectively). On average, 0.11% and 1.10% of the total sesquiterpene and camphor reservoirs in leaves of *R. officinalis* are emitted to the atmosphere per hour (percentages are calculated from field emissions). This finding constitutes a novel result. Peñuelas and Llusià (1997) had shown that the overall monoterpene emissions of this species were not correlated with its overall monoterpene content, in agreement with this study, but no data was available with regard to the relationship between emitted and stored sesquiterpenes or single monoterpenes of this species.

Qualitatively, most emitted compounds are also detected within leaves, except for (i) some minor compounds and a few major compounds of all species and (ii) *C. albidus*, which only stores sesquiterpenes, but releases both monoterpenes and sesquiterpenes and (Table 2). To date, this is to our knowledge the only known species which shows this emission strategy. From this circumstance, it might be inferred that *C. albidus* (i) possesses non-specific reservoirs for monoterpenes, as shown for some *Quercus* non-storing species (Niinemets and Reichstein, 2002) and (ii) releases monoterpenes directly after their synthesis, while only sesquiterpenes are stored prior to emission.

Quantitative and qualitative differences between emitted and stored terpenes are often attributed to the fact that the emission rate of a single compound does not depend exclusively on the concentration of this compound within the leaves according to a Raoult's Law relationship, but also on the environmental temperature and the molecular weight of each compound or its volatility (Lerdau et al., 1997). This would explain why, for instance, γ-cadinene and Δ-cadinene of *C. albidus* or β-caryophyllene of *P. halepensis* are major compounds in leaf pools (Fig. 1), while these sesquiterpenes with relatively low volatility are minor compounds in emissions (Table 2). However, the differences in volatility between terpenes cannot always explain why emitted compounds do not correspond to stored compounds (Gershenzon et al., 2000; Lerdau et al., 1994). For instance, Lerdau et al. (1994) found that while emissions of α-pinene and β-pinene from ponderosa pine where correlated with their respective concentrations within needles, emissions of Δ-carene were not correlated with concentrations of this compound. They concluded that they could not yet explain the cause of the results observed for Δ-carene. In this study, different terpene volatilities cannot explain why some minor compounds (e.g. fenchone of *R. officinalis*, bornyl acetate and β-ocimene of *P. halepensis*) and a few major compounds (e.g. linalool of *P. halepensis*) appear either in emissions or in storage organs, but not in both compartments. There are several possible explanations for this phenomenon: (i) terpenes are highly reactive, and could thereby be transformed between the leaf surface and the atmosphere, resulting in new compounds (similar but not identical to the original terpenes) that may be identified during laboratory analysis (personel communication Michael Staudt); (ii) cell membranes could be more permeable for some terpene groups (Gershenzon et al., 2000); (iii) probably, not all terpenes from a storing species are sequestered inside storage structures as suggested by Steinbrecher et al. (1999) and Tarvainen et al. (2005) for conifer species. The latter explanation implies that a fraction of the released terpenes might come from an instantaneous source. This seems to be especially true for some terpenes of *P. halepensis* since Simon et al. (2005) showed that linalool and β-ocimene emissions were directly dependent on light and these compounds were only detected in emissions in this study (Table 2).

The direct consequence of the lack of match between emitted and stored terpenes is that the terpene concentration in leaves is not sufficient to estimate the emission rate

of a compound, which would have simplified the construction of terpene emission inventories.

3. Conclusion

This study outlines the importance of studying the effect of ecological factors in terpene emissions and terpene content separately, since most often there is a lack of match between emitted and stored compounds. This lack of correlation cannot only be explained by different terpene volatilities. We suggest that (i) not all terpene compounds are equally permeable to cell membranes; (ii) some compounds are directly released by plants while others could be stored in specialized pools prior to emission; (iii) plants promote either emissions or storage according to the stress they are exposed to.

Because soil nutrients have an impact on terpene emissions of some species studied here (Ormeño et al., 2006), it would be interesting to undertake a similar experimental protocol to that described in this study by considering different substrate nutrient concentrations and also different seedling densities. This is important in order to examine the necessity of including this biotic factor in (i) terpene emission models so that terpene emission estimations will be more accurate (ii) the management of natural factors affecting vegetation fire risk.

4. Experimental

4.1. Species studied and experimental set-up

Three terpene storing species, *Rosmarinus officinalis* (R), *Pinus halepensis* (P) and *Cistus albidus*, and one terpene non-storing species, *Quercus coccifera* (Q), were selected. For storing-species, both terpene emissions and content were examined, while for the non-storing species, only terpene emissions were considered. Three-years-old seedlings of each species were randomly potted in 6 l clayed pots in summer 2003. Substrate came from the A1 horizon of a natural calcareous soil, located in a natural garrigue ecosystem.

The study was then carried out under natural environmental conditions, on 17th and 18th March 2005, from 11h30 to 15h00 (local time), that is almost 2 years after potting them together. All pots were similarly and regularly irrigated, but no nutrients were supplied throughout these 2 years, to ensure competition between plants for nutrient resources. In order to check substrate nutrient loss, the concentration of the main nutrients in substrate was measured in spring 2004 and 2005 (Table 1).

Competition treatments consisted of potting one seedling per species (i) alone (control plants): R, P, C and Q; (ii) in intraspecific competition, with other seedling of the same species (RR, PP, CC, QQ); (iii) in interspecific competition, with another seedling of a different species (e.g. RP, RC, RQ: the first letter indicates the species studied and the second the neighbour species). Six replicates per treatment were used for each species. Each replicate came from a different pot. Because each species was potted under five different treatments (e.g. R, RR, RP, RC, RQ), a total of 30 pots and 30 seedlings were used for a species.

Table 1
Physical and chemical properties of calcareous substrate

Properties	Mean ± s.e.		
pH (H_2O)	7.22 ± 0.09		
Sand (% of fine and coarse earth)	28.06 ± 3.61		
Silt (% of fine and coarse earth)	30.56 ± 4.30		
Clay (% of fine and coarse earth)	22.95 ± 3.21		
Texture	Loam		
	2004	2005	Loss (%)
TOC (total organic C) (%)	5.96 ± 0.35	5.05 ± 0.88	−15
OM (organic matter) (g kg^{-1})	102.51 ± 6.08	86.91 ± 6.98	−15
Total N (g kg^{-1}) (Kjeldhal)	3.34 ± 0.05	3.17 ± 0.09	−5
C/N	17.75 ± 0.86	16.84 ± 0.59	−7
Available P (mg kg^{-1}) (Olsen)	32.52 ± 3.25	20.57 ± 2.04	−7
Total P (mg kg^{1})	724.57 ± 40.78	716.24 ± 35.86	−1
K^+ (mg kg^{-1})	501.70 ± 43.08	333.41 ± 17.45	−33
Ca^{2+} (mg kg^{-1})	9768.20 ± 319.81	8432.95 ± 183.30	−13

Soil nutrient losses from 2004 to 2005 are also shown. $n = 8$.

4.2. Terpene emission sampling

For terpene emission sampling, a dynamic bag enclosure system, made of Teflon film (FEP), was applied to carefully enclose a single healthy twig per plant. Healthy state of leaves was determined on the basis of a visual check. Sun and shade exposed leaves, as well as primary and secondary leaves, were considered in the same twig. The system consisted of 16 bag enclosure systems. Each bag enclosure (1 l) was designed with both an air stream inlet and outlet. A blank with no twig in the gas exchange system was sampled during each measurement. A bag enclosure was always allocated for this purpose. Another bag enclosure was allocated for measurements of air temperature inside the enclosure. Thus, while 16 bag enclosures were constructed, only 14 where directly used to sample terpene emissions.

Before any sampling was undertaken, air in the bag was renewed. For this purpose, each bag was continuously flushed with non-polluted air (Alphagaz, Type 1, 99.99% purity), through a Tygon tube (Tygon® Fuel and lubricant tubing; i.d: 8 mm), specially designed for hydrocarbon transport. Inflowing air (Q_e) was precisely measured with a digital mass flow controller (Aalborg® CFC17, 0–500 ml). Q_e was 100 ± 20 ml min^{-1} and was maintained

Table 2
Mean terpene emissions and content from leaves of *R. officinalis*,*P. halepensis*, *C. albidus* and *Q. coccifera*

Emissions RI (GC–FID)	Content RI (GC–MS–FID)	Terpene type *Monoterpenes*	*R. officinalis*		*P. halepensis*		*C. albidus*		*Q. coccifera*
			Emission (ng gDM^{-1} h^{-1})	Content (μg gDM^{-1})	Emission (ng gDM^{-1} h^{-1})	Content (μg gDM^{-1})	Emission (ng gDM^{-1} h^{-1})	Content (μg gDM^{-1})	Emission (ng gDM^{-1} h^{-1})
(a)									
927	950	Tricyclene	21.7 ± 7.2	32.8 ± 3.3	20.1 ± 4.7	5.5 ± 0.6	tc	–	15.5 ± 13.3
929	951	α-Thujene	6.2 ± 4.4	40.5 ± 4.4	3.3 ± 1.7	12.0 ± 1.0	190.0 ± 149.8	–	138.9 ± 112.6
934	955	α-Pinene	848.0 ± 80.4	689.1 ± 37.4	5524.0 ± 1684.9	1488.3 ± 197.9	555.0 ± 183.5	–	918.0 ± 400.3
950	968	Camphene	252.6 ± 9.8	407.7 ± 28.7	136.7 ± 27.4	92.8 ± 75.0	89.5 ± 22.9	–	128.9 ± 54.6
967	981	Sabinene	38.6 ± 6.9	26.6 ± 2.2	164.4 ± 41.6	64.3 ± 9.2	84.5 ± 30.3	–	77.7 ± 28.6
971	983	β-Pinene	217.3 ± 38.0	251.4 ± 20.7	200.7 ± 54.4	90.9 ± 11.8	134.2 ± 45.9	–	124.8 ± 66.3
976	996	β-Myrcene	246.3 ± 30.5	208.1 ± 17.9	348.5 ± 91.6	78.0 ± 13.6	81.1 ± 24.4	–	167.8 ± 66.1
1011	1006	α-Phellandrene	28.6 ± 9.2	69.9 ± 5.4	5.3 ± 2.6	–	9.8 ± 5.6	–	8.4 ± 7.6
1015	1009	Δ^3-Carene	120.2 ± 14.1	7.7 ± 1.7	2368.7 ± 945.0	284.7 ± 37.6	102.0 ± 40.1	–	173.7 ± 65.1
1019	1015	α-Terpinene	4.7 ± 2.0	34.9 ± 2.3	0.5 ± 0.3	–	3.1 ± 2.4	–	8.0 ± 6.5
1036	1024	*p*-Cymene	6.0 ± 2.8	71.9 ± 22.6	10.9 ± 4.6	25.3 ± 3.3	8.3 ± 3.0	–	15.1 ± 12.6
1039	1025	Limonene	121.2 ± 27.4	221.7 ± 16.6	131.5 ± 36.5	4.6 ± 0.5	30.0 ± 17.3	–	9.2 ± 9.2
1040	1027	β-Phellandrene	98.6 ± 26.5	–	61.2 ± 19.5	–	8.1 ± 2.0	–	114.9 ± 90.8
1041	1027	1.8-Cineole	136.2 ± 31.7	1021.0 ± 75.1	12.5 ± 5.1	–	24.9 ± 19.3	–	7.6 ± 7.2
1044	1035	β-Ocimene	2.2 ± 1.5	–	12.3 ± 10.2	–	–	–	–
1063	1052	γ-Terpinene	16.1 ± 5.1	75.6 ± 42.3	25.9 ± 9.7	10.0 ± 0.9	tc	–	–
1073	1064	*cis*-Linalool oxide	216.3 ± 41.8	–	188.2 ± 20.2	–	221.7 ± 70.2	–	552.7 ± 340.9
1093	1078	Δ-Terpinene	22.0 ± 10.8	21.1 ± 1.5	70.6 ± 29.0	51.6 ± 8.7	27.6 ± 2.8	–	16.8 ± 13.4
1099	–	Fenchone	44.4 ± 4.7	–	65.3 ± 25.2	–	171.5 ± 96.6	–	19.6 ± 6.9
1102	1092	Linalool	191.3 ± 34.4	–	284.6 ± 32.2	–	–	–	293.6 ± 73.9
1110	–	Thujone *cis*	–	–	tc	–	167.8 ± 54.5	–	–
1123	–	Thujone *trans*	–	–	2.5 ± 2.2	–	–	–	–
1159	1130	Camphor	188.5 ± 47.0	1450.5 ± 103.3	5.6 ± 3.6	–	tc	–	0.3 ± 0.3
1179	1153	Borneol	25.8 ± 7.9	316.3 ± 17.1	tc	–	tc	–	–
1186	1168	Terpineol-4-ol	–	42.8 ± 2.3	–	3.5 ± 1.0	6.3 ± 4.6	–	–
1202	1182	Terpineol	tc	107.2 ± 6.6	0.8 ± 0.5	–	–	–	13.4 ± 8.7
1231	1200	Verbenone	tc	318.1 ± 15.9	1.7 ± 1.0	–	33.4 ± 23.4	–	4.3 ± 4.3
–	1281	Bornyl acetate	–	161.2 ± 13.6	–	32.1 ± 18.8	–	–	–

(*continued on next page*)

Table 2 (*continued*)

Emissions RI (GC–FID)	Content RI (GC–MS–FID)	Terpene type *Sesquiterpenes*	*R. officinalis*		*P. halepensis*		*C. albidus*		*Q. coccifera*
			Emission (ng gDM^{-1} h^{-1})	Content (μg gDM^{-1})	Emission (ng gDM^{-1} h^{-1})	Content (μg gDM^{-1})	Emission (ng gDM^{-1} h^{-1})	Content (μg gDM^{-1})	Emission (ng gDM^{-1} h^{-1})
(b)									
–	1350	α-Cubebene	–	–	–	–	–	14.4 ± 13.6	–
–	1370	α-Ylangene	–	21.3 ± 1.4	–	–	–	–	–
1372	1375	Copaene	27.1 ± 8.7	6.8 ± 0.5	40.5 ± 13.1	33.6 ± 2.8	20.7 ± 12.0	7.6 ± 0.4	31.0 ± 19.1
1395	1386	β-Bourbonene	15.7 ± 6.7	–	13.1 ± 6.3	–	830.9 ± 191.9	18.5 ± 2.3	25.4 ± 17.9
–	1392	β-Elemene	–	–	–	9.3 ± 1.5	–	4.1 ± 0.4	–
–	1394	β-Cubebene	–	–	–	–	–	7.2 ± 2.2	–
1416	1406	Isocaryophyllene	14.2 ± 8.6	–	1.6 ± 1.1	–	219.2 ± 45.1	–	–
–	1413	α-Gurjuene	–	–	–	–	–	8.7 ± 2.6	–
1436	1418	β-Caryophyllene	43.6 ± 11.7	87.9 ± 5.5	234.5 ± 70.8	649.8 ± 68.8	189.0 ± 724.7	70.9 ± 10.4	63.2 ± 37.8
1448	–	β-Gurjunene	38.5 ± 9.1	–	34.1 ± 12.4	–	229.2 ± 140.3	–	79.2 ± 51.3
1462	–	Unknown 1	7.3 ± 4.3	–	4.9 ± 1.0	–	305.4 ± 85.2	–	18.1 ± 12.7
1468	1452	α-Caryophyllene	66.9 ± 9.9	153.0 ± 12.1	82.3 ± 12.1	132.2 ± 23.9	329.8 ± 115.8	10.6 ± 1.1	83.4 ± 16.0
–	1460	*cis*-β-Farnesene	98.7 ± 8.5	–	93.5 ± 8.4	10.8 ± 4.1	–	–	183.4 ± 61.4
1473	–	Unknown 2							
1475	1462	*allo*-Aromadendrene	–	–	–	–	2017.2 ± 502.3	86.2 ± 12.8	–
–	1478	γ-Muurolene	–	2.9 ± 0.7	–	5.8 ± 0.7	–	7.0 ± 0.6	–
1462	1484	Δ-Germacrene	93.9 ± 36.4	12.5 ± 1.0	48.6 ± 11.3	42.2 ± 4.3	2738.9 ± 707.9	81.3 ± 9.9	29.0 ± 14.8
1480	1486	AR-curcumene	62.7 ± 32.2	–	39.6 ± 12.4	–	2761.2 ± 1293.0	79.7 ± 8.3	66.2 ± 48.7
–	1495	Valencene	–	12.8 ± 0.9	–	–	–	–	–
1489	1498	α-Zingiberene	7.4 ± 6.6	–	44.7 ± 12.0	–	633.4 ± 586.9	121.1 ± 13.8	7.3 ± 6.2
1505	1502	α-Muurolene	tc	3.9 ± 0.4	7.3 ± 4.0	66.5 ± 6.9	152.4 ± 89.6	–	9.8 ± 6.2
1507	1511	β-Bisabolene	–	5.1 ± 0.6	–	–	83.0 ± 67.1	–	–
1508	1514	γ-Cadinene	30.8 ± 15.3	9.2 ± 0.8	33.1 ± 19.5	38.6 ± 3.6	394.2 ± 98.4	129.8 ± 16.4	48.0 ± 26.8
1512	1523	Δ-Cadinene	–	–	tc	20.7 ± 2.2	59.5 ± 44.7	85.3 ± 10.3	12.2 ± 7.6
1516	1525	β-*trans*-Farnesene	–	–	–	–	25.8 ± 18.5	–	tc
1522	1526	β-Sesquiphellandrene	1.3 ± 0.9	–	2.3 ± 1.8	–	14.5 ± 8.9	–	–
–	1537	Elemol*	–	–	–	33.4 ± 2.6	–	–	–
–	1548	Caryophyllene oxide*	–	27.6 ± 1.4	–	12.0 ± 2.6	–	29.8 ± 3.9	–
–	1599	Guaiol*	–	–	–	314 ± 2.5	–	–	–
–	1601	Humulene II epoxide*	–	27.9 ± 1.9	–	–	–	–	–

[a] RT: retention index; tc: trace compounds; –: not detected compound; *: oxygenated sesquiterpenes, only studied in terpene content.
[b] Emission values correspond the emission factor (E_S).
[c] Values are means ± s.e. They are calculated from plants potted alone, in intraspecific and interspecific competition ($n = 30$).

Table 3
MANOVA test results on the effect of competition in terpene emissions and content from leaves of *R. officinalis*, *P. halepensis*, *C. albidus* and/or *Q. coccifera*

Species	No. of major compounds	No. of overall emissions	Total No. of DV	*F*	*p*
Terpene concentration					
R. officinalis	7	2	9	1.96	0.011
P. halepensis	4	2	6	1.80	0.036
C. albidus	5	2	7	0.88	0.595
Emission factor					
R. officinalis	7	3	10	1.21	0.274
P. halepensis	4	3	7	2.60	0.002
C. albidus	5	3	8	1.49	0.079
Q. coccifera	6	3	9	1.12	0.388

[a] No. of DV is the number of dependent variables used in the test.
[b] *p*: test significance.
[c] *F*: test value.

during 30 min, so that air bag was renewed three times. After air renewal, terpene sampling took place with continued inflow of clean air at the same inflow rate. The outgoing terpenes from each bag enclosure were collected on glass sorbent tubes, filled with preconditioned Tenax TA (Varian®), using pumping system (Edwards®), placed downstream of the adsorbent tubes. One Tenax TA per bag enclosure was used. Outflowing air (Q_s) at each bag enclosure was precisely measured with a bubble flow meter (0–280 ml/min, GPE Meterate 314-140/084), placed immediately after each Tenax. Flow meters were specially designed to fit into the flow system between the sampling tube and the pump, via a reinforced flexible and antioxidant PVC tube (i.d: 0.8 mm). Q_e applied during terpene sampling was higher than Q_s to enable the air bag to be slightly overpressurized. Bag enclosure overpressurization also prevented constant leaf contact with the Teflon film and penetration of outside ambient air.

Terpene sampling took place during 10 min at Q_s=80 ± 30 ml min^{-1}. Q_s at each bag enclosure was verified to remain constant every 2 min. The sampled air volume (~1 l) was calculated in order to optimize the ratio signal/threshold without exceeding the breakthrough volumes of each compound. After sampling, all Tenax TA were immediately placed in a refrigerator at +4 °C until being stored at −20 °C in the laboratory.

In parallel to terpene emission sampling, Photosynthetically Active Radiation (PAR) was measured (Portable photo system, plant and canopy transmission meter, Surechem©, EMS-7 Model) outside bag enclosures. PAR values were automatically recorded every minute (Organizer II, Psion©, Digitron instrumentation, LZ64 Model). Ambient temperature and air temperature inside the bag enclosure were measured. Bag air temperature was used to compute emission factors, as shown in other studies (Hansen et al., 1997; Llusià and Peñuelas, 2000; Sabillon and Cremades, 2001). This is justified since emissions were sampled from whole twigs instead of single leaves and Owen et al. (1997) demonstrated that the difference between leaf and enclosure air temperatures is greater higher than 1 °C. Moreover, Arey et al. (1995) underlined that for purposes of scaling-up to an emissions inventory, the ambient light levels and the temperature within the enclosure, rather than individual leaf measurements of temperature and PAR, are appropriate. During sampling, environmental temperature and PAR values ranged from 20 to 23 °C and 770 and 910 μmol s^{-1} m^{-2}, respectively.

After emission sampling, leaves of each twig were cut off and stored at +4 °C until being stored at −20 °C in the laboratory. They were then lyophilized (Liovac-GT-2E Sterys®) to calculate their foliage dry matter (DM). Total foliage DM per twig ranged from 2 to 5 g.

4.3. Terpene emission analyses (GC–FID) and factor emission (E_s) calculation

Tenax TA with adsorbed terpenes were analyzed by gas chromatography (GC) fitted with a Flame ionization detector (FID) (HP®5890 series II). Prior to thermal desorption, a preflush phase was run (3 min, 10 ml min^{-1}, 60 °C) to reduce humidity in the Tenax. Then, thermal desorption (Thermal Desorption Cold Trap injector, Varian®, CP4020-TCT, model) was carried out under nitrogen flow (10 min, 50 ml min^{-1}, 250 °C) and cryogenic concentration in a silica capillary trap, cooled with liquid nitrogen at 100 °C. Compounds were then separated in the non-polar chromatographic column (Ultra 2, 50 m, 0.2 mm) through a programme temperature from 60 °C to 220 °C at a rate of 3 °C min^{-1}, then held at 220 °C for 5 min.

The identity of peaks was checked by comparing their retention times with those of the commercial standards (purity higher than 90%) (Sigma–Aldrich and Firminich). Also, the retention index of each compound was calculated and compared with those found in the literature (Adams, 1989; Jennings and Shibamoto, 1980). Calibration of monoterpene and sesquiterpene factor response from commercial standards was performed periodically throughout the sampling period. Calibration curves were always highly significant (r^2>0.98). In a few cases, when standards were not available, peak identification was achieved by injection

of previously extracted terpenes from each species, in Tenax TA. The identity of these compounds was determined through a GC–MS (see method below for terpene content analysis). Their quantitative analysis (GC–FID) was achieved by considering the average of individual response factors of compounds whose standards were available.

Terpene emissions considered were oxygenated and non-oxygenated monoterpenes and non-oxygenated sesquiterpenes, since Helmig et al. (2004) demonstrated that the bag enclosure technique was not optimal for oxygenated sesquiterpenes. OVOC (other/unknown volatile organic compounds) were considered in total emitted terpenes. Thus, total emitted terpenes are the sum of monoterpenes, sesquiterpenes and OVOC.

Monoterpene, sesquiterpene and total emission factors (E_{SM}, E_{SS}, E_{ST}) of *R. officinalis, P. halepensis* and *C. albidus* were calculated by the empirical algorithm proposed by Tingey et al. (1980) (normalization to 30 °C temperature) since temperature is the main environmental parameter controlling emissions from storing species (Lerdau et al., 1997). Emission factors (E_S) of *Q. coccifera* were calculated following the algorithm of Guenther et al. (1995) (normalization to 30 °C temperature and 1000 μmol m^{-2} s^{-1} PAR), since terpene emissions of this non-storing Mediterranean oak are currently considered to be temperature and light dependent (Niinemets et al., 2002). Although PAR values were measured outside Teflon bag enclosures, PAR values were not corrected before normalizing terpene emissions since Teflon is fully light permeable between 300 and 900 nm (Kesselmeier et al., 1996), though Bertin and Staudt (1996) showed that Teflon retained less than 10% of PAR. Throughout the paper, emissions shown are emission factors (E_S) unless the contrary is specified.

4.4. Terpene content extraction

Terpenes were extracted from lyophilized leaves. Prior to terpene extraction, leaves were crushed. The method used for terpene extraction was similar to that used by Llusià and Peñuelas (2000). It consisted in dissolving 1 g of DM in 10 ml of an organic solvent (cyclohexane), for 20 min, under constant shaking at room temperature. A non-terpene volatile internal standard (undecane) was added to the cyclohexane extraction to avoid interference with terpenes. The addition was carried out before grinding. The extract was immediately analyzed or stored at −20 °C until analysis (within 24–72 h). Repeatability, efficacy of the terpene extraction (i) before and after lyophilization (Table 4), (ii) with and without soaking, (iii) with different extracting times (20 min, 2 h, 2 days) and temperatures (ambient temperature, +4 °C), were previously tested for each species. Under these conditions, terpene extraction results were similar unless the terpene extraction was stored at +4 °C. Recovery through this method was between 90% and 95%.

Table 4
Mean terpene concentration (mg gDM^{-1}) obtained from fresh and dry leaves (after lyophilization) of *R. officinalis*, *P. halepensis* and *C. albidus*

	Fresh leaves	Dry leaves	*W*/*t*	*p*
Rosmarinus officinalis				
Monoterpenes	6.85 ± 0.44	8.00 ± 1.15	0.93	0.39
Sesquiterpenes	0.26 ± 0.05	0.16 ± 0.03	3.00	0.38
Total	7.11 ± 0.42	8.12 ± 1.16	0.81	0.45
Pinus halepensis				
Monoterpenes	1.49 ± 0.05	1.54 ± 0.06	0.64	0.54
Sesquiterpenes	1.02 ± 0.15	1.24 ± 0.04	12.00	0.23
Total	2.50 ± 0.20	1.67 ± 0.68	1.00	0.35
Cistus albidus				
Sesquiterpenes	0.79 ± 0.02	0.66 ± 0.09	16.00	0.17

Student test (*t*) was applied to test differences for monoterpenes and total terpenes, while Mann Whitney (*W*) test was applied to test differences for sesquiterpene concentrations since data did not show equal variances.
[a] *p*: test significance.
[b] Values shown are means ± s.e. (n =5).

4.5. Terpene content analyses (CG–MS–FID)

Analyses were performed through GC (gas chromatography, Hewlett Packard GC6890®) coupled to a mass selective detector (MSD, HP 5973N). The system was fitted with a dual column set-up. One of the columns, the HP-5MS capillary column (30 m, 0.25 μm – JW Scientific), in constant flow mode, was connected directly to MSD (qualitative analysis). The other column, HP5 capillary column (30 m, 0.25 μm – JW Scientific), was directly connected to the flame ionization detector (FID) (quantitative analyses). Sampled volumes (2 μl) were injected through an automatic injector (ALS 7683). Injection was performed in splitless mode at 50 min ml^{-1} for 1 min. Injection temperature was maintained at 250 °C. Helium (99.99%) was used as carrier gas. A constant flow rate of 1 ml min^{-1} was set throughout the analysis run. The oven temperature was initially set at 50 °C and then increased to 160 °C at a rate of 2 °C min^{-1}. It then remained constant for 5 min. The MSD transfer line heater was maintained at 280 °C. The parameters of the MSD for electron impact (EI) mode were: ion source: 230 °C; MS quadrupole: 150 °C; electron energy: 70 eV; electron multiplayer energy: 1200 V. Data were acquired in scan mode from 40 to 500 amu.

Identity of stored terpenes was established by comparison of the retention time and the mass spectrum of detected compounds with those of the authentic reference samples. Terpene identity was then confirmed with generated libraries of retention indexes (Adams, 1989; Jennings and Shibamoto, 1980). Similarly to terpene emissions, each terpene was quantified by calculating its response factor. For this purpose, each standard was calibrated before injecting terpene extractions. Also, when the standard was not available, the average monoterpene or sesquiterpene emission factor was used, respectively.

Stored terpenes were oxygenated and non-oxygenated monoterpenes and sesquiterpenes. In contrast to emitted

terpenes, unknown stored terpenes (OVOC) were not considered, since they were always negligible.

4.6. Statistical analyses

The potential relationship between stored and emitted terpenes was determined in the basis of linear and non-linear regression analyses. This test was applied to (i) the overall field emissions (not to the emission factor) and overall content, (ii) each major compound. In the case of sesquiterpenes, the study of the relationship between stored and emitted sesquiterpenes was performed by considering non-oxygenated stored sesquiterpenes, since oxygenated sesquiterpenes were excluded from emission analysis. The overall effect of competition (independent variable) on emitted and stored terpenes (dependent variables) was tested through a Multivariate analysis of variance (MANOVA). Dependent variables used in the MANOVA (i) for emissions are: the emission factor (E_S) of each major emitted compound and that of the overall monoterpenes, sesquiterpenes and total terpenes (E_{SM}, E_{SS}, E_{ST}, respectively) (ii) for content are: the concentration of each major stored compound and that of the overall monoterpenes. Monoterpene concentration, instead of sesquiterpene concentration, was chosen arbitrarily for the MANOVA test, since these two variables are highly correlated. This is due to the fact that, in contrast to emitted terpenes, unknown terpenes (OVOC) were under detection limits, and consequently, OVOC could not be quantified in stored terpenes. Consequently, the sum of the overall monoterpene and sesquiterpene content always amounted to 100%.

In all cases, emission factors (E_S) were previously log-transformed in order to conform to the normal data distribution required for the MANOVA test. After the overall effect of competition was tested through MANOVA, a post hoc Tukey test (following one-way ANOVA) was applied for each dependent variable to compare pairs of means between the different competition treatments.

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