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# Temporal effects of prescribed burning on terpene production in Mediterranean pines

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Prescribed burning is used to reduce fuel hazard but underburning can damage standing trees. The effect of burning on needle terpene storage, a proxy for secondary metabolism, in fire-damaged pines is poorly understood despite the protection terpenes confer against biotic and abiotic stressors. We investigated variation in needle terpene storage after burning in three Mediterranean pine species featuring different adaptations to fire regimes. In two pure-stands of *Pinus halepensis* Mill. and two mixed-stands of *Pinus sylvestris* L. and *Pinus nigra* ssp. *salzmanni* (Dunal) Franco, we compared 24 h and 1 year post-burning concentrations with pre-burning concentrations in 20 trees per species, and evaluated the relative contribution of tree fire severity and physiological condition ( $\delta^{13}\text{C}$  and N concentration) on temporal terpene dynamics (for mono- sesqui- and diterpenes). Twenty-four hours post-burning, monoterpene concentrations were slightly higher in *P. halepensis* than at pre-burning, while values were similar in *P. sylvestris*. Differently, in the more fire-resistant *P. nigra* monoterpene concentrations were lower at 24 h, compared with pre-burning. One year post-burning, concentrations were always lower compared with pre- or 24 h post-burning, regardless of the terpene group. Mono- and sesquiterpene variations were negatively related to pre-burning  $\delta^{13}\text{C}$ , while diterpene variations were associated with fire-induced changes in needle  $\delta^{13}\text{C}$  and N concentration. At both post-burning times, mono- and diterpene concentrations increased significantly with crown scorch volume in all species. Differences in post-burning terpene contents as a function of the pine species' sensitivity to fire suggest that terpenic metabolites could have adaptive importance in fire-prone ecosystems in terms of flammability or defence against biotic agents post-burning. One year post-burning, our results suggest that in a context of fire-induced resource availability, pines likely prioritize primary rather than secondary metabolism. Overall, this study contributes to the assessment of the direct and indirect effects of fire on pine terpene storage, providing valuable information about their vulnerability to biotic and abiotic stressors throughout time.

**Keywords:** conifers, fire ecology, *Pinus halepensis*, *Pinus nigra*, *Pinus sylvestris*, plant volatiles, prescribed fire, secondary metabolism.

## Introduction

Prescribed burning (PB) is the planned use of fire under mild weather conditions to meet defined management objectives (Wade et al. 1989). Prescribed burning is executed mostly for fire risk reduction, but also for forest management, restoring habitats or improving grazing. Generally, prescribed burns are low intensity fires, but certain management objectives require a higher burning intensity to effectively achieve specific goals,

such as significantly removing understory or slash. In this case, PB can partially damage trees and affect their vitality in the short-term. Some studies have analysed the effects of PB on post-burning growth (Battipaglia et al. 2014, Valor et al. 2015) and tree vitality (see Woolley et al. 2012 for review). Less attention has been dedicated to understanding the effect of PB on secondary metabolites produced by pines (Lavoit et al. 2013), despite the protection they confer against biotic and abiotic stressors,

and their potential to increase plant flammability (Ormeño et al. 2009, Loreto and Schnitzler 2010).

The quantity and composition of terpenes produced against a stressor can be constrained by the plant's physiological status (Sampedro et al. 2011) and genetics (Pausas et al. 2016), but also by the nature and severity of the stress, and the species affected. The main secondary metabolites biosynthesized in conifers are terpenes and phenols (Langenheim 2003). In *Pinus* species, oleoresin is a mixture of terpenes including monoterpenes (volatile metabolites), sesquiterpenes (metabolites with intermediate volatility) and diterpenes (semi-volatile compounds), which are stored in resin ducts of woody and needle tissues (Phillips and Croteau 1999). Upon stress, plants follow a constitutive or induced strategy to defend themselves from a stressor. Although most *Pinus* spp. favour the production of constitutive terpenes under stress conditions, they can also synthesize new induced defences (Phillips and Croteau 1999). The induction timing may be different depending on the chemical groups of terpenes, type of stress, and the species or tissue attacked (Lewinsohn et al. 1991, Achotegui-Castells et al. 2015).

Direct effects of fire such as rising temperatures or heat-induced needle damage can alter terpene production. Increases in air and leaf temperature trigger the emission of volatile terpenes (Alessio et al. 2004) but their synthesis can also be stimulated if the optimal temperature of enzymes is not exceeded (Loreto and Schnitzler 2010). Benefits of such stimulation include thermoprotection against heat, since terpene volatiles neutralize the oxidation pressure encountered by chloroplasts under thermal stress (Vickers et al. 2009). As the emission of volatile terpenes in several Mediterranean pines cease 24 h after fire (Alessio et al. 2004) or wounding (Pasqua et al. 2002), we hypothesized that the accumulation of monoterpenes will be higher 24 h post-burning, than before PB.

Indirect effects of fire can affect terpene concentrations by means of increasing resource availability (Certini 2005). In turn, terpene variations induced by fire could change needle flammability (Ormeño et al. 2009) and susceptibility to insects (Hood et al. 2015). The 'growth differentiation balance hypothesis' (GDBH) (Herms and Mattson 1992, Stamp 2003) predicts that under poor water and nutrient availabilities, growth is more limited than photosynthesis. Since carbon assimilation is maintained, the excess of carbohydrates favours the synthesis of carbon-based secondary metabolites. On the contrary, when resource availability is high, the growth of plants is not expected to be limited and plants allocate a greater proportion of assimilates to growth rather than to defence traits (Herms and Mattson 1992, Stamp 2003). Accordingly, a short-term response following PB should be an increasing demand on the plant for chemical defence if trees are damaged, but with time, if trees heal, increased fertilization and reduced water competition induced by PB (Feeney et al. 1998) could favour carbon allocation to growth rather than chemical defences.

Time-course terpene responses of the direct and indirect effects of PB could differ between tree species depending on their fire resistance strategies. In this study, we used pines with contrasting tolerance to surface fires: *Pinus halepensis*, a fire sensitive species, *Pinus sylvestris*, moderately fire-resistant and the fire-resister *Pinus nigra*, which is supposed to be less vulnerable to fire tissue damage due to its pyro-resistant traits (e.g. thicker bark, higher crown base height) (Fernandes et al. 2008). In agreement with these strategies, we previously found that radial growth was reduced the year of PB in the most fire-sensitive species and unaffected in *P. nigra*, while 1 year post-burning, growth was augmented in *P. nigra* and *P. halepensis*, and reduced in *P. sylvestris* (Valor et al. 2015). In consequence, we hypothesized that 1 year post-burning, the concentration of terpenes would be, as a whole, lower than before PB, if fire induces a decrease in nutrient and water competition; this reduction would be lower on damaged trees and in pines defined as having lower fire resistance (e.g., *P. halepensis* and *P. sylvestris*).

The objectives of this study were to evaluate the effects of relatively high-intensity PB (enough to remove understory and ladder fuels) on mono-, sesqui- and diterpene storage in *Pinus* spp., comparing 24 h and 1 year post-burning concentrations with pre-burning concentrations. We modelled the relative change of terpene concentrations at two sampling times: (i) 24 h post-burning, as a function of fire severity and pre-burning physiological condition and (ii) 1 year post-burning, as a function of fire severity and PB-induced changes in pine physiological conditions. Additionally, we aimed to identify the most representative terpenes of each sampling time since burning.

## Materials and methods

The study was established in three sites situated in the NE Iberian Peninsula (Catalonia, Spain): two plots in mixed-stands of *P. nigra* ssp. *salzmanni* (Dunal) Franco and *P. sylvestris* L. at Miravé and Lloreda localities, situated in the foothills of the Pyrenees; and two other plots in a pure-stand of *P. halepensis* Mill. at El Perelló locality, in the Southern part of Catalonia. The *P. halepensis* stand is located in areas of dry Mediterranean climate while the mixed-stands of *P. nigra* and *P. sylvestris* are situated in temperate cold sub-Mediterranean climate with milder summers and colder winters (Table 1). In the sub-Mediterranean sites, soils are developed from calcareous colluviums (0.5–1 m deep) and thus classified as Calcaric cambisols (FAO 2006); in the Mediterranean site, they are developed from limestones (0.4–0.5 m deep) and classified as Leptic Regosol (FAO 2006). The understory is dominated by *Buxus sempervirens* L. and *Viburnum lantana* L., in the *P. nigra* and *P. sylvestris* mixed-stands, and by *Pistacia lentiscus* L. and *Quercus coccifera* L. in the *P. halepensis* stand.

Table 1. Topographical and climate characteristics of the study localities.

Study sites			Topography			Climate <sup>1</sup>	
Localities	Lat. (°)	Long. (°)	Aspect	Slope (%)	Elevation (m.a.s.l.)	Annual rainfall (mm)	Mean annual temperature (°C)
Lloreda	1.5706	42.0569	N	30	715	731.6	11.7
Miravé	1.4494	41.9515	NE	25	723	677.3	11.5
El Perelló	0.6816	40.9068	NW	10	244	609.9	15.5

<sup>1</sup>Climate variables, annual rainfall and annual mean temperature, were estimated using a georeferenced model (Ninyerola et al. 2000).

### Experimental design: tree selection and prescribed burning

A total of four plots (30 × 30 m) were set up: one in each of the mixed-stand of *P. nigra* and *P. sylvestris*, and two in the pure *P. halepensis* stand. Each plot was burnt in spring 2013 (Table 2). Prescribed burns were conducted by the Forest Actions Support Group (GRAF) of the Autonomous Government (Generalitat de Catalunya) using a strip headfire ignition pattern. Prescribed burning aimed to decrease fuel hazard by reducing surface and ladder fuel loads. Between 90% and 100% of the surface fuel load was consumed in all plots. Needle terpene concentration, fire features and tree physiological condition were studied in 9/10 dominant or co-dominant pines per species in each plot. Each tree was sampled on three occasions for analysing terpene concentration: 24 h before PB (pre-burning), 24 h and 1 year after PB (24 h post-burning and 1 year post-burning, respectively).  $\delta^{13}\text{C}$  and N concentrations of 1-year-old needles were also analysed as a proxy of physiological condition in pre-burning and 1 year post-burning samples.

Before PB, selected trees were identified with a metal tag. Their diameter at breast height (DBH), total height and height to live crown base were measured. During fires, the fire residence time (minutes) above 60 °C and the maximum temperature at the base of the trunk were measured for the selected trees with K-thermocouples (4 mm) connected to dataloggers (Testo 175), packed with a fireproof blanket and buried into the soil. Temperatures were recorded every 10 s. The maximum temperatures registered at the soil surface occurred in the *P. nigra* and *P. sylvestris* plots, while the highest residence time above 60 °C was recorded in the *P. halepensis* plots (Table 2). One week after PB, the crown volume scorched was visually estimated to the nearest 5% as an indicator of fire severity. Foliage scorch was defined as a change in needle colour resulting from direct foliage ignition or indirect heating (Catry et al. 2010).

### Needle sampling

In each plot, we cut an unscorched branch from the top of the south-facing crown in the 9/10 trees selected per species for each sampling time studied: pre-burning, 24 h and 1 year post-burning. Five twigs with unscorched healthy needles were cut immediately, covered with aluminium paper and stored in a portable refrigerator at 4 °C until being stored at –20 °C in the laboratory for terpene analysis. The time period between the field and the laboratory did not exceed 2 h. Additionally, about

five twigs were transported to the laboratory, dried at 60 °C and stored in tins before  $\delta^{13}\text{C}$  and N concentration analysis.

### Needle terpene concentration

In the studied pine species, needles reached up to 3 years old. Before terpene extraction, we collected the 1-year-old needles from each twig to control for the effect of age for each sampling time. Needles were cut in small parts (~5 mm) and placed in well-filled, tightly closed amber glass vials to avoid exposure to light and oxygen (Guenther 1949, in Farhat et al. 2001). The extraction method consisted in dissolving 1 g of cut 1-year-old unscorched green needles in 5 ml of organic solvent (cyclohexane + dichloromethane, 1:9), containing a constant amount of undecane, a volatile internal standard to quantify terpene concentrations which was not naturally stored in the needles. Extraction occurred for 20 min, under constant shaking at room temperature, similar to extractions shown in Ormeño et al. (2007). The extract was stored at –20 °C and then analysed within the following 3 weeks. Analyses were performed on a gas chromatograph (GS-Agilent 7890B, Agilent Technologies, Les Ulis, France) coupled to a mass selective detector (MSD 5977A, Agilent Technologies, Les Ulis, France). Compound separation was achieved on an HP-5MS (Agilent Technologies, Les Ulis, France) capillary column with helium as the carrier gas. After sample injection (1 µl), the start temperature (40 °C for 5 min) was ramped up to 245 °C at a rate of 3 °C min<sup>-1</sup>, and then until 300 °C a rate of 7 °C min<sup>-1</sup>. Terpene identifications were based on the comparison of terpene retention times and mass spectra with those obtained from authentic reference samples (Sigma-Aldrich®, Sigma-Aldrich, Saint-Quentin-Fallavier, France) when available, or from databases (NIST2008, Adams 2007) when samples were unavailable. Also, we calculated the Kovats retention index and compared it with bibliographical data. Terpenes were quantified based on the internal standard undecane (36.6 ng µl<sup>-1</sup> of injected solution). Thus, based on calibrations of terpene standards of high purity (97–99%), also prepared using undecane as internal standard, chromatographic peak areas of an extracted terpene were converted into terpene masses based on the relative response factor of each calibrated terpene. Results were expressed on a needle dry mass (DM) basis. The identified terpenes were grouped in mono-, sesqui- and diterpenes. At each post-burning time, we calculated the relative change of terpene concentration as the

Table 2. Characteristics of prescribed burnings and forest experimental units (mean  $\pm$  std).

Experimental unit	Meteorological conditions				PB characteristics				Forest characteristics <sup>3</sup>					
	Burning date (day/month/year)	Air temp. (°C)	Rel. humidity (%)	Wind speed <sup>1</sup> (km h <sup>-1</sup> )	Burn surf. (ha)	Tmax <sup>2</sup> (°C)	RT60 <sup>2</sup> (min)	Soil water content (%)	Bole scorched height (cm)	Sp.	Phyto- volume (m <sup>3</sup> ha <sup>-1</sup> )	DBH (cm)	Density (number trees ha <sup>-1</sup> )	Basal area (m <sup>2</sup> ha <sup>-1</sup> )
Perelló1	13/05/2013	19.5 $\pm$ 1.6	49.2 $\pm$ 2.9	2.5 $\pm$ 1.0	1.0	65–750	2–89	7.5 $\pm$ 0.5	84.2 $\pm$ 57.1	Ph	597	20.6 $\pm$ 3.5	533	18.4
Perelló2	13/05/2013	19.9 $\pm$ 1.6	66.7 $\pm$ 7.7	4.0 $\pm$ 0.5	0.7	127–561	7–241	7.5 $\pm$ 0.5	50.6 $\pm$ 30.5	Ph	611	18.5 $\pm$ 5.9	922	27.6
Miravé	12/06/2013	25.8 $\pm$ 2.1	54.9 $\pm$ 4.4	17.6 $\pm$ 3.6	0.9	60–718	6–28	30.3 $\pm$ 12.0	71.2 $\pm$ 53.3	Pn/Ps	1637	14.1 $\pm$ 5.1	1711	30.3
Lloreda	5/06/2013	21.8 $\pm$ 1.4	60.4 $\pm$ 4.9	6.75 $\pm$ 5.9	1.1	107–834	7–30	34.3 $\pm$ 10.3	65.1 $\pm$ 48.2	Pn/Ps	949	12.4 $\pm$ 3.1	2411	30.9

<sup>1</sup>Wind speed was measured outside the forest.

<sup>2</sup>Range of maximum temperatures (Tmax) and residence time above 60 °C (RT60) in 10 trees in each of the Perelló experimental units and in 20 trees in Miravé and Lloreda.

<sup>3</sup>Ph, *Pinus halepensis*; Pn/Ps, *P. nigra* and *P. sylvestris*; phytovolume calculated using the cover and height of the understory shrubs; diameter at breast height (DBH), density and basal area of trees with DBH  $\geq$  7.5 cm.

difference between the pre- and post-burning concentration of each terpene group expressed as percentage.

### Tree physiological condition: $\delta^{13}\text{C}$ and N analysis

$\delta^{13}\text{C}$  and N analysis were carried out on 1-year-old unscorched needles in pre-burning and 1 year post-burning samples. For  $\delta^{13}\text{C}$  and N, needles were oven-dried at 60 °C for 48 h, ground and analysed at the Stable Isotope Facility of the University of California at Davis (USA) using an ANCA interfaced to a 20–20 Europa<sup>®</sup> isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK).

### Climatic data before and during sampling years

Monthly precipitation ( $P$ ) and temperature ( $T$ ) from March 2012 to August 2014 were downloaded from the three nearest meteorological stations to the sub-Mediterranean and the Mediterranean plots. Monthly potential evapotranspiration ( $PET$ ) was estimated using the Thornthwaite (1948) method. For each sampling year ( $t$ ), 2013 and 2014, accumulated values of  $P$  and  $PET$  of different periods were calculated for each meteorological station. Seven periods of accumulated climate data were compiled: annual, from June before the sampling year ( $t - 1$ ) to May of the sampling year ( $t$ ); spring, summer, fall and winter before the sampling year ( $t - 1$ ); spring and summer of the sampling year ( $t$ ). For each period, we calculated the difference between  $P$  and  $PET$  ( $P - PET$ ) for each meteorological station and sampling year.

Linear mixed models (LMM), considering plot as a random factor, were used to:

- (i) Analyse potential differences in pre-burning tree physiological condition and fire parameters among pine species.
- (ii) Test for differences in total terpene and terpene group concentrations (expressed in a needle mass basis and as the percentage of the terpene group from the total) between times since burning for each pine species.
- (iii) Model 24 h and 1 year impact of PB on the relative concentration change of mono-, sesqui- and diterpenes with respect to pre-burning concentration. The 24 h and 1 year post-burning models considered pine species as a fixed factor, needle  $\delta^{13}\text{C}$  and N concentration pre-burning, and the proportion of crown scorched and fire residence time above 60 °C as covariables. In addition, in the 1 year post-burning model,  $\delta^{13}\text{C}$  and N concentration changes were also included (1 year post-burning minus pre-burning levels of  $\delta^{13}\text{C}$  and N concentration). Second interactions of pine species with each co-variable were included.

Terpene concentrations were log-transformed to accomplish normal distribution requirement. When the relative concentration change of terpenes was modelled, 100 was summed as a constant before taking the logarithm. Therefore, log-transformed data higher than 2 indicate higher terpene concentrations than pre-burning,

while values lower than 2 mean lower terpene concentrations. Residuals presented no pattern and highly correlated explanatory variables were avoided. The variance explained for the fixed effects was obtained by comparing the final model with the null model (containing only the random structure). A Tukey post-hoc test was used for multiple comparisons when needed.

For each pine species, terpene profiles were evaluated using a principal component analysis to show potential qualitative and quantitative variation in needle terpene within and between plots and time since burning. Terpene concentrations were centred and the variance–covariance matrix used to understand how terpene profiles varied. Moreover, for each pine species, we used a multi-level sparse partial least squares discriminant analysis (sPLS-DA) to select the terpenes that best separated each time since burning in terms of their concentration. The sPLS-DA is a supervised technique that takes the class of the sample into account, in this case time since burning, and tries to reduce the dimension while maximizing the separation between classes. To conduct the analysis, we selected those compounds that were present in at least 75% of the sampled trees, resulting in a total of 48, 37 and 35 compounds in *P. halepensis*, *P. nigra* and *P. sylvestris*, respectively. We used the multilevel approach to account for the repeated measures on each tree to highlight the PB effects within trees separately from the biological variation between trees. The classification error rate was estimated with leave-one-out cross validation with respect to the number of selected terpenes on each dimension. Lastly, differences in  $P - PET$  between sampling years were tested by a Student's *t*-test for the Mediterranean and sub-Mediterranean plots. All analyses were conducted with the software R (v. 3.2.1, The R Foundation for Statistical Computing, Vienna, Austria) using the package *nlme* for linear mixed-effects modelling and the package *mixOmics* for the sPLS-DA analysis. The model variances explained by fixed effects (marginal  $R^2$ ) and

by both fixed and random effects (conditional  $R^2$ ) are provided (Nakagawa and Schielzeth 2013).

## Results

### Tree, fire and climate characteristics

The proportion of crown scorched was significantly higher in *P. halepensis* than in the other species despite the fact that the three pine species presented similar height to live crown base (Table 3). By contrast, no differences in fire residence time above 60 °C were encountered among species (Table 3). Needle  $\delta^{13}C$  decreased significantly 1 year post-burning in the three species while N concentration was similar (Table 3).

This decrease in  $\delta^{13}C$  contrasted with the drier conditions found 1 year post-burning ( $P - PET = 200$  mm and = 135 mm in Mediterranean and sub-Mediterranean plots, respectively) in comparison with pre-burning ( $P - PET = 481$  mm and = 290 mm in Mediterranean and sub-Mediterranean plots, respectively; see Figure S1 available as Supplementary Data at *Tree Physiology* Online).

A total of 56, 59 and 49 terpenes were identified and quantified in *P. halepensis*, *P. nigra* and *P. sylvestris*, respectively (see Table S1 available as Supplementary Data at *Tree Physiology* Online). Pre-burning, *P. nigra* showed the highest terpene concentration ( $65.6 \pm 7.1$  mg  $g_{DM}^{-1}$ ) followed by *P. halepensis* and *P. sylvestris* ( $41.2 \pm 5.8$  mg  $g_{DM}^{-1}$  and  $21.4 \pm 2.6$  mg  $g_{DM}^{-1}$ , respectively). Before PB, more than 45% of total terpene concentration was represented by diterpenes in *P. halepensis* while sesquiterpenes represented about 59% in *P. nigra* and monoterpenes represented 83% in *P. sylvestris* (see Table S2 available as Supplementary Data at *Tree Physiology* Online). Considering all sampling times,

Table 3. Studied pine trees and fire characteristics (mean  $\pm$  std) before and after prescribed burnings grouped by species.

Tree and fire characteristics	<i>P. halepensis</i>	<i>P. nigra</i>	<i>P. sylvestris</i>
<i>n</i> (trees)	20	19	19 <sup>1</sup>
DBH (cm)	20.0 $\pm$ 6.9a	13.6 $\pm$ 5.5b	12.7 $\pm$ 5.3b
Total height (m)	9.1 $\pm$ 2.4a	8.3 $\pm$ 2.4a	8.6 $\pm$ 1.9a
Height to live crown base (m)	5.2 $\pm$ 1.0a	4.8 $\pm$ 1.3a	6.6 $\pm$ 13.2b
Crown scorched (%)	44.0 $\pm$ 32.1a	6.6 $\pm$ 13.2b	5.5 $\pm$ 9.5b
Fire residence time >60 °C (min)	38.2 $\pm$ 54.1a	16.6 $\pm$ 6.9a	15.2 $\pm$ 6.4a
Needle $\delta^{13}C$ (‰)			
Pre-burning	−25.8 $\pm$ 0.5Aa	−26.6 $\pm$ 1.0 Ab	−26.5 $\pm$ 0.6Ab
1 year post-burning	−27.6 $\pm$ 0.9Ba	−28.5 $\pm$ 1.4Ba	−28.0 $\pm$ 0.8Ba
Needle N content (mg $g_{DM}^{-1}$ )			
Pre-burning	14.8 $\pm$ 1.9Aa	10.1 $\pm$ 0.8Ab	12.3 $\pm$ 1.6Ac
1 year post-burning	14.9 $\pm$ 3.2Aa	9.1 $\pm$ 2.7Ab	11.0 $\pm$ 3.1Aa

<sup>1</sup>Sample size is 18 for 1 year post-burning data because of death of one tree.

Different small letters within a row indicate statistical significant differences ( $P < 0.05$ ) among pine species using LME (where fixed factor = species, random factor = plot) followed by Tukey post-hoc test. Different capital letters within a column indicate statistical significant differences ( $P < 0.05$ ) between pre-burning and 1 year post-burning for each pine species using LMM (where fixed factor = time since burning, random factor = plot) followed by Tukey post-hoc test.



the diterpene thunbergol in *P. halepensis*, the sesquiterpene  $\beta$ -caryophyllene in *P. nigra* and the monoterpene  $\alpha$ -pinene in *P. sylvestris* were the major compounds found, representing an average of 22%, 22% and 40% of the total terpene concentration, respectively (see Figure S2 available as Supplementary Data at *Tree Physiology* Online). Terpene concentration and composition strongly varied within plots in all species, with no clear differences in terpene composition among plots (see Figures S3a, S4a and S5a available as Supplementary Data at *Tree Physiology* Online). The variation in terpene concentrations was high within pre- and 24 h post-burning samples, while variation for 1 year post-burning concentrations was much lower (see Figures S3b, S4b and S5b available as Supplementary Data at *Tree Physiology* Online). In all species, the quantity of dominant compounds in pre- and 24 h post-burning samples were clearly different from those of 1 year post-burning samples (see Figures S3b, S4b and S5b available as Supplementary Data at *Tree Physiology* Online). For instance, the quantity of  $\alpha$ -pinene was higher in pre- and 24 h post-burning times in all pine species in opposition with 1 year post-burning samples. Limonene was characteristic 24 h post-burning in the needles of *P. halepensis* and *P. nigra*, while the quantity of camphene and myrcene was higher in pre- and 24 h post-burning needles samples of *P. sylvestris*.

Differences in total terpene concentration between pre- and 24 h post-burning were only detected in *P. nigra*, which decreased ~39% (Figure 1a). When analysing terpene groups, the 24 h post-

burning needle concentration of both mono- and sesquiterpenes were, in comparison with pre-burning, slightly higher in *P. halepensis*, lower in *P. nigra* and similar in *P. sylvestris* (Figure 1b and c). No differences were detected in the diterpene concentration between pre- and 24 h post-burning times (Figure 1d).

One year after burning, total terpene concentration was lower compared with the levels observed pre- and 24 h post-burning in the three species (Figure 1a). In *P. halepensis* this reduction was similar for each terpene group while, in the two sub-Mediterranean species, it was mostly due to a decrease in the proportion of monoterpenes (see Table S2 available as Supplementary Data at *Tree Physiology* Online). In contrast, an increase in the relative contribution of the sesquiterpene group to the total terpenes was found 1 year post-burning in both sub-Mediterranean species.

The relative changes of mono- and diterpene concentrations 24 h post-burning were directly related to the proportion of crown scorched (Table 4). However, crown scorch volume interacted with pine species to explain the relative changes in mono- and diterpene concentrations (Table 4). Thus, in both *P. halepensis* and *P. sylvestris*, the 24 h post-burning concentration of monoterpenes was higher than pre-burning and increased with crown scorched (Figure 2a.1 and a.3); only individual pines with low proportion of the crown scorched (<15–20%) showed similar or lower concentration than pre-burning. In

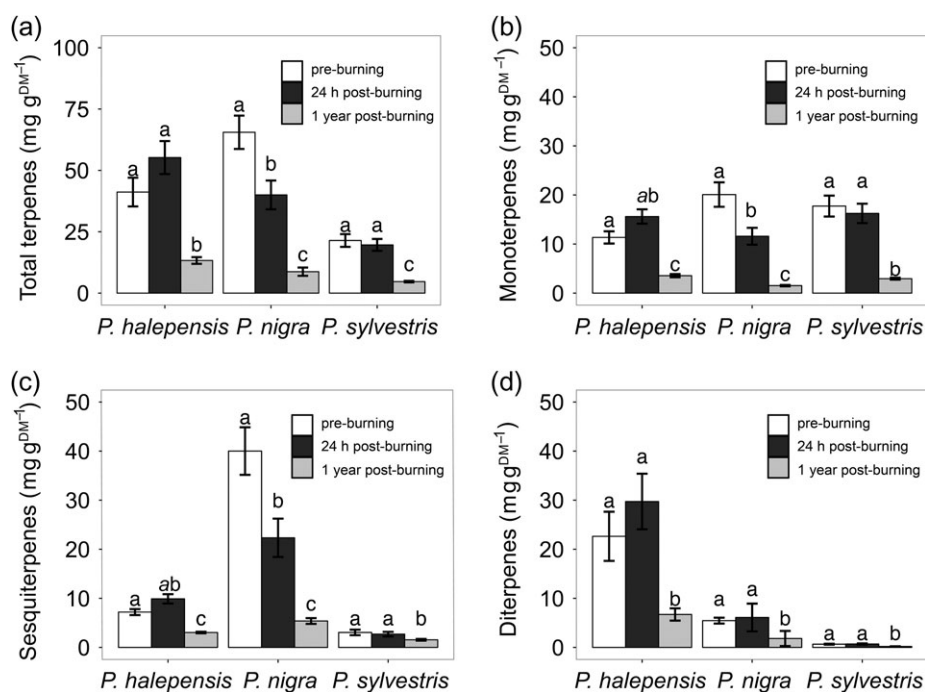


Figure 1. Concentration (mean  $\pm$  SE) of total terpene (a), monoterpenes (b), sesquiterpenes (c) and diterpenes (d) across time since burning (TSB) for each pine species (*P. halepensis*,  $n = 20$ ; *P. sylvestris*,  $n = 19$ ; *P. nigra*,  $n = 19$  and  $n = 18$  in 1 year-post-burning). Differences in the concentration between TSB within each pine species were tested using LMM considering plot as a random factor. Within each pine species, different letters indicate differences between TSB using a Tukey post-hoc, where regular letters indicate significant differences at  $P < 0.05$ ; italic letters represent a marginal significant difference ( $0.05 < P < 0.1$ ).

Table 4. Summary of the models characterizing the impact of prescribed burning and tree vitality on the 24 h post-burning relative concentration change of mono-, sesqui- and diterpenes, calculated as the standardized difference between 24 h post-burning and pre-burning concentration expressed as percentage (logarithmically transformed). Only the significant interaction terms are shown. Bold characters indicate significant effects ( $P < 0.05$ ).

	24 h post-burning relative concentration change								
	Monoterpenes			Sesquiterpenes			Diterpenes		
	Est.	SE	<i>P</i>	Est.	SE	<i>P</i>	Est.	SE	<i>P</i>
Intercept	<b>1.146</b>	<b>0.203</b>	<b>0.00</b>	<b>2.780</b>	<b>0.633</b>	<b>0.00</b>	<b>1.585</b>	<b>0.074</b>	<b>0.00</b>
CVS	<b>0.009</b>	<b>0.003</b>	<b>0.00</b>	0.001	0.001	0.48	<b>0.027</b>	<b>0.005</b>	<b>0.00</b>
RT60 <sup>1</sup>	0.000	0.000	0.29	<b>0.002</b>	<b>0.001</b>	<b>0.04</b>	0.001	0.001	0.33
$\delta^{13}\text{C}$	-0.035	0.030	0.25	0.000	0.040	0.99	-0.011	0.054	0.83
HLCB <sup>2</sup>	<b>0.041</b>	<b>0.020</b>	<b>0.04</b>	0.008	0.026	0.73	-0.052	0.035	0.14
Foliar N	<b>0.033</b>	<b>0.016</b>	<b>0.03</b>	<b>-0.112</b>	<b>0.062</b>	<b>0.07</b>	0.019	0.026	0.47
Sp. = <i>P. halepensis</i> ( <i>Ph</i> )	0.228	0.128	0.21	-1.358	0.735	0.20	<b>0.476</b>	<b>0.132</b>	<b>0.07</b>
Sp. = <i>P. sylvestris</i> ( <i>Ps</i> )	<b>0.209</b>	<b>0.069</b>	<b>0.00</b>	<b>-1.604</b>	<b>0.688</b>	<b>0.02</b>	<b>0.395</b>	<b>0.106</b>	<b>0.00</b>
CVS $\times$ <i>Ph</i>	<b>-0.007</b>	<b>0.003</b>	<b>0.02</b>				<b>-0.025</b>	<b>0.005</b>	<b>0.00</b>
CVS $\times$ <i>Ps</i>	-0.005	0.005	0.32				<b>-0.024</b>	<b>0.009</b>	<b>0.00</b>
Foliar N $\times$ <i>Ph</i>				<b>0.155</b>	<b>0.067</b>	<b>0.02</b>			
Foliar N $\times$ <i>Ps</i>				<b>0.174</b>	<b>0.065</b>	<b>0.01</b>			
AIC	24.48			32.81			67.39		
$R^2$ (fixed)	0.54			0.46			0.44		
$R^2$ (random + fixed)	0.59				0.57				0.44

CVS, crown volume scorched (%).

<sup>1</sup>RT60, fire residence time above 60 °C (min).

<sup>2</sup>HLCB, height to live crown base (m).

contrast, the relative change of monoterpene concentration in *P. nigra* was generally lower than pre-burning, at least in the range of crown scorch measured (0–50%) (Figure 2a.2). The relationship between the relative concentration change in diterpenes and crown scorch followed a similar trend as in monoterpenes for *P. halepensis* and *P. sylvestris* (Figure 2b.1 and b.2), while in *P. nigra*, the ratio of change in crown scorch was higher and shifted from lower to higher concentrations than pre-burning in the middle of the measured crown scorch range (Figure 2b.3).

The relative concentration change of monoterpene was also directly related to the needle N concentration and the height to live crown base (Table 4). In the case of sesquiterpenes, needle N concentration interacted with pine species (Table 4, Figure 2). Thus, the relative concentration change of sesquiterpenes 24 h post-burning was higher in *P. halepensis* and *P. sylvestris*, and augmented as needle N concentration increased (Figure 2c.1 and c.3), whereas it was always lower in *P. nigra*, decreasing inversely with increasing needle N concentration (Figure 2c.2). Finally, fire residence time above 60 °C directly affected the relative change of sesquiterpene concentration in all species (Table 4).

One year after PB, the relative change of mono- and sesquiterpene concentrations were always lower than pre-burning and inversely related with  $\delta^{13}\text{C}$  of pre-burning needles (Table 5, Figure 3a.1). The 1 year post-burning relative change concentration of diterpenes were also lower than pre-burning, but

variations were associated with changes in  $\delta^{13}\text{C}$  or N concentration of needles (Figures 3a.2 and a.3).

Similar to 24 h post-burning, the proportion of crown scorched had a direct effect on the relative concentration change of all terpene groups, although marginally significant in mono- and sesquiterpene models (Table 5). This variable interacted with pine species in the case of diterpenes (Figure 3b) and showed that as crown scorch increased, the relative concentration change in *P. nigra* was more acute than in the other species (Table 5, Figure 3b.2).

#### Discriminant terpenes across time since burning for each pine species

The multilevel sPLS-DA in *P. halepensis* led to optimal selection of six and one terpenes on the first two dimensions with a classification error rate of 0.26 and 0.06, respectively, reflecting a clear separation between times since burning (Figure 4). Among compounds, terpinen-4-ol separated pre-burning (Cluster 2) from both post-burning times; whereas E- $\beta$ -ocimene and  $\alpha$ -thujene discriminated the 24 h post-burning sampling time from the others (Cluster 1). Four sesquiterpenes characterized the 1 year post-burning needle samples (Cluster 3).

In *P. nigra*, we chose three dimensions and the corresponding terpenes selected for each were four, one and one (Figure 5). The classification error rates were 0.35, 0.33 and 0.18, respectively, for the first three dimensions. Two clusters were differentiated: pre-burning was discriminated, mainly, by three sesquiterpenes (Cluster 1) and bornyl acetate and  $\beta$ -springene represented, post-burning samplings (Cluster 2) (Figure 5).



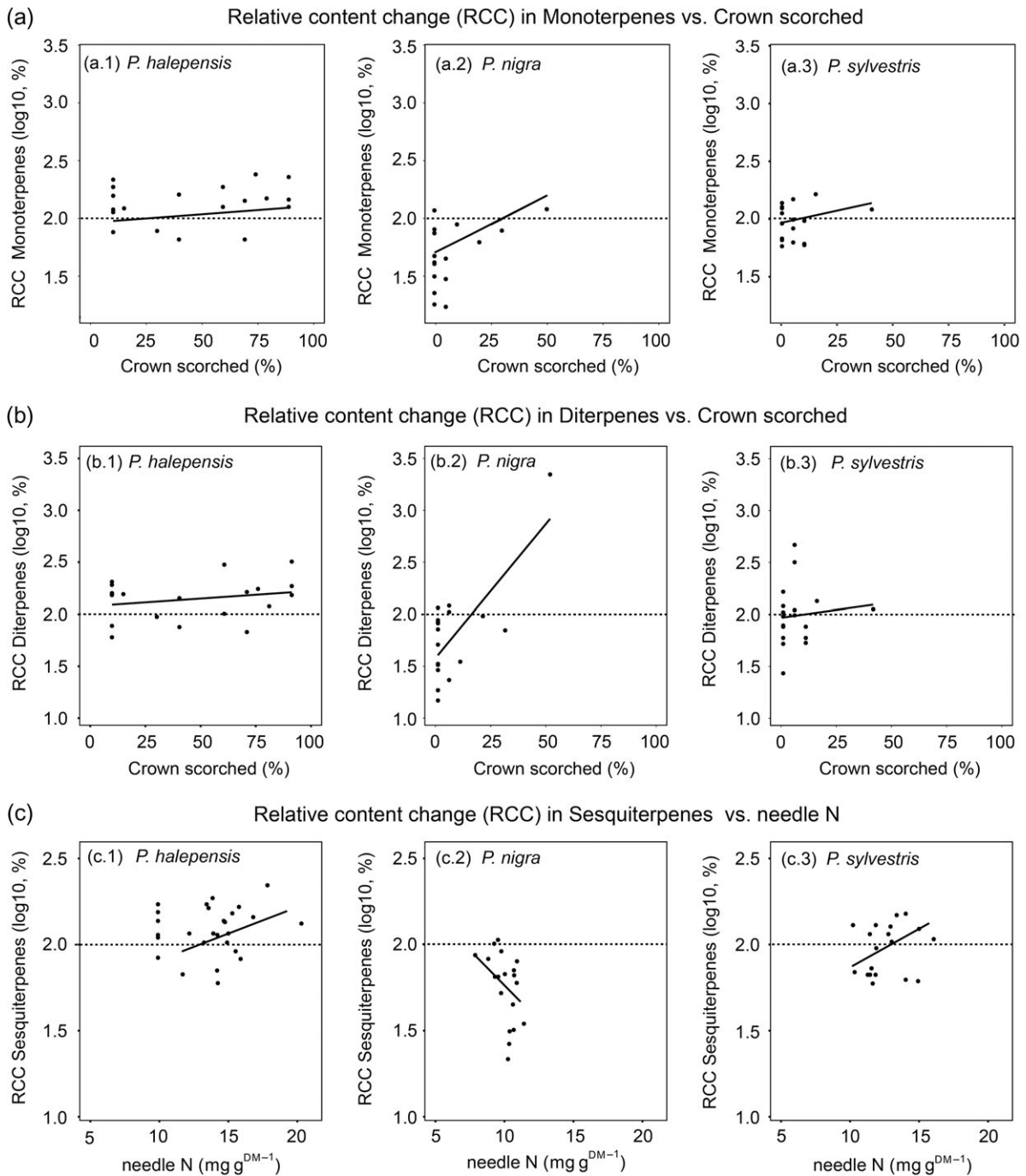


Figure 2. Measured and predicted (line) relative concentration change (log-transformed) using 24 h post-burning models (see Table 4) of monoterpenes and diterpenes against crown scorched (a and b) and for sesquiterpenes against needle N (c). Before the log-transformation, 100 was summed. The dashed line indicates no changes between pre- and post-burning terpene concentrations: higher values indicate a higher terpene concentration than those of pre-burning, while the opposite is indicated by lower values.

Finally, two dimensions were selected for *P. sylvestris* (Figure 6) with 11 terpenes on each component. The classification error rates were 0.66 and 0.33. As in *P. nigra*, two clusters were distinguished: sesquiterpenes characterized the pre-burning sampling time, whereas both post-burning times were characterized mainly by mono- and sesquiterpenes (Figure 6).

## Discussion

*Pinus nigra* is a species considered to be resistant to medium-low fire intensities, *P. sylvestris* a moderately fire-resistant species and *P. halepensis* a fire-sensitive species (Agee 1998, Fernandes et al. 2008). While the concentration of the semi-

Table 5. Summary of the models characterizing the impact of prescribed burning and tree vitality on the 1 year post-burning relative concentration change of mono-, sesqui- and diterpenes, calculated as the standardized difference between 1 year post-burning and pre-burning content expressed as percentage (logarithmic transformed). Only the significant interaction terms are shown. Bold characters indicate significant effects ( $P < 0.05$ ).

	1 year post-burning relative concentration change								
	Monoterpenes			Sesquiterpenes			Diterpenes		
	Est.	SE	<i>P</i>	Est.	SE	<i>P</i>	Est.	SE	<i>P</i>
Intercept	<b>-1.879</b>	<b>0.812</b>	<b>0.05</b>	<b>-2.699</b>	<b>0.907</b>	<b>0.00</b>	<b>0.812</b>	<b>0.107</b>	<b>0.00</b>
CVS	<b>0.002</b>	<b>0.001</b>	<b>0.07</b>	<b>0.002</b>	<b>0.001</b>	<b>0.06</b>	<b>0.035</b>	<b>0.005</b>	<b>0.00</b>
RT60 <sup>1</sup>	-0.000	0.000	0.50	-0.000	0.000	0.53	-0.000	0.001	0.80
$\delta^{13}\text{C}$	<b>-0.103</b>	<b>0.030</b>	<b>0.00</b>	<b>-0.144</b>	<b>0.034</b>	<b>0.00</b>	-0.065	0.063	0.30
Foliar N	0.019	0.017	0.26	0.012	0.019	0.53	0.001	0.029	0.96
HLCB <sup>2</sup>	-0.003	0.020	0.86	0.001	0.023	0.94	-0.058	0.035	0.11
Sp = <i>P. halepensis</i> ( <i>Ph</i> )	<b>0.603</b>	<b>0.073</b>	<b>0.01</b>	<b>0.611</b>	<b>0.067</b>	<b>0.01</b>	<b>0.696</b>	<b>0.150</b>	<b>0.04</b>
Sp = <i>P. sylvestris</i> ( <i>Ps</i> )	<b>0.351</b>	<b>0.056</b>	<b>0.00</b>	<b>0.647</b>	<b>0.063</b>	<b>0.00</b>	<b>0.847</b>	<b>0.110</b>	<b>0.00</b>
Change $\delta^{13}\text{C}$ <sup>3</sup>	-0.029	0.030	0.33				<b>0.101</b>	<b>0.041</b>	<b>0.01</b>
Change N <sup>4</sup>	0.009	0.013	0.47				<b>-0.062</b>	<b>0.021</b>	<b>0.00</b>
CVS × <i>Ph</i>							<b>-0.031</b>	<b>0.006</b>	<b>0.00</b>
CVS × <i>Ps</i>							<b>-0.029</b>	<b>0.009</b>	<b>0.00</b>
AIC	0.87			-0.30			81.79		
$R^2$ (fixed)	0.70			0.69			0.67		
$R^2$ (random+fixed)	0.70			0.69			0.67		

CVS, crown volume scorched (%).

<sup>1</sup>RT60, fire residence time above 60 °C (min).

<sup>2</sup>HLCB, height to live crown base (m).

<sup>3</sup>Change  $\delta^{13}\text{C}$ , change in  $\delta^{13}\text{C}$  (difference between 1 year post-burning and pre-burning  $\delta^{13}\text{C}$ ).

<sup>4</sup>Change N, change in foliar N content (difference between 1 year post-burning and pre-burning N content).

volatile diterpenes was not affected 24 h post-burning, the concentration of mono- and sesquiterpenes seems to decrease in *P. nigra*, was sustained in *P. sylvestris* and tended to increase in *P. halepensis*. Although massive needle terpene emissions have been reported at ambient temperatures often reached during PB (Alessio et al. 2004, Loreto and Schnitzler 2010, Zhao et al. 2012), various explanations may justify the different terpene contents observed 24 h post-burning between species. For instance, terpenes stored in needle resin ducts are likely to encounter different resistance to volatilization due to differences in the specific characteristics of the epistomatal chambers which are, respectively, unsealed, sealed and buried in needles of *P. nigra*, *P. sylvestris* and *P. halepensis* (Hanover and Reicosky 1971, Boddi et al. 2002, Kim et al. 2011). These differences in needle morphology may contribute to explaining the reduction of terpenes observed 24 h post-burning in *P. nigra*. Another reason for variable terpene contents may be different respiration sensitivity between species. As the consumption of assimilates increases relative to the photosynthetic production at high temperatures (Farrar and Williams 1991), this could bring about a decrease in the weight of carbohydrates and, thus, an apparent increase in needle terpene concentrations. If the respiration sensitivity to increasing temperature is higher in *P. halepensis* than in the other two species, this may explain the slight increase in terpene concentration in this species 24 h post-burning. Alternatively, the increase in monoterpene concentration in unscorched

needles of *P. halepensis* 24 h post-burning may partly reflect systemic induced resistance, triggered by burning needles from lower parts of the canopy, although no data was found in literature to support this hypothesis. Finally, although we carefully selected only 1-year-old unscorched needles and from the same part of the crown, we cannot fully exclude that terpene variation between pre- and post-burning are reflecting differences in light availability between the sampled needles.

Terpene dynamics within the species were modulated by fire severity. Thus, relative concentration changes of mono- and diterpenes increased with the proportion of crown scorched 24 h post-burning. This trend was evident 1 year post-burning, suggesting that the damaged pines were still investing in chemical defences. According to the GDBH (Herns and Mattson 1992, Stamp 2003) and the reduction in radial growth detected in *P. halepensis* and *P. sylvestris* (Valor et al. 2015), we hypothesized that the increase in monoterpenes by *P. halepensis* and, to a lesser extent, in *P. sylvestris*, may constrain primary metabolism. Although the rate of increase in diterpenes post-burning was greater in *P. nigra* than in the other two species, *P. nigra* required a greater proportion of scorched crown in order to achieve higher concentrations than those observed pre-burning. Therefore, trees with a greater proportion of scorched crown could be investing in secondary metabolism rather than primary metabolism, although this potential trade-off on carbon investment deserves further research.

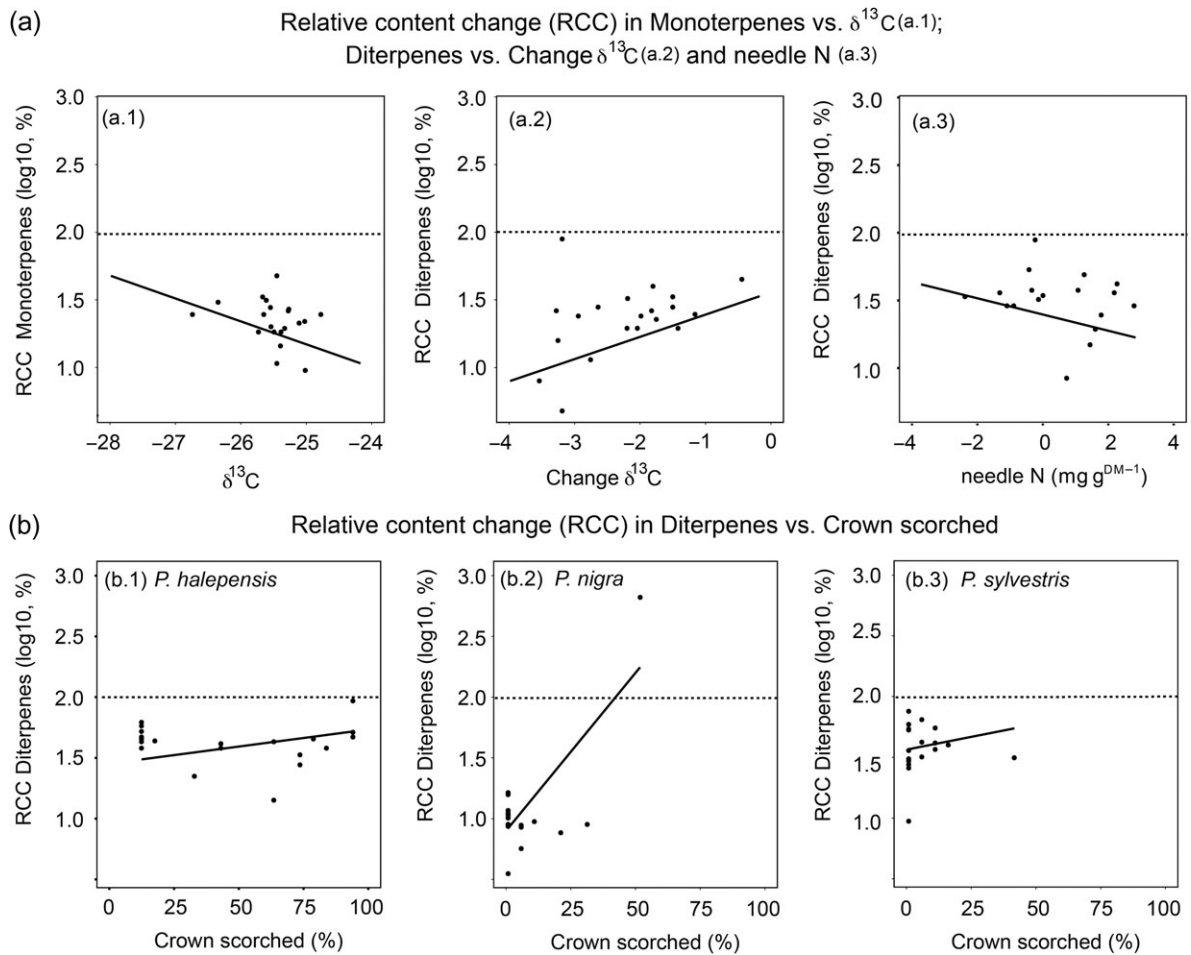


Figure 3. Measured and predicted (line) relative concentration change (log-transformed) using 1 year post-burning models (see Table 5) for monoterpenes against  $\delta^{13}\text{C}$  (a.1), for diterpenes against change in  $\delta^{13}\text{C}$  (a.2), change in needles N (a.3), and the interaction between species and crown scorch (b). Before the log-transformation, 100 was summed. The dashed line indicates no changes between pre- and post-burning terpene concentrations: higher values indicate a higher terpene concentration than those of pre-burning while the opposite is indicated by lower values.

Needle N concentration was positively associated with the relative concentration change of monoterpenes in the three species and of sesquiterpenes in the case of *P. halepensis* and *P. sylvestris*. As resin canal ducts are limited by N (Björkman et al. 1998), these positive relationships may be explained by an increase in the number and size of the ducts in needles with higher N content. In contrast, we did not detect any effect of pre-burning water status, as estimated by  $\delta^{13}\text{C}$ , for 24 h post-burning terpene concentration change in individual pines.

According to our study, tree-to-tree variation in terpene concentration is known to be naturally high, even over short spatial distances, or when plants grow in the same soil in the same geographic area (Ormeño et al. 2008, Kännaste et al. 2013). Our study reveals, however, that this variation is reduced 1 year post-burning within and between plots. One year post-burning, the terpene concentration was lower than pre-burning, while an increase could be expected given the drier meteorological conditions during the year after burning (Loreto and Schnitzler 2010). In

contrast, lower needle  $\delta^{13}\text{C}$  values, compared with pre-burning, suggest a decrease in water competition 1 year post-burning, an increase in the photosynthetic rate or stomatal conductance (Battipaglia et al. 2014), or an improvement in water conditions in the remaining needles of highly scorched trees (Wallin et al. 2003). A lower terpene concentration 1 year after burning differs from other studies (Cannac et al. 2009, Lavoit et al. 2013) comparing burned versus unburned plots. These studies concluded that needle terpene concentration returns to normal values 1 year after fire. They suggested that short-term increases in nutrient availability had minor effects on terpene concentration. The discrepancies with our investigation may be explained by the higher burning intensity in our study, which impacted water availability as indicated by  $\delta^{13}\text{C}$  values. In agreement with the GDBH (Herms and Mattson 1992, Stamp 2003), our results showed that the relative concentration change of diterpenes was lower in trees that had an improvement in their physiological condition 1 year post-burning, as suggested by needle  $\delta^{13}\text{C}$  change and changes in needle N concentration. Despite the fact that no relationships

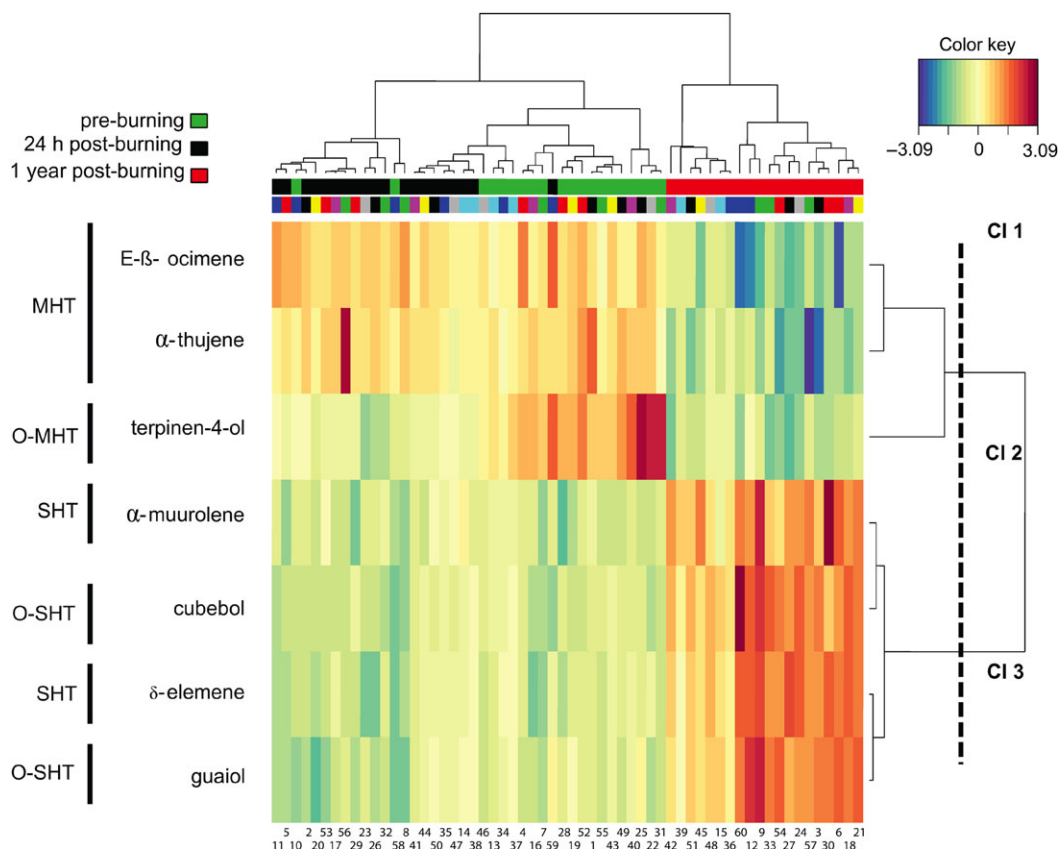


Figure 4. Hierarchical clustering for *P. halepensis* of the seven terpenes selected with multilevel sPLS-DA using terpene content. Samples are represented in columns and terpenes in rows. MHT, monoterpene hydrocarbon; SHT, sesquiterpene hydrocarbon; O, oxygenated compounds; der, derivative compounds.

were found between mono- or sesquiterpenes regarding the change in  $\delta^{13}\text{C}$  or N, the direct relationship between the relative terpene concentration change and the pre-burning  $\delta^{13}\text{C}$  suggested that the decrease in both terpene groups occurred in pines that were more stressed pre-burning.

The ecological functions of many mono-, sesqui- and diterpene compounds are still not well understood, although in recent years significant achievements have been made via genetic engineering (Cheng et al. 2007, Loreto and Schnitzler 2010). Likewise, research on terpenes and flammability is generally scarce, though there are some studies that have shown a correlation between both variables (Owens et al. 1998, Alessio et al. 2004, Ormeño et al. 2009). The reduction in terpene concentration 24 h post-burning in the fire-resister *P. nigra* could imply a reduction of needle flammability with respect to pre-burning, strengthened by a reduction in the highly flammable  $\alpha$ -caryophyllene (also known as  $\alpha$ -humulene) and the increase in bornyl acetate, which is inversely related to flammability (Owens et al. 1998). By contrast, increases of mono- and sesquiterpene concentrations in *P. halepensis* may involve greater flammability, which would favour fire reaching the canopy to effectively open the serotinous cones. Specifically, the sPLS-DA showed E- $\beta$ -ocimene, which is correlated with

flammability (Page et al. 2012), as representative of 24 h post-burning samples. In *P. sylvestris*, the poor terpene discrimination in relation to time since burning limits the interpretation of any compound in terms of flammability.

Fire-damaged trees are more vulnerable to insects, especially bark beetles, and infections by root fungus, which contribute to trees susceptibility to beetle attack (Sullivan et al. 2003, Parker et al. 2006). The accumulation of high amounts of monoterpenes 24 h post-burning in the lower fire resistant species (*P. halepensis* and *P. sylvestris*) when fire partially scorches the crowns, might accomplish several functions, such as effective transport of diterpenes to the affected tissues (Phillips and Croteau 1999), better protection of the photosynthetic apparatus (Vickers et al. 2009) or ensuring the needs for chemical defence against pathogens (Phillips and Croteau 1999). According with this last function, E- $\beta$ -ocimene and  $\alpha$ -thujene with antifungal activity (Bajpai et al. 2007, Deba et al. 2008) appear to correctly classify 24 h post-burning needle samples of *P. halepensis*. Although the discriminant analysis in *P. sylvestris* showed poor classification power, the presence of E- $\beta$ -ocimene and  $\gamma$ -terpinene also suggests that trees possess a higher resistance to fungus compared with pre-burning (Espinosa-García et al. 1993). In the case of the fire-resistant *P. nigra*, the pre-burning concentration of monoterpenes may be sufficient to

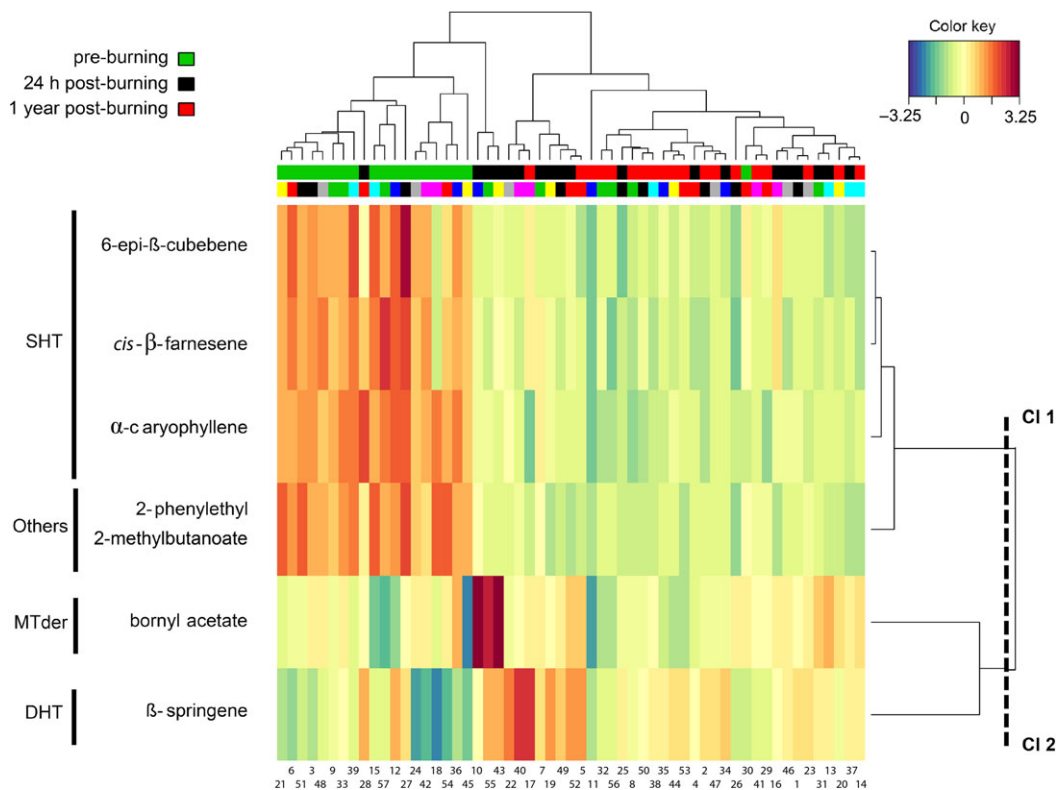


Figure 5. Hierarchical clustering for *P. nigra* of the six terpenes selected with multilevel sPLS-DA using terpene content. Samples are represented in columns and terpenes in rows. MT, monoterpene hydrocarbon; SHT, sesquiterpene hydrocarbon; DHT, diterpene hydrocarbon; der, derivative compounds; others, compounds other than terpenes.

cope with biotic stresses related with medium intensity fires. Nonetheless, bornyl acetate seems to represent 24 h post-burning samples conferring resistance to defoliators immediately after fire (Zou and Cates 1995). The high accumulation of diterpenes 24 h post-burning in *P. nigra* as the proportion of the scorched crown increases in respect to the other species, and possibly indicates a better chemical protection against xylophagous insects (LaFever et al. 1994). In *P. nigra* and *P. sylvestris*, the fact that the percentage of sesquiterpenes was augmented significantly 1 year post-burning with respect to pre-burning, together with the increase in the relative concentration change as crown scorch augmented, might indicate the importance of sesquiterpenes as indirect defences to a wide range of biotic stressors (Phillips and Croteau 1999, Schnee et al. 2006) and, as reported in Lavoie et al. (2013), were representative in repeatedly burned plots. Similarly, our classification found the sesquiterpenes guaiol,  $\alpha$ -muurolene and  $\delta$ -elemene as being characteristic in 1 year post-burning *P. halepensis* needle samples. These compounds might have defensive roles in defoliated trees against insects (Wallis et al. 2008, Liu et al. 2013).

After fire, bark beetles pose a significant threat to trees, especially when a significant amount of the crown has been scorched (Lombardero et al. 2006). Several volatile terpenes such as  $\alpha$ -pinene, camphene and myrcene can be released during PB

and facilitate the attack of bark beetles (Coyle and Lott 1976). Twenty-four hour post-burning *P. sylvestris* tended to present higher amounts of these terpene compounds, suggesting higher susceptibility to bark beetle attack with respect to the other species. Finally, limonene, which is highly toxic for several types of beetle (Raffa et al. 2005), was present in higher amounts in *P. nigra* and *P. halepensis*, suggesting a higher resistance to bark beetle attack for both species 24 h post-burning.

The concentration of mono- and sesquiterpenes 24 h post-burning was similar to the pre-burning ones in the more fire-sensitive species (*P. halepensis* and *P. sylvestris*) and lower in the fire-resistant *P. nigra* species. Terpene dynamics were modulated within the species by fire severity, as indicated by the direct relation between the proportion of scorched crown and the concentration of terpenes 24 h post-burning. As discussed, a combination of morphological and physiological mechanisms may be operating during and in the short-term after PB, but no clear conclusions may be stated. However, differences in terpene contents as a function of the pine species sensitivity to fire suggest that terpenic metabolites could have adaptive importance in fire-prone ecosystems, in terms of flammability and defence against biotic agents short-term



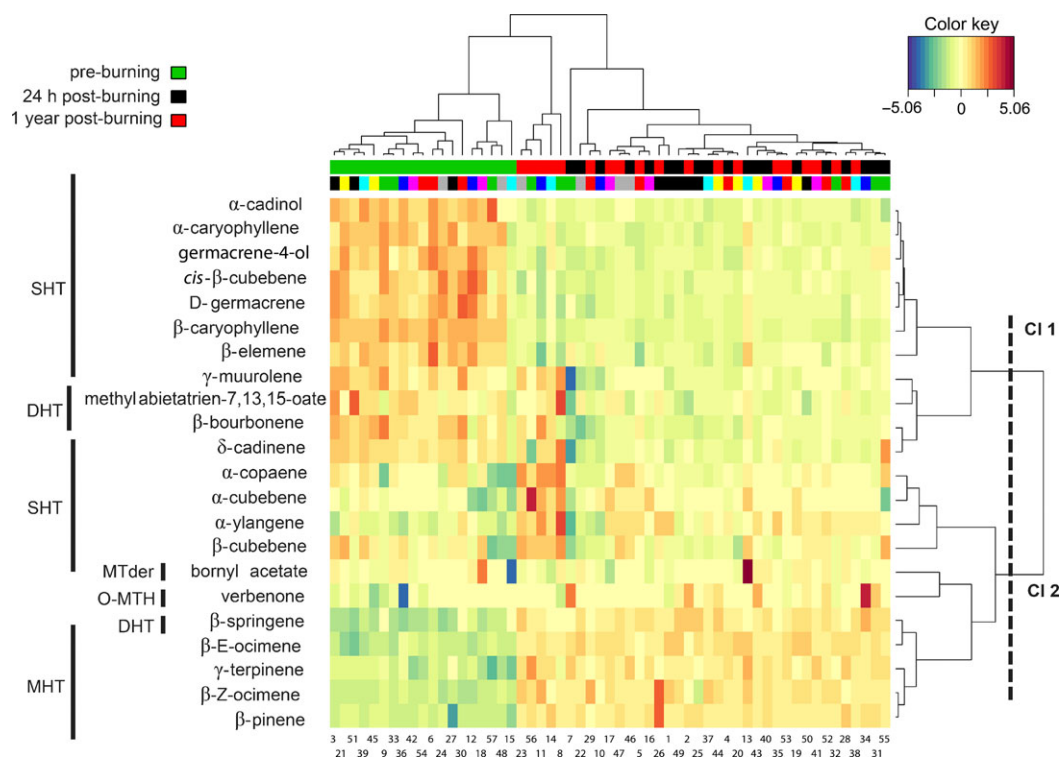


Figure 6. Hierarchical clustering for *P. sylvestris* of the 22 terpenes selected with multilevel sPLS-DA using terpene content. Samples are represented in columns and terpenes in rows. MHT, monoterpene hydrocarbon; SHT, sesquiterpene hydrocarbon; DHT, diterpene hydrocarbon; O, oxygenated compounds; MTder, derivative monoterpenes.

after fire. In agreement with the GDBH (Herms and Mattson 1992, Stamp 2003) trees may be allocating assimilates to growth rather than to defence, as suggested by the remarkable decrease in terpene concentration and the negative relation between terpene concentration and the change in needle  $\delta^{13}\text{C}$ . This decrease in terpene concentration, in turn, could imply a higher susceptibility to fire-related pathogens and insects.

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## Conflict of interest

None declared.

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