# Strong Induction of Minor Terpenes in Italian Cypress, *Cupressus sempervirens*, in Response to Infection by the Fungus *Seiridium cardinale*

Ander Achotegui-Castells · Roberto Danti · Joan Llusià ·
Gianni Della Rocca · Sara Barberini · Josep Peñuelas

**Abstract** *Seiridium cardinale*, the main fungal pathogen responsible for cypress bark canker, is the largest threat to cypresses worldwide. The terpene response of canker-resistant clones of Italian cypress, *Cupressus sempervirens*, to two differently aggressive isolates of *S. cardinale* was studied. Phloem terpene concentrations, foliar terpene concentrations, as well as foliar terpene emission rates were analyzed 1, 10, 30, and 90 days after artificial inoculation with fungal isolates. The phloem surrounding the inoculation point exhibited *de novo* production of four oxygenated monoterpenes and two unidentified terpenes. The concentrations of several constitutive mono- and diterpenes increased strongly (especially α-thujene, sabinene, terpinolene, terpinen-4-ol, oxygenated monoterpenes, manool, and two unidentified diterpenes) as the infection progressed. The proportion of minor terpenes in the infected cypresses increased markedly from the first day after inoculation (from 10 % in the control to 30–50 % in the infected treatments). Foliar concentrations showed no clear trend, but emission rates peaked at day 10 in infected trees, with higher δ-3-carene (15-fold) and total monoterpene (10-fold) emissions than the control. No substantial differences were found among cypresses infected by the two fungal isolates. These results suggest that cypresses activate several direct and indirect chemical defense mechanisms after infection by *S. cardinale*.

## Introduction

Fungal pathogens infect trees by using enzymes, toxins, growth regulators, and by obtaining nourishment from the substances produced by the host. Conifers make use of chemical defenses, primarily terpenes and phenols (Franceschi et al. 2005; Phillips and Croteau 1999) that ward against pathogenic fungi and other threats. Terpenes are used in conifers as constitutive defenses (a first line of defense against any enemy) but also as induced defenses against pathogens; increases in absolute amounts, proportional changes, phytoalexin production, and general or specific responses to an antagonist can appear at different time points following infection (Michelozzi 1999). Oleoresin is secreted from injured or infected tissues, thus deterring fungal pathogens or insects and sealing the wound at the same time (Trapp and Croteau 2001). Hundreds of studies have demonstrated that terpenes can strongly inhibit fungal spore germination and mycelial growth (see reviews by Bakkali et al. 2008; Boulogne et al. 2012 and references therein) by disrupting internal structures and permeabilizing fungal cells (Bakkali et al. 2008).

Plants can respond generally to pathogenic infections but may also react specifically to specific pathogens. Conifers can have distinct terpene reactions to different fungal pathogens (Raffa and Smalley 1995; Schiller and Madar 1991; Zamponi et al. 2007), but usually exhibit similar reactions to different fungal isolates or strains of the same fungus (Bonello et al. 2008; Faldt et al. 2006; Schiller and Madar 1991). In addition to the local terpene reactions to fungal infection, systemic responses have been found in non-infected tissues. Systemic

A. Achotegui-Castells · J. Llusià · J. Peñuelas
CREAF, Cerdanyola del Vallès 08193, Catalonia, Spain

A. Achotegui-Castells (✉) · J. Llusià · J. Peñuelas
CSIC, Global Ecology Unit CREAF-CEAB-UAB, Cerdanyola del Vallès 08193, Catalonia, Spain
e-mail: a.achotegui@creaf.uab.es

R. Danti · G. D. Rocca · S. Barberini
IPSP-CNR, Via Madonna del Piano 10, I-50019 Sesto Florentino, FI, Italy

changes in phloem terpene concentrations (Viiri et al. 2001), foliar terpene concentrations (Schiller and Madar 1991), and foliar terpene emission rates (Faldt et al. 2006) have been observed in conifers infected by fungi. These phenomena could enhance the defense of undamaged plant tissues, prepare the plant for new attacks related to the infection, or activate indirect defense strategies (Bonello et al. 2008).

Cypress bark canker caused by the mitosporic fungus *Seiridium cardinale* (Wagener) Sutton & Gibson is the most severe and widespread disease affecting Italian cypress (*Cupressus sempervirens* L.) worldwide (Battisti et al. 1999; Della Rocca et al. 2011; Graniti 1998). This disease affects the cortical tissues (phloem and cambium but not xylem) of several members of the Cupressaceae family, causing severe diebacks and often death of the cankered trees over a time span of months to years (Graniti 1998). After the first outbreak reported in California in 1929 (Wagener 1939), cypress bark canker has spread rapidly to other regions of the world, having a relevant impact in the Mediterranean Basin (Graniti 1998; Panconesi 1991; Xenopoulos 1990). The disease spreads by dissemination, mainly by rainwater, of asexual spores of the fungus (conidia) produced in fruiting bodies on the surface of affected trees or by windborne raindrops and vectors (Battisti et al. 1999; Covassi et al. 1975; Zocca et al. 2008). Results from a 40-year genetic improvement program have revealed a moderate variability in the response of some Mediterranean native and naturalized *C. sempervirens* populations to *S. cardinale* infections, with 1–2 % of trees being resistant. Several resistant genotypes have been selected, and some varieties have been patented and successfully commercialized (Danti et al. 2006, 2013; Panconesi and Raddi 1991).

Italian cypress has an oleoresin rich in terpenoids and reacts to wounds or fungal infection by producing traumatic resin ducts in the phloem (Hudgins et al. 2004; Krokene et al. 2008). The composition of basic terpenes in several tissues and the reaction to some environmental changes have been studied for this tree (Gallis et al. 2007; Mazari et al. 2010; Piovetti et al. 1980, 1981; Yani et al. 1993; Yatagai et al. 1995). Two terpene phytoalexins, cupressotropolone A and B, were detected in Italian cypresses inoculated with *Diplodia pinea* f. sp. *cupressi*, another canker-causing fungal pathogen (Madar et al. 1995a, b). These phytoalexins showed substantial activity against several fungal pathogens of cypress, including *S. cardinale* (Madar et al. 1995a). Moderate antifungal activity of the essential oil of *C. sempervirens* leaves was observed against fungal pathogens of other hosts (Mazari et al. 2010). The proportions of terpene contents of leaves of healthy and naturally infected *C. sempervirens* trees (by *D. pinea* f. sp. *cupressi* and *S. cardinale*) were studied by Schiller and Madar (1991), and although proportions differed among treatments, no specific compound was associated with fungal infection or resistance, and no clear differences in tree response among the two fungal pathogens were found.

In summary, little is known about conifer phytoalexin production, systemic reactions, or foliar emissions under fungal infection, especially for families other than Pinaceae. As for the *C. sempervirens* – *S. cardinale* pathosystem, little is known about changes in the terpene composition of Italian cypress as a response to infection by the main cypress bark canker agent.

The goals of this study were thus: (*i*) to monitor the locally induced terpene response of the phloem of canker-resistant cypress clones to wounds and infection by two *S. cardinale* isolates during the first 90 days after artificial inoculation; (*ii*) to investigate the systemic response of cypress leaves to fungal infection, analyzing foliar concentration and emission rates and; (*iii*) to study the differential responses in cypress tissues induced by the two isolates of *S. cardinale* characterized by different pathogenicity.

## Methods and Materials

*Study Site* The study was performed in an experimental field of the Institute of Sustainable Protection of Plants – National Research Council (IPSP-CNR, in italian) in Cannara, Perugia, central Italy (42°58′29″ N, 12°36′38″ E). The field was at an elevation of 192 m a.s.l. and provided equal light, nutrient, and water availability for all trees. We used 64 four-yr-old grafted plants of *C. sempervirens*, planted with a 3×3 m spacing and belonging to four genotypes patented by IPSP-CNR for their resistance to cypress bark canker: Italico, Bolgheri, Agrimed and Mediterraneo (16 trees of each genotype) (Danti et al. 2006; Panconesi and Raddi 1991). Cypresses were watered twice a week during the first month after planting. Soil was a clayey reclaimed alluvial. The climate is moderately continental, with hot summers and cold winters with sporadic snowfall. The average rainfall is 815 mm yr$^{-1}$ distributed on 80 rainy days with a peak in autumn. The yearly average annual temperature is 13.8 °C. The coldest month is January with an average minimum of 0 °C, and the warmest month is July with an average maximum temperature of 30 °C.

*Experimental Design* To monitor tree reactions against fungal infection, we applied four treatments to the cypresses: 1) control (no damage); 2) mildly virulent (Mv, wound+inoculation with a moderately aggressive *S. cardinale* isolate (ref. submitted)); 3) highly virulent (Hv, wound+infection with a more aggressive *S. cardinale* isolate); and 4) Wounded (wound only, without inoculation). Trees were inoculated following a standard procedure (Danti et al. 2006, 2013), which consists of removing a disc of bark from the stem with a sterile cork borer of 4 mm diam and filling the wound with a plug of the same size of malt extract agar (MEA). This plug was taken from the margin of a colony of the fungus grown on MEA 2 %

in the dark for 15 d at 25 °C. The inoculation site was covered with wet cotton wool and wrapped with Parafilm®.

Tissue samples were collected from 26 April to 25 July 2012, 1, 10, 30, and 90 d after applying the above treatments. The sampling method was destructive, so trees were used only once to avoid any effects from the wounds. Each treatment, for each sampling date, had four replicates (four treatments× four time points×four replicates=64). Within the treatments, each of the four replicates contained each of the four tree genotypes.

*Field sampling. Tissue Sampling*  Three types of samples were collected from each tree: *i*) phloem removed from a segment of the inoculated stem containing the infected tissues (samples were taken from a height of ca. 80 cm); *ii*) foliar tissue from the closest branch to the inoculation point and; *iii*) foliar volatile organic compound (VOC) emission, from the same branch where foliar tissue was taken. Emissions were sampled first to avoid tree reactions to wounding. All sampled tissues were stored in liquid nitrogen in the field and then at −20 °C in the laboratory.

*VOC Sampling*  Twigs immediately above the inoculation point (3.5–21 cm) were sampled to analyze VOC emissions. The selected twigs were wrapped first with Teflon ribbon a few days before the sampling to minimize effects of mechanical manipulation and alteration of the emissions.

The VOC emissions were sampled from 09:00 to 15:00 h (solar time) using the conifer chamber (a 230 cm$^3$ cuvette) of the LiCor 6400 Portable Photosynthesis System (Li-Cor Inc, Lincoln, NE, USA). The twig was carefully inserted into the chamber, placing its closure on the Teflon ribbon. Air flow rate inside the conifer chamber was set to 600 μmol s$^{-1}$. The chamber was allowed to stabilize for 15 min, as monitored by environmental and physiological parameters such as temperature, photosynthetic active radiance (PAR), photosynthesis, and stomatal conductance. When the twig had physiologically stabilized, we placed one end of a metallic VOC trap (Markes International Inc. Wilmington, DE, USA), filled with 115 mg of Tenax and 230 mg of Unicarb, in the chamber to collect the VOCs exhausted from the twig chamber. A QMAX pump (Supelco, Bellefonte, PA, USA) attached to the other end of the metallic trap pulled the air from the conifer chamber. A Defender 510 fluxometer (Bios International Corporation, Butler, NJ, USA) was placed between the QMAX and the VOC trap to control the air flux. Sampling time was 5 min, with an absorption flux of ca. 7 ml s$^{-1}$. The sampled VOC traps were stored in the field in a 4 °C portable refrigerator until transferred to a -20 °C freezer in the laboratory. Blank samples were collected after every two twig samples, as described above, but without a twig inside the conifer chamber. The VOC-sampled

leaves also were stored, and once in the laboratory dried until constant weight, in order to refer the emission rates to g of dry weight (μg g$^{-1}$ of foliar dry weight h$^{-1}$).

*Sample Analyses and Terpene Identification*  Phloem and leaves were ground separately inside 50-ml Teflon tubes filled with liquid nitrogen to avoid the evaporation of VOCs and to facilitate their crushing. After samples had been pulverized, 1 ml of pentane containing 0.5 μl of dodecane (used as an internal standard) was added, and the Teflon tubes were stored for at least 12 h at −20 °C. After extract stabilization to laboratory temperature, 300 μl of the supernatant were stored in vials, for subsequent analysis in a gas chromatograph/mass spectrometer (GC/MS). The tubes, now containing only the unused extract, were dried to a constant weight and then weighed in a precision balance. Tubes were later exhaustively cleaned, dried and reweighed to tare them. One blank was analyzed after every five samples.

Two μl of the biomass extract were injected into a capillary column (HP 5MS, 30 m×0.25 μm×0.25 mm) in a GC (7890A, Agilent Technologies, Santa Clara, CA, USA) with a MS detector (5975C inert MSD with Triple-Axis Detector, Agilent Technologies). The temperature was maintained first at 35 °C for 2 min, increased at 15 °C min$^{-1}$ to 150 °C and maintained for 5 min, increased at 30 °C min$^{-1}$ to 250 °C and maintained for 5 min, and finally increased at 30 °C min$^{-1}$ to 280 °C and maintained for 5 min. Total run time was 29 min, and the helium flow was set to 1 ml min$^{-1}$.

Terpenes were identified by comparing the mass spectra with published spectra (libraries NIST 05 and Wiley 7n) and the spectra of known standards. Calibration curves for the quantification of each terpene were prepared with commercial standards of the most abundant compounds found in the samples. Four monoterpenes (α-pinene, sabinene, limonene, and γ-terpinene), three sesquiterpenes (caryophyllene, caryophyllene oxide, and cedrol), two diterpenes (phytol and totarol), and one non-terpene internal standard (dodecane) were used (Fluka Chemie AG, Buchs, Switzerland). All terpene calibration curves were highly significant ($r^2 \geq 0.99$) for the relationship between signal strength and terpene concentration. The most abundant terpenes exhibited similar sensitivities (differences <5 %). Terpenes identified only by published spectra that were considered important for the experiment were later verified with standards: α-thujene (Chemos GmbH, Regenstauf, Germany) terpinolene, terpinen-4-ol, sabinene hydrate, camphor, α-terpineol (Fluka Chemie AG, Buchs, Switzerland), and manool (Sequoia Research Products Limited, Pangbourne, United Kingdom).

*Terpene Emission Rates*  The terpene emissions collected by the VOC traps were released with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes International Inc. Wilmington, DE, USA) and desorbed using an injector

(Unity, Series 2, Markes International Inc. Wilmington, DE, USA) in the GC/MS described above. A full-scan method was used for the chromatographic analyses. The desorbed sample was retained in a cryotrap at −20 °C. The split was 1:10. The sample was redesorbed at 250 °C for 10 min, injected into the column with a transfer line at 250 °C, and submitted to the same chromatographic process described above for the analysis of terpene concentrations.

No diterpenes were used as standards for the analyses of emission rates because they are not volatile at ambient temperature. The terpene emission rates were expressed in $\mu g\ g^{-1}$ (dry weight (dw)) $h^{-1}$. Even though the days of sampling were similar (sunny and warm), the terpene emission rates were standardized at 30 °C using an algorithm for terpene-storing species (Guenther et al. 1993):

$$E = E_s\{\exp[\beta(T-T_s)]\}$$

where $E$ represents the emission rate in $\mu g\ g^{-1}$ (dw) $h^{-1}$ of monoterpenes at temperature $T$ (in degrees Kelvin, K), $E_s$ is the emission factor in $\mu g\ g^{-1}$ (dw) $h^{-1}$ at standard temperature $T_s$ (303 K), and $\beta$ represents an empirically determined coefficient, 0.09 K.

*Statistical Analyses* Data were analyzed using restricted maximum likelihood (REML), with the treatment (control, Wounded, Mv and Hv) as the fixed factor and the genotype (Agrimed, Bolgheri, Italico and Mediterraneo) as the random factor. Pairwise comparisons between treatments were performed using a Tukey's *post-hoc* test. Data that did not fit normality requirements were log transformed. Statistical analyses were conducted using R software version 2.15.2 (R Foundation for Statistical Computing, 2012) and Statistica version 8.0 (Statsoft Inc. Tulsa, OK, USA) and the graphics were generated using SigmaPlot version 11.0 (Systat Software, Chicago, IL, USA).

## Results

*Local Phloem* Phloem samples of cypresses had similar concentrations of monoterpenes and diterpenes, and sesquiterpenes represented only ca. 10 % of the total terpene concentration. Sixty-eight terpenes represented more than 0.1 % of the total peak area of the chromatograms, and those detected in more than 40 % of all samples (27 terpenes) were selected for statistical analyses. The most abundant monoterpenes were α-pinene and δ-3-carene (ca. 90 % of total monoterpenes in the control). α-Cubebene and longifolene were the principal sesquiterpenes, and totarol was the most abundant diterpene (ca. 60 % of total diterpenes in the control).

*Qualitative Differences among Treatments* Six terpenes appeared exclusively in the infected treatments (Mv and Hv) 30 and 90 days after inoculation. These six *de novo* terpenes were found in all four cypress genotypes. Four of these were oxygenated monoterpenes: oxygenated monoterpene *de novo 1* (detected in 15 of 16 samples of Mv and Hv at days 30 and 90, 0.093±0.02 mg $g^{-1}$, mean±SE), sabinene hydrate (16/16; 0.17±0.03 mg $g^{-1}$), camphor (10/16; 0.16±0.04 mg $g^{-1}$), and α-terpineol (13/16; 0.36±0.1 mg $g^{-1}$). The monoterpene *de novo 2* (14/16; 0.11±0.04 mg $g^{-1}$) and the diterpene *de novo 3* (6/16; 5.4±1.7 mg $g^{-1}$) could not be identified. No differences in concentration were detected between treatment or time for the *de novo* compounds (REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, $P<0.05$). Thymyl methyl ether (another oxygenated monoterpene) did not appear in the control but was detected in some of the Wounded samples and in all infected treatments from day 10 to day 90, reaching a mean concentration of 2.9±1.2 mg $g^{-1}$ in Hv at day 30 (Table 1).

*Quantitative Differences among Treatments* Total concentrations were lower in the infected treatments than in the control at days 1 and 10 but increased substantially after day 30 (Table 1). Total terpenes were nearly 4-fold higher in the infected treatments compared to control at day 30, and reached a maximum of 140 mg $g^{-1}$ at day 90 (Table 1). This increase in total terpenes was due partly to increased concentrations of some of the most abundant compounds (α-pinene, diterpene 1) but also to the strong increases in concentrations of several minor compounds. These changes led to a decrease in the proportions of the main compounds. α-Thujene was among the most induced compounds in the infected treatments (up to a 57-fold increase relative to the control), and presented differences from day 10, with concentrations and proportions rising steadily until day 90. Next, in order of retention time was sabinene, whose concentrations (60-fold increase) had begun to differentiate by day 10 and whose proportions peaked between days 10–30, and then dropped slightly by day 90 (Fig. 1). Terpinolene concentrations (18-fold increase) had higher proportions in the infected treatments throughout the experiment, reaching maximum proportion at day 1. Terpinen-4-ol (622-fold increase) retained a high concentration and proportional difference between treatments from days 10 to 90. Diterpene 2 was the most induced diterpene (164-fold increase) and increased its concentration steadily from day 1 to day 90 (Fig. 2). Diterpene 5 (43-fold), diterpene 6 (42-fold ), and manool (11-fold ) increased in concentration and proportions from day 10 to 90. Limonene (12-fold ) and α-terpinene (15-fold) also notably increased, but the concentrations were significantly higher than the control only at day 90. Oxygenated monoterpenes (the sum of terpinen-4-ol, thymyl methyl ether, and bornyl acetate) were the most induced terpene class, with up to 1063-fold higher

**Table 1** Mean concentrations (±SE) in mg g⁻¹ dry weight and mean proportions (±SE) in %, relative to the terpene category, of the terpenes in the local phloem of cypresses infected with *S. cardinale*

| Name | RT (min) | | DAY 1 | | | | DAY 10 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Control | Wounded | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| α-thujene | 7.73 | [] | 0.027±0.006 | 0.025±0.013 | 0.038±0.016 | 0.011 | 0.035±0.012 | 0.085±0.053 | 0.035±0.013 | 0.072±0.035 |
| | | % | 0.23±0.01 | 0.32±0.1 | 0.55±0.24 | 0.18 | **0.24±0.05b** | **0.75±0.33ab** | **0.82±0.19ab** | **1.1±0.3a** |
| α-pinene | 7.83 | [] | 6.3±0.1 | 2.9±1.2 | 2.7±2.1 | 1.1±1.0 | **8.8±2.3a** | **6.0±2.4ab** | **2.5±1.1b** | **3.6±1.9b** |
| | | % | 55±10 | 53±7 | 42±10 | 37±6 | 59±9 | 71±5 | 47±14 | 46±11 |
| α-fenchene | 8.04 | [] | 0.33±0.13 | 0.15±0.07 | 0.27±0.21 | 0.18 | **0.38±0.15a** | **0.11±0.07b** | **0.048±0.024b** | **0.063±0.038b** |
| | | % | 2.7±0.7 | 2.3±0.5 | 2.4±0.3 | 2.8 | **2.2±0.6a** | **0.98±0.38a** | **0.76±0.24b** | **0.77±0.23b** |
| Sabinene | 8.33 | [] | 0.074±0.002 | 0.039±0.014 | 0.064±0.049 | 0.015±0.009 | 0.11±0.04 | 0.41±0.25 | 0.21±0.09 | 0.43±0.2 |
| | | % | 0.65±0.09 | 0.9±0.17 | 2.1±0.8 | 2.7±1.4 | **0.68±0.08b** | **3.4±1.6a** | **3.9±1.0a** | **5.3±0.5a** |
| β-pinene | 8.39 | [] | 0.11±0.07 | 0.060±0.019 | 0.079±0.07 | 0.049±0.042 | **0.16±0.03a** | **0.15±0.07ab** | **0.053±0.016b** | **0.088±0.049ab** |
| | | % | 0.88±0.43 | 1.7±0.6 | 2.5±1.2 | 3.8±1.8 | 1.4±0.5 | 1.7±0.2 | 1.1±0.2 | 1.0±0.3 |
| β-myrcene | 8.49 | [] | 0.14±0.07 | 0.059±0.021 | 0.097±0.084 | 0.042±0.035 | **0.22±0.06a** | **0.14±0.08ab** | **0.069±0.031b** | **0.14±0.08ab** |
| | | % | 1.1±0.4 | 1.2±0.2 | 2.7±1.0 | 3.8±2.2 | 1.4±0.1 | 1.3±0.5 | 1.3±0.2 | 1.3±0.5 |
| δ-3-carene | 8.78 | [] | 4.3±1.5 | 1.7±0.8 | 2.3±2.0 | 1.2±1.2 | **5.4±2.5a** | **1.1±1.1b** | **0.29±0.26b** | **0.76±0.63b** |
| | | % | **35±7ab** | **29±6a** | **19±10c** | **19±12bc** | **29±8a** | **7.9±7.9b** | **4.0±3.1b** | **8.7±4.1b** |
| Limonene | 9.01 | [] | 0.11±0.04 | 0.056±0.015 | 0.072±0.05 | 0.029±0.021 | 0.13±0.03 | 0.080±0.030 | 0.27±0.21 | 0.11±0.04 |
| | | % | 0.90±0.20 | 1.5±0.4 | 3.7±1.5 | 4.2±2.8 | 0.90±0.11 | 0.90±0.07 | 6.4±5.6 | 1.0±0.2 |
| Terpinolene | 9.67 | [] | 0.34±0.2 | 0.3±0.09 | 0.32±0.18 | 0.19±0.09 | **0.68±0.21b** | **0.76±0.27ab** | **0.71±0.19ab** | **2.01±0.98a** |
| | | % | **2.7±1.3ab** | **11±6b** | **31±15a** | **34±16ab** | **5.0±1.0b** | **10±2ab** | **21±8a** | **19±7ab** |
| Terpinen-4-ol | 10.74 | [] | NA | 0.011 | 0.0060 | NA | **0.016±0.005b** | **0.051±0.036a** | **0.055±0.017a** | **0.13±0.02a** |
| | | % | NA | 0.13 | 2.2 | NA | **0.08±0.01b** | **0.55±0.25ab** | **2.1±1.1a** | **1.8±0.8ab** |
| Thymyl methyl ether | 11.50 | [] | NA | NA | NA | NA | NA | 1.2 | 0.97±0.78 | 1.1±0.4 |
| | | % | NA | NA | NA | NA | NA | 10 | 26±13 | 19±13 |
| α-terpinene | 13.35 | [] | 0.044±0.023 | 0.018 | 0.062 | | 0.087±0.045 | 0.044±0.004 | 0.015±0.005 | 0.037±0.028 |
| | | % | 0.42±0.26 | 0.22 | 0.35 | NA | 0.40±0.14 | 0.35±0.05 | 0.21±0.04 | 0.24±0.17 |
| Minor Monoterpenes | | [] | 1.2±0.5 | 0.7±0.2 | 0.8±0.6 | 0.4±0.2 | **1.8±0.5b** | **2.2±1ab** | **1.9±0.8ab** | **3.8±1.6a** |
| | | % | **9.6±2.6ab** | **19±6b** | **44±17a** | **50±18ab** | **12±2b** | **24±2ab** | **49±15a** | **45±15a** |
| Oxygenated Monoterpenes | | [] | NA | 0.012 | 0.006 | NA | **0.016±0.005b** | **0.46±0.45ab** | **1.0±0.8a** | **1.2±0.4a** |
| | | % | NA | 0.13 | 2.0 | NA | **0.08±0.01b** | **4.0±3.7ab** | **28±13a** | **21±14a** |
| Total Monoterpenes | | [] | 12±2 | 5.2±2.3 | 5.1±4.2 | 2.3±2.1 | **16±5a** | **9±3.7b** | **4.6±1.5b** | **8.1±3.7b** |
| | | % | 55±2 | 60±4 | 62±13 | 56±14 | 44±7 | 48±2 | 57±7 | 48±13 |
| α-cubebene | 13.35 | [] | 0.51 | 0.35±0.18 | 0.39±0.29 | 0.17±0.13 | **1.5±0.3a** | **0.59±0.25b** | **0.29±0.11b** | **0.33±0.19b** |
| | | % | 25 | 43±5 | 51±25 | 68±24 | **45±6b** | **44±8ab** | **68±11ab** | **66±10a** |
| Longifolene | 14.99 | [] | 0.84±0.45 | 0.45±0.23 | 1.1±0.9 | 1.4 | **1.4±0.4a** | **0.51±0.42b** | **0.18±0.1b** | **0.14±0.12b** |
| | | % | 64±2 | 44±6 | 41±18 | 63 | 39±9 | 27±17 | 20±6 | 20±7 |
| β-cedrene | 15.10 | [] | 0.17±0.05 | 0.15±0.03 | 0.18±0.13 | 0.23 | **0.45±0.08a** | **0.16±0.05b** | **0.064±0.025b** | **0.10±0.07b** |
| | | % | 15±5 | 10±2 | 8.5±0.8 | 10.4 | 11±3 | 16±6 | 11±1 | 12±2 |
| Cedrol | 17.49 | [] | **0.080±0.010ab** | **0.051±0.015b** | **0.21±0.09a** | **0.056±0.053b** | **0.35±0.11a** | **0.14±0.08b** | **0.076±0.02b** | **0.057±0.042b** |
| | | % | 9±5 | 19±10 | 24±21 | 8±3 | 9.2±3.7 | 13±5 | 18±6 | 11±2 |
| Total Sesquiterpenes | | [] | 1.3±0.7 | 0.81±0.41 | 1.4±1 | 0.77±0.72 | **3.4±0.7a** | **1.4±0.5b** | **0.48±0.22b** | **0.57±0.39b** |
| | | % | 6.0±2.8 | 8.0±2.3 | 11±1 | 16±5 | **10±1a** | **7.9±0.6b** | **5.7±1.4b** | **3.6±1.2b** |
| Manool | 20.82 | [] | 0.17 | 0.11±0.06 | 0.20±0.06 | 0.14±0.13 | 0.33±0.07 | 0.47±0.32 | 0.37±0.22 | 0.37±0.13 |
| | | % | 2.1±0.1 | 3.9±1.2 | 4.0±2.2 | 4.3±2.3 | **2.3±0.3b** | **6.1±2.7ab** | **17±5a** | **10±4ab** |
| Diterpene 1 | 22.26 | [] | 1.4±0.8 | 0.71±0.43 | 1.8±1.5 | 0.60±0.60 | **2.2±0.7a** | **0.70±0.51b** | **0.22±0.17b** | **0.44±0.36b** |
| | | % | **18±11ab** | **18±5a** | **18±6ab** | **10±0b** | 15±5 | 8.0±3.8 | 5.0±2.5 | 8.7±4.8 |
| Diterpene 2 | 22.84 | [] | 0.042±0.040 | 0.042±0.005 | 0.11±0.07 | 0.038 | 0.091±0.03 | 1.2±0.9 | 0.77±0.5 | 0.87±0.28 |

**Table 1** (continued)

| Name | RT (min) | | DAY 1 | | | | DAY 10 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Control | Wounded | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| Diterpene 3 | 22.96 | % | **0.53±0.45b** | **0.78±0.06ab** | **1.4±0.1a** | **0.31** | **0.64±0.23b** | **15±8a** | **27±9a** | **24±9a** |
| | | [] | 0.11±0.02 | 0.072±0.034 | 0.13±0.09 | 0.37 | **0.37±0.07a** | **0.15±0.05b** | **0.12±0.04b** | **0.060±0.022b** |
| Totarol | 23.3 | % | 1.4±0.2 | 1.7±0.2 | 1.6±0.1 | 2.8 | **2.7±0.3a** | **2.3±0.3ab** | **2.2±0.7ab** | **1.8±0.4b** |
| | | [] | 4.8±1.0 | 1.6±0.7 | 4.3±2.8 | 4.1±4.0 | **7.7±1.3a** | **2.7±1.5b** | **0.92±0.64b** | **0.99±0.49b** |
| Diterpene 4 | 23.45 | % | 59±11 | 54±4 | 58±7 | 49±12 | 54±2 | 38±10 | 28±9 | 29±14 |
| | | [] | 0.25±0.09 | 0.074±0.032 | 0.19±0.13 | 0.46 | **0.51±0.11a** | **0.31±0.14ab** | **0.14±0.04b** | **0.11±0.03b** |
| Diterpene 5 | 23.77 | [] | 3.1±1.1 | 2.8±0.8 | 2.4±0.1 | 3.5 | **3.7±0.8ab** | **4.8±1.2a** | **3.5±0.4b** | **2.9±0.5b** |
| | | [] | 0.018±0.005 | 0.028 | 0.061±0.011 | 0.029 | 0.063±0.01 | 0.18±0.15 | 0.16±0.07 | 0.41±0.16 |
| Diterpene 6 | 24.64 | % | 0.22±0.07 | 6.4 | 1.4±0.9 | 0.22 | **0.43±0.11c** | **2.1±1.4bc** | **5.8±2.8ab** | **11±6a** |
| | | % | 0.044±0.018 | 0.041 | 0.15 | 0.042 | **0.091±0.023a** | **0.10±0.01a** | **0.033±0.003bc** | **0.040±0.025c** |
| | | [] | 0.55±0.24 | 0.93 | 1.1 | 0.27 | 0.65±0.27 | 1.0±0.1 | 0.54±0.03 | 0.72±0.27 |
| Diterpene 7 | 24.93 | [] | 0.73±0.06 | 0.34±0.16 | 0.75±0.62 | 0.82±0.81 | **1.9±0.4a** | **0.50±0.30b** | **0.27±0.2b** | **0.22±0.17b** |
| | | % | 9.1±0.9 | 11±1 | 8±2 | 12±0 | **14±2a** | **5.1±2.5b** | **8.6±3.3ab** | **4.7±2.1b** |
| Totarolone | 25.45 | [] | 0.040±0.012 | 0.032 | 0.043±0.025 | 0.13 | 0.09±0.013 | 0.17±0.08 | 0.06±0.03 | 0.079±0.03 |
| | | % | 0.49±0.07 | 0.75 | 0.65±0.16 | 0.78 | **0.64±0.11b** | **3.9±2.1a** | **1.9±0.6ab** | **2.4±1.0ab** |
| Hinokione | 25.55 | [] | 0.45±0.08 | 0.20±0.09 | 0.46±0.39 | 0.46±0.42 | **0.95±0.21a** | **0.83±0.28ab** | **0.32±0.11b** | **0.34±0.112b** |
| | | % | 5.6±0.1 | 6.8±0.4 | 4.7±1.5 | 21±14 | 6.5±0.4 | 14±4 | 8.4±0.4 | 9.4±3.0 |
| Total diterpenes | | [] | 8.0±0.1 | 3.1±1.4 | 8.1±5.8 | 6.6±6.5 | **14±2a** | **7.2±2.8b** | **3.1±1.4b** | **3.8±1.2b** |
| Total terpenes | | % | 39±5 | 32±2 | 40±0.1 | 42±19 | 46±7 | 42±2 | 28±6 | 41±14 |
| | | [] | 21±3 | 9.3±3.9 | 10±8 | 7.5±7.1 | **34±6a** | **18±7b** | **9±3.3b** | **13±5b** |

| Name | DAY 30 | | | | DAY 90 | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Wounded | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| α-thujene | **0.018±0.008b** | **0.11±0.02a** | **0.49±0.07a** | **0.68±0.33a** | **0.032±0.011b** | **0.11±0.05b** | **1.5±0.5a** | **1.8±0.5a** |
| | **0.16±0.04b** | **0.52±0.11b** | **1.4±0.3a** | **1.3±0.3a** | **0.30±0.06b** | **0.70±0.06b** | **2.4±0.4a** | **2.2±0.6a** |
| α-pinene | 7.8±2.4 | 18±4 | 26±9 | 32±15 | **8.7±3.1b** | **8.8±3.8bc** | **45±19ab** | **54±12a** |
| | **78±6ab** | **82±9a** | **61±6b** | **67±6ab** | 77±10 | 67±13 | 68±4 | 68±5 |
| α-fenchene | 0.18±0.09 | 0.21±0.06 | 0.81±0.4 | 1.2±0.9 | 0.15±0.07 | 0.21±0.15 | 0.8±0.3 | 0.8±0.2 |
| | 1.4±0.5 | 1.1±0.4 | 1.6±0.6 | 1.7±0.6 | 1.4±0.5 | 1.3±0.4 | 1.1±0.2 | 1.1±0.1 |
| Sabinene | **0.052±0.021b** | **0.48±0.06a** | **2.0±0.3a** | **2.9±1.5a** | **0.066±0.027b** | **0.42±0.21b** | **3.0±0.5a** | **2.6±0.5a** |
| | **0.51±0.11b** | **2.3±0.4b** | **6.1±1.6a** | **5.3±1.6a** | **0.58±0.12c** | **2.3±0.7b** | **6.7±2.0a** | **3.5±0.5ab** |
| β-pinene | 0.13±0.07 | 0.38±0.04 | 0.35±0.08 | 0.48±0.22 | **0.22±0.1bc** | **0.30±0.14b** | **0.97±0.31ab** | **1.1±0.3a** |
| | **1.3±0.5ab** | **1.8±0.4a** | **1.0±0.2b** | **0.92±0.11b** | | | 1.7±0.2 | 1.4±0.3 |
| β-myrcene | 0.12±0.07 | 0.36±0.02 | 0.67±0.16 | 0.98±0.52 | **0.26±0.09b** | **0.36±0.18b** | **2.1±0.7a** | **2.4±0.7a** |
| | 1.1±0.3 | 1.7±0.3 | 1.7±0.1 | 1.5±0.4 | **2.2±0.4ab** | **2.0±0.6b** | **3.5±0.3a** | **3.0±0.7a** |
| δ-3-carene | 1.9±1.0 | 1.3±1.3 | 2.6±1.8 | 1.1±0.9 | 1.3±1.1 | 3.5±3.3 | 1.1±0.8 | 1.7±0.9 |
| | 14±7 | 7.7±7.3 | 5.1±3.2 | 2.0±1.4 | 12±8 | 19±11 | 4.6±4.2 | 3.4±2.3 |
| Limonene | 0.066±0.032 | 0.19±0.02 | 0.41±0.11 | 0.67±0.43 | **0.12±0.04b** | **0.20±0.10b** | **1.0±0.4a** | **1.3±0.3a** |

**Table 1** (continued)

| Name | DAY 30 | | | | DAY 90 | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Wounded | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| Terpinolene | 0.68±0.18 | 0.93±0.18 | 0.97±0.08 | 0.97±0.27 | **0.98±0.25b** | **1.2±0.2b** | **1.7±0.1a** | **1.6±0.3a** |
| | **0.22±0.04b** | **0.41±0.1a** | **3.29±1.04a** | **3.9±2.2a** | **0.3±0.06b** | **0.56±0.33b** | **2.5±1ab** | **4.0±0.8a** |
| | **2.6±0.8b** | **2.0±0.6b** | **7.6±1.4a** | **6.5±1.0a** | **3.1±0.8b** | **3.4±0.6ab** | **4.5±0.8ab** | **6.3±2.3a** |
| Terpinen-4-ol | **0.006±0.001b** | **0.018±0.006b** | **1.4±0.4a** | **3.5±2.7a** | **0.019±0.005b** | **0.058±0.014b** | **2.5±1.2a** | **3.5±1a** |
| | **0.09±0.04b** | **0.08±0.02b** | **3.3±0.4a** | **4.0±2.2a** | **0.20±0.07b** | **0.27±0.01b** | **3.2±1.0a** | **4.2±1.2a** |
| Thymyl methyl ether | NA | NA | 2.9±1.2 | 2.5±0.9 | NA | 0.032 | 1.5±1.3 | 2.8±0.9 |
| | NA | NA | 9.8±4.2 | 8.8±4.6 | NA | 0.19 | 1.8±1.5 | 4.1±1.3 |
| α-terpinene | 0.025±0.01 | 0.030±0.002 | 0.25±±0.07 | 0.27±0.14 | **0.052±0.012b** | **0.13±0.1b** | **0.73±0.28a** | **0.80±0.19a** |
| | 0.17±0.06 | 0.14±0.04 | 0.58±0.09 | 0.43±0.09 | 0.52±0.12 | 0.71±0.29 | 1.1±0.1 | 1.0±0.1 |
| Minor Monoterpenes | **0.81±0.30c** | **2.2±0.1bc** | **13±3a** | **17±8ab** | **1.2±0.4b** | **2.3±1.2b** | **17±6a** | **21±4a** |
| | **8.2±1.1b** | **11±2b** | **34±6a** | **31±5a** | **11±2b** | **14±2b** | **28±3a** | **28±5a** |
| Oxygenated Monoterpenes | **0.006±0.001b** | **0.018±0.006b** | **4.3±1.2a** | **6.0±2.7a** | **0.019±0.005b** | **0.074±0.002b** | **4.1±2.4a** | **6.3±1.6a** |
| | **0.088±0.043b** | **0.080±0.02b** | **13±4a** | **13±4a** | **0.20±0.07b** | **0.37±0.08b** | **5.0±2.4a** | **8.3±2.0a** |
| Total Monoterpenes | 11±4 | 22±3 | 41±11 | 50±24 | **11±3b** | **15±7b** | **63±23a** | **77±13a** |
| | 41±3 | 45±4 | 49±5 | 49±4 | **22±1c** | **42±5b** | **54±2a** | **55±3a** |
| α-cubebene | 0.56±0.23 | 0.76±0.08 | 2.3±0.9 | 1.7±0.9 | 1.8±0.5 | 1.3±0.5 | 3.8±1.6 | 3.3±0.5 |
| | **31±5ab** | **22±3b** | **38±5a** | **29±5ab** | 40±7 | 40±7 | 45±7 | 40±7 |
| Longifolene | 0.68±0.29 | 2.0±0.7 | 1.8±1.0 | 1.7±1.3 | 1.6±0.8 | 2.2±1.1 | 2.8±1.7 | 2.1±0.6 |
| | **37±8ab** | **56±17a** | **28±6b** | **27±11b** | **35±12ab** | **44±16a** | **36±10ab** | **25±6b** |
| β-cedrene | **0.26±0.05b** | **0.74±0.3ab** | **1.1±0.2a** | **1.3±0.6a** | **0.62±0.2b** | **0.23±0.19b** | **0.80±0.40b** | **1.9±0.4a** |
| | 20±6 | 21±12 | 22±3 | 28±6 | **14±3ab** | **11±3b** | **15±1ab** | **21±3a** |
| Cedrol | 0.14±0.01 | 0.25±0.16 | 0.61±0.09 | 0.77±0.38 | **0.53±0.18ab** | **0.13±0.09b** | **0.44±0.23b** | **1.2±0.2a** |
| | 12±6 | 7.1±5.1 | 13±2 | 16±3 | 12±2 | 9±6 | 8.1±2.8 | 14±2 |
| Total Sesquiterpenes | 1.6±0.6 | 3.5±0.6 | 5.9±2.1 | 5.6±2.3 | 4.5±1.1 | 3.7±1.7 | 7.7±2.9 | 8.5±0.8 |
| | 6.5±0.6 | 7.6±1.3 | 6.8±1.1 | 5.9±0.8 | **9.3±1.5ab** | **9.4±0.8a** | **7.0±1.0bc** | **6.4±0.8b** |
| Manool | **0.23±0.09b** | **0.61±0.16ab** | **2.2±0.69ab** | **2.5±0.9a** | **1.7±0.9ab** | **0.72±0.31b** | **3.4±1.7a** | **3.1±0.8a** |
| Diterpene 1 | **1.7±0.1 c** | **2.6±0.3b** | **7.9±2.7a** | **8.0±0.7a** | **4.3±1.0ab** | **3.7±0.2b** | **6.5±1.4a** | **6.0±0.8a** |
| | 1.0±0.4 | 2.2±0.6 | 3.9±1.7 | 3.4±1.8 | **2.0±0.6ab** | **1.7±0.8b** | **3.1±1.9ab** | **7.0±1.7a** |
| | 8.0±3.0 | 9.2±1.5 | 11±3 | 9.0±2.1 | 5.8±1.5 | 9.7±2.3 | 9.2±3.3 | 14±2 |
| Diterpene 2 | **0.05±0.034b** | **1.6±0.5a** | **6.4±1.8a** | **8.2±2.4a** | **0.27±0.20b** | **1.4±0.7b** | **8.5±3.7a** | **9.6±2.6a** |
| | **0.80±0.70b** | **6.6±1.2a** | **23±8a** | **29±2a** | **0.57±0.33b** | **6.1±3.1b** | **17±3a** | **19±3a** |
| Diterpene 3 | 0.34±0.11 | 0.62±0.12 | 0.54±0.09 | 0.51±0.16 | **1.2±0.4a** | **0.49±0.27b** | **0.95±0.38ab** | **1.0±0.2ab** |
| | **2.8±0.2a** | **2.7±0.1a** | **1.9±0.3b** | **1.7±0.1b** | **3.4±0.2a** | **2.5±0.3ab** | **2.2±0.2b** | **2.2±0.3b** |
| Totarol | 7.8±2.6 | 12±2 | 10±3 | 8.5±3 | 19±5 | 9±4 | 16±6 | 18±3 |
| | **62±2a** | **52±1a** | **33±6b** | **27±2b** | **56±2a** | **51±4a** | **40±4b** | **38±4b** |

**Table 1** (continued)

| Name | DAY 30 | | | | DAY 90 | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Wounded | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| Diterpene 4 | **0.36±0.07b** | **0.94±0.13a** | **0.65±0.1ab** | **0.79±0.2ab** | 1.5±0.6 | 0.95±0.67 | 1.5±0.6 | 1.3±0.4 |
| | 3.2±0.5 | 4.6±1.4 | 2.3±0.3 | 3.2±1.0 | 4.2±0.4 | 4.3±1.5 | 3.5±0.4 | 2.7±0.4 |
| Diterpene 5 | **0.027±0.005b** | **0.42±0.2a** | **0.91±0.32a** | **1.1±0.4a** | **0.31±0.21b** | **0.48±0.05b** | **0.60±0.31ab** | **1.2±0.4a** |
| | **0.27±0.10bc** | **1.6±0.6b** | **3.0±0.7ab** | **4.0±0.7a** | 0.70±0.32 | 1.9±0.5 | 1.7±0.5 | 2.5±0.6 |
| Diterpene 6 | 0.032±0.01 | 0.22±0.07 | 1.1±0.5 | 1.3±0.9 | **0.053±0.016b** | **0.27±0.1ab** | **1.0±0.4ab** | **1.4±0.7a** |
| | **0.42±0.23b** | **0.92±0.16ab** | **3.5±1.4a** | **3.1±1.4a** | **0.12±0.01b** | **1.1±0.6ab** | **2.0±0.3ab** | **2.7±1.3a** |
| Diterpene 7 | 1.4±0.7 | 1.7±0.2 | 1.9±0.7 | 1.6±0.7 | **4.2±1.3a** | **1.8±0.8b** | **3.2±1.1ab** | **2.7±0.3ab** |
| | **10±2a** | **7.4±0.3ab** | **5.8±1.6ab** | **4.4±1.2b** | **12±0a** | **11±1a** | **8.2±0.7b** | **5.9±0.8c** |
| Totarolone | **0.13±0.04b** | **0.41±0.09a** | **0.36±0.1ab** | **0.42±0.04a** | **0.74±0.21b** | **0.39±0.11a** | **0.51±0.19ab** | **0.55±0.15ab** |
| | 1.5±0.7 | 1.8±0.1 | 1.5±0.6 | 2.0±0.7 | **2.1±0.1a** | **1.4±0.1ab** | **1.9±0.6ab** | **1.1±0.2b** |
| Hinokione | 1.1±0.2 | 2.3±0.4 | 2.0±0.2 | 2.4±0.6 | **3.8±1.3b** | **1.7±0.8a** | **3.4±1.1ab** | **3.1±0.6ab** |
| | 9.3±1.3 | 10±1 | 7.2±0.9 | 9.2±1. | **11±1a** | **9.0±0.5a** | **8.6±1.1ab** | **6.3±0.4b** |
| Total diterpenes | 13±4 | 23±4 | 30±7 | 31±11 | **35±10ab** | **18±9b** | **42±16a** | **49±9a** |
| | **52±4a** | **47±4ab** | **38±4b** | **38±4b** | **69±3a** | **49±5b** | **36±1c** | **34±2c** |
| Total terpenes | 25±8 | 48±6.7 | 82±18 | 93±39 | **51±14b** | **37±16c** | **117±43ab** | **140±22a** |

RT retention time. [ ] = concentration, % = proportion, NA not available. Numbers and letters in bold type indicate statistically significant differences (REML, fixed = treatment, 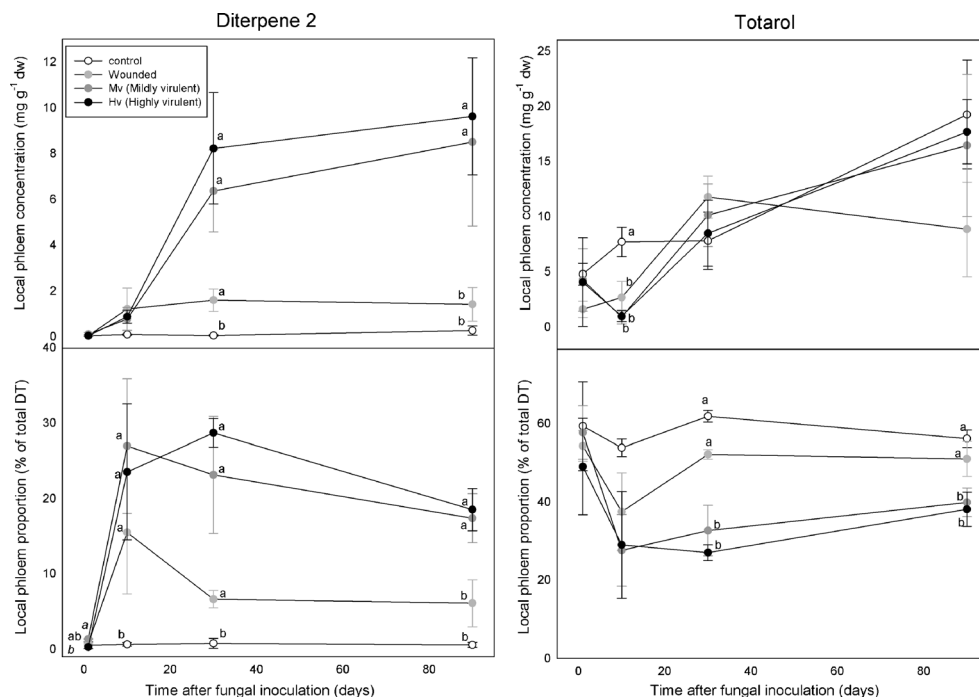random = genotype, paired Tukey's *post-hoc* test, *P*<0.05) and marginally significant differences (*P*<0.10, in *italics*)

**Fig. 1** Mean phloem concentrations (±SE) and mean proportions (±SE) relative to total monoterpenes (*MT*) of sabinene and oxygenated monoterpenes (sum of terpinen-4-ol, thymyl methyl ether, and bornyl acetate), some of the most induced compounds in the infected treatments (Mv and Hv) relative to the control and Wounded. Different letters indicate statistically significant differences (REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, *P*<0.05)
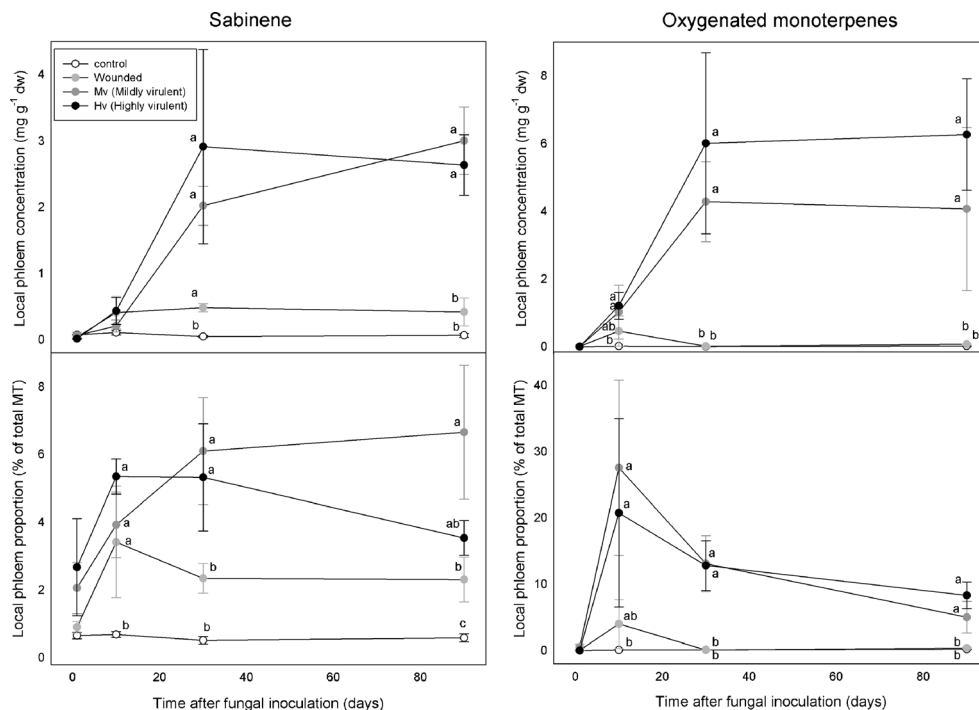


concentrations in the infected treatments than in the control (Fig. 1).

At day 1 post inoculation, total terpenes tended to decrease relative to control, as did all terpene classes (mono-, sesqui-, and diterpenes), despite the lack of statistical differences among treatments. Only cedrol exhibited differences, with Mv higher than Wounded and Hv

(REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, *P*<0.05) (Table 1). δ-3-Carene had a higher proportion in Wounded than in all other treatments, and terpinolene, the minor monoterpenes (sum of all monoterpenes except α-pinene and δ-3-carene), and diterpene 2 had higher proportions in the infected treatments than in the control or Wounded (Table 1, Figs. 2 and 3).

**Fig. 2** Mean phloem concentrations (±SE) and mean proportions (±SE) relative to total diterpenes (*DT*) of diterpene 2, and totarol. Different letters indicate statistically significant differences (REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, *P*<0.05) and marginally significant differences (*P*<0.10, in *italics*)
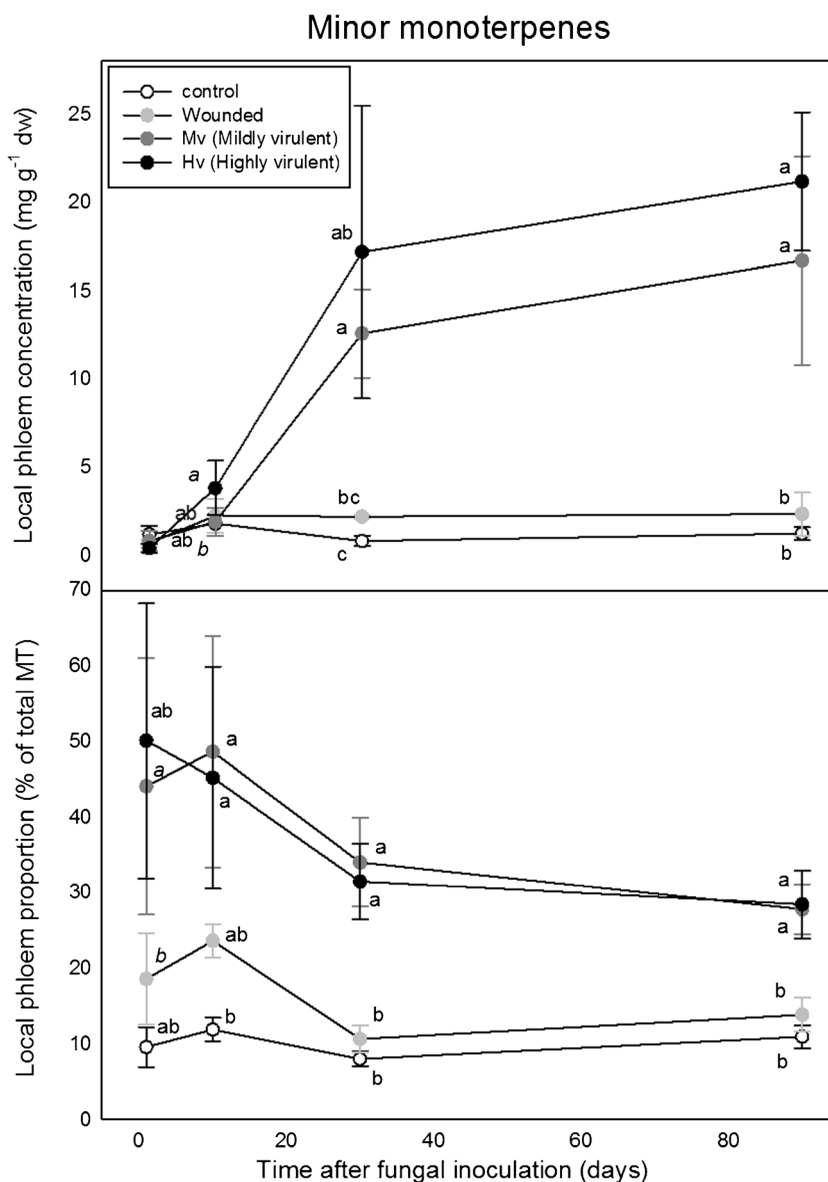
Terpene concentrations decreased significantly at day 10 in both infected treatments relative to control for total terpenes and all terpene classes, except the oxygenated monoterpenes, that increased 75-fold. α-Pinene, α-fenchene, β-pinene, β-myrcene, δ-3-carene, total monoterpenes, all sesquiterpenes (including total sesquiterpenes), the majority of diterpenes (including total diterpenes), and total terpenes had the highest concentrations in the control. Terpinolene, terpinen-4-ol, minor monoterpenes, and oxygenated monoterpenes, however, increased significantly in infected treatments compared to the control and Wounded (Table 1).

α-Fenchene, δ-3-carene, total sesquiterpenes, and diterpenes 3, 4, and 7 also decreased in proportion in the infected treatments relative to the control. In contrast, α-thujene, sabinene, terpinolene, terpinen-4-ol, oxygenated monoterpenes, minor monoterpenes, α-cubebene, manool,

diterpenes 2 and 5, and totarolone had higher proportions in infected treatments than in the control or Wounded (Table 1).

By day 30, concentrations tended to change relative to those at day 10, with total terpene, total mono-, total sesqui-, and total diterpene concentrations increasing non-significantly in the infected treatments. Concentrations of α-thujene, sabinene, terpinolene, terpinen-4-ol, minor and oxygenated monoterpenes, β-cedrene, manool, diterpenes 2 and 5, and totarolone were higher in infected treatments than control or Wounded (Table 1). Proportions showed similar trends, with the monoterpenes listed above increasing in proportion in the infected treatments. α-Cubebene, manool, and diterpenes 2, 5, and 6 also increased in proportion. In contrast, α-pinene, β-pinene, longifolene, totarol, diterpenes 3 and 7, and total diterpenes decreased in proportion (Table 1).

Finally, the largest contrasts appeared by day 90, with concentrations in the infected treatments being the highest reported in the study. Concentrations of α-thujene, α-pinene, sabinene, β-pinene, β-myrcene, limonene, terpinolene, terpinen-4-ol, α-terpinene, oxygenated, minor and total monoterpenes, β-cedrene, cedrol, manool, diterpenes 1, 2, 5, and 6, totarolone, hinokione, total diterpenes, and total terpenes were all higher in infected treatments than in Wounded and/or the control. The proportions also were higher in the infected trees for α-thujene, sabinene, β-myrcene, limonene, terpinolene, terpinen-4-ol, oxygenated, minor and total monoterpenes, β-cedrene, manool, and diterpenes 2 and 6. In contrast, longifolene, total sesquiterpenes, totarol, diterpenes 3 and 7, totarolone, hinokione, and total diterpenes showed the opposite trend, having higher proportions in the control or Wounded than in the infected treatments (Table 1). No differences were found among the control trees from days 1 to 90, except for total diterpene concentrations at day 90, which were higher than on other sampling days.

Two PCAs (Fig. 4) were conducted with phloem monoterpene concentrations and monoterpene proportions on days 30 and 90 as variables, to provide a general overview of the differences among treatments and infection times. In the concentration PCA, the first two PCs accounted for 69.1 % and 11.0 % of the total variance, respectively. PC1 distributed the cases by terpene concentration, separating Hv and Mv from Wounded and control treatments (two-way ANOVA of the PC scores, $P < 0.05$) and PC2 significantly separated the cases of day 30 from those of day 90 ($P < 0.05$). In the proportion PCA, the first two PCs accounted for the 36.3 % and 20.4 % of the total variance, respectively. PC1 significantly ($P < 0.05$) separated the cases with decreased proportion of main terpenes and increased proportion of minor terpenes, and PC2 also separated the cases of day 30 and day 90 ($P < 0.05$).

*Fungal Isolates* Mv and Hv did not elicit clearly different reactions. Statistically significant differences between terpene concentrations in the infected treatments were observed only for two sesquiterpenes. Cedrol was significantly higher in Mv than in Hv at day 1, and cedrol and β-cedrene were higher in Hv than in Mv at day 90 (Table 1).



**Fig. 4** Principal Component Analysis (*PCA*) for the concentrations (mg g$^{-1}$ of dry weight) (*left panels*) and proportions (% of total monoterpenes; *right panels*) of the 12 monoterpenes studied at days 30 and 90 after infection. The biplots depict loadings of PCA variables (*above*) and scores of PCA cases (*below*). T-4-ol = terpinen-4-ol, tme = thymyl methyl ether. Letters indicate the different treatments applied: *C* Control (*green*), *W* Wounded (*yellow*), *M* Mildly virulent (*red*), *H* Highly virulent (*red*). Samples of day 90 are marked with an asterisk (*), and samples of day 30 have no asterisk ( )
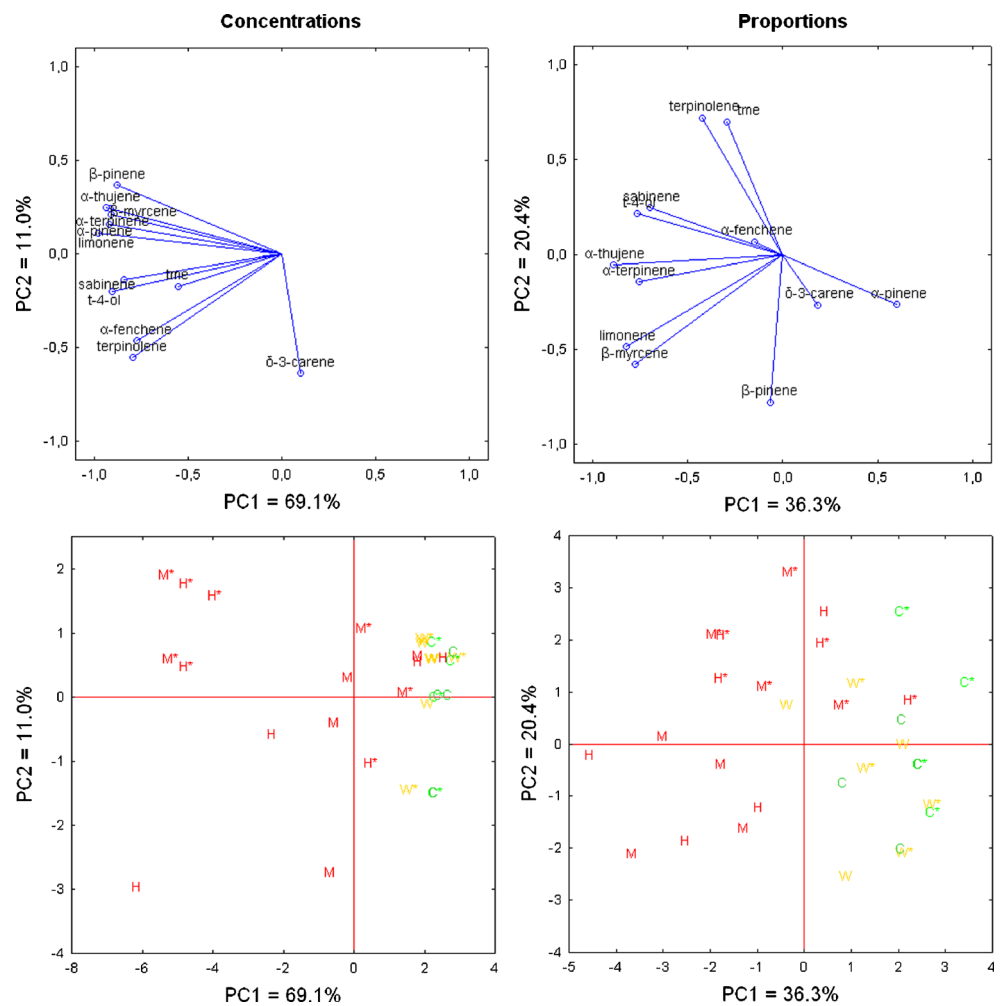
*Foliar Terpene Concentration* Leaves also presented abundant terpenes, with high concentrations of monoterpenes, moderate abundances of sesquiterpenes, and traces of diterpenes. No qualitative differences were found among treatments, and few quantitative differences in concentrations were observed (Table 2).

No differences in concentration were detected at day 1 (Table 2). At day 10, the control had higher concentrations of the sesquiterpenes α-cubebene, caryophyllene, germacrene D, α-muurolene, and total sesquiterpenes than did Hv. At day 30, no differences among treatments were found (Table 2). At day 90, the control had higher concentrations of β-myrcene, limonene, terpinolene, bornylene, and α-cubebene than did Wounded.

No correlation was found between the concentrations (Table 2) and proportions (data not shown) of the terpene species analyzed. No direct differences were found between the fungal isolates. Hv had lower concentrations than the control in several occasions on day 10 (Table 2), while Mv concentrations were not different from the control or Wounded.

*Foliar Emission Rates* The foliar emissions contained eight monoterpenes and two sesquiterpenes (Table 3, Fig. 5). No qualitative differences were found, but some quantitative differences appeared. The largest differences were in total monoterpene emissions and δ-3-carene (REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, $P<0.05$), which were higher for the infected trees at day 10 than the control and Wounded. The proportions did not show any clear trend (data not shown).

At day 1, the emission rates of β-myrcene and limonene were higher in Wounded than in the control (Table 3). At day 10, δ-3-carene had a higher emission rate in Hv than the control and a marginally higher emission rate than in Wounded. α-Cedrene also had a marginally higher emission rate in Hv than in the control. Total monoterpenes showed higher emission rates in infected treatments than in the control. In contrast, the emission rate of β-pinene was marginally higher in the control than in Wounded. All compounds, except β-myrcene and δ-3-carene, had the highest emission rates in the Hv treatment at day 10. At day 30, differences were observed only in emission rates of sesquiterpenes; Hv had a higher foliar emission rate of longifolene than did Mv, and Wounded had a marginally significant higher emission rate of α-cedrene than did Mv. Finally, at day 90, α-cedrene had a higher emission rate in the control than in Wounded, and Mv, and β-pinene had a higher emission rate in Mv than in Hv (Table 3). Hv tended to elicit higher emissions and larger differences (sometimes statistically significant) relative to the control and Wounded than did Mv (Table 3, Fig. 5).

Foliar concentrations and emissions appeared to be negatively correlated, but the correlations were not statistically significant. Only the correlation between total monoterpene concentration and total monoterpene emission was significant for day 10 (simple regression; $R^2=0.435$, $P<0.05$).

## Discussion

*Qualitative and Quantitative Changes in Local Phloem* Despite genotypic differences among trees and the different levels of pathogenicity of the fungal isolates, the same six terpenes appeared *de novo* only in the inoculated treatments at days 30 and 90, for all genotypes studied. Notably, four of these six compounds were oxygenated monoterpenes (oxygenated monoterpene 1, sabinene hydrate, camphor, and α-terpineol), a class of terpenoids noted for strong antifungal activity, usually more fungistatic than non-oxygenated monoterpenes. (Bakkali et al. 2008; Hussain et al. 2011; Jiao et al. 2012; Zouari et al. 2011). Most of the *de novo* compounds were detected in relatively low concentrations (0.09–0.36 mg g$^{-1}$ dw) except for *de novo 3*, a diterpene that had a mean concentration of 5.4 mg g$^{-1}$ but was rarely detected. We were not able to detect cupressotropolone A and B, two sesquiterpene phytoalexins of fungal-infected cypresses discovered by Madar et al. (1995a) using thin layer chromatography (TLC).

The scarce information that is available for the role of sabinene hydrate in tree defense and fungal inhibition (Ramos et al. 2011; Tomlin et al. 2000) suggests that this compound might have moderate defensive and antifungal activity. The role of camphor (Kotan et al. 2007; Marei et al. 2012; Pragadheesh et al. 2013; Ramsewak et al. 2003) is ambiguous, being inhibitory for some fungi but not others, suggesting slight fungal toxicity. α-Terpineol, however, is a powerful fungal inhibitor ( Cakir et al. 2004; Hammer et al. 2003; Kossuth and Barnard 1983; Kotan et al. 2007; Kusumoto et al. 2014; Zhou et al. 2014). Thymyl methyl ether is among the least inhibitive chemical structures of thymol to several fungi (Kumbhar and Dewang 2001).

The only *de novo* terpenes known to be produced by Italian cypress in response to a fungal pathogen are the oxygenated sesquiterpenes cupressutropolone A and B, produced under infection by *Diplodia pinea*, another canker-causing fungus (Madar et al. 1995a). These two sesquiterpenes are considered *C. sempervirens* phytoalexins, because they cause strong or total inhibition of mycelial growth and spore germination for *S. cardinale* and other cypress pathogens (Madar et al. 1995a).

The *de novo* compounds we found could, thus, likely be antifungal phytoalexins because *i*) sabinene hydrate, camphor, and α-terpineol appeared exclusively in the infected treatments, *ii*) they are oxygenated monoterpenes, *iii*) their antifungal activity has been reported in literature (especially α-terpineol), and *iv*) the report by Madar et al. (1995a). The possibility that these *de novo* compounds (especially α-terpineol and camphor) are a product or a biotransformation

**Table 2** Mean concentrations (±SE) in mg g$^{-1}$ dry weight of the terpenes in the leaves of cypresses infected with *S. cardinale*

| Name | RT (min) | DAY 1 Control | DAY 1 Wounded | DAY 1 Mildly virulent | DAY 1 Highly virulent | DAY 10 Control | DAY 10 Wounded | DAY 10 Mildly virulent | DAY 10 Highly virulent |
|---|---|---|---|---|---|---|---|---|---|
| Tricyclene | 7.68 | 0.053±0.022 | 0.041±0.017 | 0.059±0.005 | 0.054±0.014 | 0.074±0.041 | 0.032±0.014 | 0.058±0.025 | 0.028±0.020 |
| α-thujene | 7.72 | 0.20±0.15 | 0.24±0.21 | 0.051±0.023 | 0.044±0.012 | 0.84±0.79 | 0.40±0.37 | 0.34±0.31 | 0.16±0.15 |
| α-pinene | 7.82 | 12±5 | 8.7±3.9 | 11±4 | 12±5 | 15±7 | 7.8±3.0 | 13±5 | 6.2±4.1 |
| α-fenchene | 8.01 | 0.18±0.08 | 0.12±0.04 | 0.15±0.06 | 0.14±0.08 | 0.21±0.07 | 0.12±0.05 | 0.17±0.07 | 0.087±0.017 |
| Sabinene | 8.32 | 0.63±0.48 | 0.67±0.59 | 0.23±0.05 | 0.17±0.04 | 1.9±1.7 | 1.0±0.9 | 1.1±0.9 | 0.46±0.39 |
| β-pinene | 8.39 | 0.17±0.06 | 0.11±0.04 | 0.17±0.05 | 0.15±0.06 | 0.23±0.08 | 0.13±0.04 | 0.18±0.06 | 0.081±0.044 |
| β-myrcene | 8.47 | 0.28±0.11 | 0.16±0.07 | 0.27±0.07 | 0.22±0.08 | 0.42±0.24 | 0.18±0.09 | 0.29±0.13 | 0.088±0.037 |
| δ-3-carene | 8.77 | 6.6±3.0 | 3.7±1.7 | 5.3±2.0 | 4.8±2.7 | 6.0±2.9 | 3.5±1.6 | 5.6±2.5 | 1.8±0.7 |
| Limonene | 8.98 | 0.34±0.13 | 0.27±0.13 | 0.27±0.10 | 0.26±0.11 | 0.34±0.19 | 0.20±0.10 | 0.33±0.15 | 0.12±0.05 |
| γ-terpinene | 9.31 | 0.041±0.014 | 0.025±0.014 | 0.020±0.003 | 0.021±0.006 | 0.083±0.064 | 0.053±0.040 | 0.050±0.036 | 0.034±0.022 |
| Terpinolene | 9.66 | 0.33±0.16 | 0.17±0.10 | 0.29±0.10 | 0.31±0.15 | 0.37±0.18 | 0.18±0.09 | 0.30±0.13 | 0.085±0.036 |
| Monoterpene 1 | 11.68 | 0.19±0.11 | 0.081±0.064 | 0.016±0.005 | 0.009±0.004 | 0.041±0.026 | 0.028±0.014 | 0.067±0.031 | 0.054±0.034 |
| Bornylene | 13.13 | 0.062±0.038 | 0.037±0.03 | 0.045±0.018 | 0.046±0.026 | 0.061±0.034 | 0.037±0.021 | 0.046±0.04 | 0.011±0.005 |
| α-terpinene | 13.36 | 0.67±0.42 | 0.33±0.25 | 0.94±0.47 | 0.59±0.28 | 0.84±0.31 | 0.45±0.23 | 0.72±0.35 | 0.14±0.05 |
| Total monoterpenes | | 21±9 | 15±9 | 18±7 | 19±8 | 26±9 | 14±5 | 22±7 | 9±4 |
| α-cubebene | 13.43 | 0.15±0.05 | 0.094±0.047 | 0.12±0.04 | 0.14±0.06 | 0.32±0.17a | 0.17±0.09ab | 0.25±0.06ab | **0.043±0.018b** |
| β-cedrene | 15.10 | 0.12±0.03 | 0.070±0.032 | 0.17±0.09 | 0.22±0.06 | 0.18 | 0.14±0.12 | 0.14±0.06 | 0.11 |
| Caryophyllene | 15.18 | 0.56±0.19 | 0.38±0.23 | 0.34±0.15 | 0.40±0.15 | 0.85±0.46a | 0.59±0.31ab | 0.66±0.28ab | **0.19±0.10b** |
| α-caryophyllene | 15.74 | 1.3±0.6 | 0.89±0.58 | 0.81±0.40 | 1.2±0.5 | 2.2±1.2 | 1.8±1.0 | 2.2±1.2 | 0.56±0.29 |
| Germacrene D | 16.13 | 2.6±0.9 | 1.6±0.9 | 2.0±0.8 | 3.3±1.1 | 5.6±2.3a | 3.4±1.4ab | 4.0±1.3ab | **1.5±0.8b** |
| α-muurolene | 16.31 | 0.12±0.03 | 0.089±0.035 | 0.075±0.032 | 0.089±0.030 | 0.25±0.10a | 0.17±0.07ab | 0.18±0.04ab | **0.072±0.001b** |
| Cedrol | 17.48 | 0.32±0.06 | 0.16±0.07 | 0.40±0.16 | 0.44±0.22 | 0.41±0.25 | 0.36±0.20 | 0.32±0.18 | 0.15±0.12 |
| Total sesquiterpenes | | 4.9±1.8 | 3.1±1.9 | 3.8±1.2 | 5.5±1.8 | 9.5±4a | 6.3±3ab | 7.1±2.6ab | **2.5±1.2b** |
| Total terpenes | | 26±10 | 18±7 | 22±8 | 24±9 | 36±12 | 20±7 | 29±9 | 11±5 |

| Name | DAY 30 Control | DAY 30 Mildly virulent | DAY 30 Highly virulent | DAY 90 Control | DAY 90 Wounded | DAY 90 Mildly virulent | DAY 90 Highly virulent |
|---|---|---|---|---|---|---|---|
| Tricyclene | 0.071±0.035 | 0.13±0.026 | 0.11±0.04 | 0.091±0.008 | 0.083±0.021 | 0.092±0.01 | 0.060±0.027 |
| α-thujene | 0.29±0.23 | 0.070±0.022 | 0.062±0.020 | 0.21±0.16 | 0.063±0.021 | 0.040±0.003 | 0.040±0.021 |
| α-pinene | 16±8 | 23±8 | 19±8 | 19±5 | 17±6 | 24±2 | 17±8 |
| α-fenchene | 0.23±0.09 | 0.31±0.12 | 0.25±0.11 | 0.24±0.03 | 0.15±0.05 | 0.21±0.05 | 0.18±0.08 |
| Sabinene | 1.1±0.9 | 0.40±0.03 | 0.34±0.13 | 0.83±0.61 | 0.26±0.06 | 0.19±0.03 | 0.15±0.06 |
| β-pinene | 0.24±0.10 | 0.36±0.12 | 0.35±0.16 | 0.28±0.06 | 0.15±0.07 | 0.30±0.03 | 0.23±0.11 |
| β-myrcene | 0.36±0.12 | 0.40±0.17 | 0.32±0.16 | 0.41±0.04a | 0.25±0.07b | 0.37±0.04ab | **0.28±0.12ab** |
| δ-3-carene | 8.8±3.7 | 9.5±4.2 | 6.5±2.9 | 7.2±1.1 | 4.1±1.5 | 6.1±1.7 | 5.0±2.4 |
| Limonene | 0.46±0.17 | 0.53±0.26 | 0.45±0.20 | 0.51±0.10a | 0.29±0.08b | 0.41±0.12ab | **0.36±0.18ab** |
| γ-terpinene | 0.039±0.025 | 0.032±0.011 | 0.024±0.007 | 0.054±0.022 | 0.021±0.004 | 0.027±0.004 | 0.018±0.008 |
| Terpinolene | 0.38±0.14 | 0.50±0.22 | 0.33±0.16 | 0.48±0.05a | 0.26±0.07b | 0.39±0.07ab | **0.31±0.15ab** |
| Monoterpene 1 | 0.019±0.004 | 0.013±0.001 | 0.018±0.011 | 0.013±0.004 | 0.015±0.003 | 0.034±0.024 | 0.014±0.013 |

**Table 2** (continued)

| Name | DAY 30 | | | DAY 90 | | | |
|---|---|---|---|---|---|---|---|
| | Control | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| Bornylene | 0.074±0.032 | 0.094±0.046 | 0.053±0.025 | **0.080±0.017a** | **0.029±0.014b** | **0.063±0.024ab** | **0.048±0.026ab** |
| α-terpinene | 0.93±0.39 | 1.6±0.8 | 1.2±0.7 | 1.0±0.2 | 0.44±0.15 | 0.89±0.24 | 0.57±0.27 |
| Total monoterpenes | 29±11 | 37±14 | 29±12 | 30±5 | 23±7 | 33±3 | 18±10 |
| α-cubebene | 0.25±0.03 | 0.27±0.12 | 0.29±0.28 | **0.16±0.03a** | **0.087±0.068b** | **0.15±0.03ab** | **0.13±0.06ab** |
| β-cedrene | 0.19±0.10 | 0.42±0.29 | 0.38±0.29 | 0.19±0.04 | 0.11±0.05 | 0.25±0.09 | 0.15±0.09 |
| Caryophyllene | 0.43±0.21 | 0.63±0.40 | 0.39±0.25 | 0.63±0.25 | 0.40±0.14 | 0.49±0.16 | 0.27±0.19 |
| α-caryophyllene | 1.3±0.7 | 1.6±0.9 | 1.0±0.6 | 1.9±0.8 | 1.2±0.4 | 1.2±0.5 | 0.83±0.62 |
| Germacrene D | 2.2±0.9 | 3.7±1.3 | 2.4±0.8 | 3.7±1.0 | 3.3±1.0 | 3.2±0.8 | 1.7±0.8 |
| α-muurolene | 0.078±0.032 | 0.14±0.05 | 0.095±0.035 | 0.12±0.04 | 0.097±0.028 | 0.10±0.02 | 0.066±0.037 |
| Cedrol | 0.69±0.32 | 1.3±0.7 | 1.2±0.9 | 0.56±0.14 | 0.24±0.13 | 0.55±0.26 | 0.41±0.32 |
| Total sesquiterpenes | 5.1±1.9 | 7.9±2.9 | 5.5±2 | 7.3±1.9 | 5.3±1.5 | 5.7±1.1 | 2.7±1.5 |
| Total terpenes | 34±12 | 45±17 | 34±14 | 38±5 | 29±9 | 38±4 | 28±15 |

*RT* retention time. Numbers and letters in bold type indicate statistically significant differences (REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, $P<0.05$)

of the infecting fungal pathogen, however, cannot be discarded (Kusumoto et al. 2014; Leufvén et al. 1988; Siddhardha et al. 2011; Tan and Day 1998). Furthermore, any terpene concentration found in the infected treatments could have been altered by fungal biotransformation or production.

The increased terpene concentrations in the local phloem tissues of the infected treatments were expected because resinosis from the cracks of infected tissues is a common symptom of cankered cypresses (Graniti 1998). This phenomenon has been observed in numerous studies that address the reaction of conifer phloem and xylem to infection by fungal pathogens (Blodgett and Stanosz 1998; Bonello et al. 2008; Faldt et al. 2006; Raffa and Smalley 1995; Viiri et al. 2001). In our study, the monoterpenes, well-known inhibitors of fungi mycelial growth and spore germination (Bakkali et al. 2008; Kalemba and Kunicka 2003), and diterpenes, which also have strong antifungal activity (Eberhardt et al. 1994; Kopper et al. 2005; Kusumoto et al. 2014), were the most reactive terpenoid groups in the phloem. The oxygenated monoterpenes were the most induced terpenoid category (Table 1, Fig. 1), increasing their concentrations up to 1000-fold in infected trees relative to control and up to 333-fold relative to Wounded. The concentration decreases observed at day 10 for some of the major monoterpenes, all sesquiterpenes, and several abundant diterpenes (Table 1, Fig. 1) were unexpected. Concentration decreases for several compounds also have been observed, however, in other pathosystems (Boone et al. 2011; Davis and Hofstetter 2011), and at least one general decrease in terpene concentration also has been reported (Bonello et al. 2008). At day 10, the few compounds that increased in concentration showed an abrupt increase in proportion, and they were the same compounds that were most induced throughout this study, such as α-thujene, sabinene, terpinolene, manool, diterpene 2, and diterpene 5. By decreasing concentrations of the main compounds and by slightly increasing the concentrations of some induced terpenes, proportions of the induced compounds can increase drastically (see terpinolene and diterpene 2 in Table 1). This strategy might be a fast and cheap way of producing the desired terpene proportions rapidly, rather than by strongly increasing the concentrations of these induced compounds.

α-Thujene, sabinene, terpinolene, terpinen-4-ol, manool, and diterpenes 2 and 5 responded most to *S. cardinale* infection. The information available for α-thujene (Raffa and Berryman 1982a; Zhao et al. 2010) suggests that conifers do not use it as a defensive compound, but it may have some antifungal activity (Bajpai et al. 2007). Sabinene (De Alwis et al. 2009; Espinosa-garcia and Langenheim 1991; Kohzaki et al. 2009) and terpinolene (Davis and Hofstetter 2011; Viiri et al. 2001) are among the most induced compounds in some conifers under fungal attack, and possess antifungal properties against several phytopathogens and fungal endophytes

**Table 3** Mean terpene emission rates (±SE) in µg g⁻¹ dry weight h⁻¹ of terpenes emitted by leaves of cypresses infected with *S. cardinale*

| Name | RT (min) | Day 1 | | | | Day 10 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | Wounded | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| α-thujene | 6.53 | 0.015±0.005 | 0.18±0.15 | 0.098±0.082 | 0.072±0.036 | 0.16 | 0.055±0.012 | 0.086±0.046 | 1.23±0.92 |
| α-pinene | 6.70 | 0.69±0.54 | 2.1±0.9 | 0.70±0.23 | 5.0±4.7 | 1.8±1.7 | 2.3±0.5 | 6.8±0.3 | 13±12 |
| Camphene | 6.82 | 0.022±0.020 | 0.10±0.05 | 0.050±0.003 | 0.045±0.031 | 0.13±0.11 | 0.078±0.022 | 0.21±0.11 | 1.2±1.1 |
| Sabinene | 7.15 | 0.031±0.017 | 0.32±0.28 | 0.29±0.28 | 0.28±0.22 | 0.12 | 0.15±0.11 | 0.084±0.032 | 1.1±1.0 |
| β-pinene | 7.17 | 0.077 | 0.089±0.011 | 0.059±0.023 | 0.18 | **0.96±0.65a** | **0.22±0.15b** | **0.56±0.44ab** | **1.4±0.7ab** |
| β-myrcene | 7.22 | **0.012±0.004b** | **0.26±0.08a** | 0.15 | 0.20±0.13 | 0.024 | 0.089±0.002 | 0.41±0.31 | 0.31 |
| δ-3-carene | 7.64 | 0.43±0.23 | 2.0±1.1 | 0.55±0.52 | 1.5±0.6 | **0.30±0.13b** | **1.2±0.6b** | **1.5±0.9ab** | **4.5±1.7a** |
| Limonene | 7.70 | **0.029±0.019b** | **0.69±0.36a** | 0.21 | 0.069 | 0.24±0.22 | 1.0±0.6 | 1.5±1.5 | 5.4±4.2 |
| Longifolene | 13.31 | 0.056±0.023 | 0.14±0.12 | 0.030 | NA | NA | 0.30±0.23 | 0.94 | 0.92±0.71 |
| α-cedrene | 13.42 | 0.37±0.34 | 0.51±0.38 | 0.11 | 0.139 | **0.19±0.16b** | 1.0 | 1.8 | **1.7±1.2a** |
| Total monoterpenes | | 1.2±0.7 | 5.6±1.7 | 2.1±0.8 | 6.5±5.3 | **2.5±1.5b** | **5.1±1.1ab** | **12±4a** | **27±17a** |
| Total terpenes | | 1.4±0.6 | 6.1±1.7 | 2.2±0.9 | 6.5±5.3 | 2.6±1.5 | 5.6±1.5 | 13±5 | 30±19 |

| Name | RT (min) | Day 30 | | | | Day 90 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | Wounded | Mildly virulent | Highly virulent | Control | Wounded | Mildly virulent | Highly virulent |
| α-thujene | 6.53 | 0.13±0.06 | 0.046±0.031 | 0.14±0.13 | 0.10±0.087 | 0.001 | 0.022 | 0.020 | NA |
| α-pinene | 6.70 | 1.7±0.8 | 0.75±0.29 | 1.3±0.82 | 5.3±5.1 | NA | 1.9±1.8 | 0.76±0.55 | 0.30±0.29 |
| Camphene | 6.82 | 0.27±0.24 | 0.027±0.015 | 0.031±0.026 | 0.14±0.12 | 0.053±0.05 | 0.016±0.014 | 0.027 | 0.013±0.012 |
| Sabinene | 7.15 | 0.49±0.46 | 0.084±0.043 | 0.27±0.23 | 0.26±0.23 | 0.015±0.011 | 0.049±0.035 | 0.029±0.019 | 0.003±0.002 |
| β-pinene | 7.17 | 0.041±0.008 | 0.15 | 0.083±0.042 | 0.16±0.14 | **0.029±0.027ab** | 0.025 | **0.027±0.025b** | **0.011±0.008a** |
| β-myrcene | 7.22 | 0.22±0.11 | 0.25±0.10 | 0.15±0.021 | 0.47±0.45 | 0.010 | NA | 0.04±0.038 | 0.005 |
| δ-3-carene | 7.64 | 1.0±0.2 | 2.6±2.3 | 1.3±0.6 | 6.5±6.3 | 0.64±0.48 | 0.16±0.06 | 0.33±0.21 | 0.14±0.12 |
| Limonene | 7.70 | 0.16±0.03 | 0.46 | 0.27±0.01 | 0.49±0.41 | 0.011±0.009 | 0.037 | 0.012±0.009 | 0.14±0.04 |
| Longifolene | 13.31 | **0.12±0.02ab** | 0.052 | **0.018±0.007b** | **0.25±0.22a** | 0.024 | 0.006±0.001 | 0.16±0.16 | 0.008±0.007 |
| α-cedrene | 13.42 | **0.19±0.11ab** | **0.27±0.13a** | **0.069±0.052b** | **0.57±0.49ab** | **0.064±0.004a** | **0.016±0.001b** | **0.012±0.002b** | 0.026 |
| Total monoterpenes | | 3.8±0.8 | 3.0±1.8 | 2.9±1.5 | 11.3±10.8 | 3.7±3.5 | 1.5±1.3 | 0.69±0.42 | 0.40±0.30 |
| Total terpenes | | 3.9±0.7 | 4.6±1.9 | 3.0±1.5 | 12±11 | 3.7±3.5 | 1.5±1.3 | 0.82±0.46 | 0.42±0.30 |

*RT* retention time. *NA* not available. Numbers and letters in bold type indicate statistically significant differences (REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, *P*<0.05) and marginally significant differences (*P*<0.10, in *italics*)

**Fig. 5** Mean rates of emission (±SE) of main monoterpenes emitted by leaves. Different letters indicate statistically significant differences (REML, fixed = treatment, random = genotype, paired Tukey's *post-hoc* test, *P*<0.05)
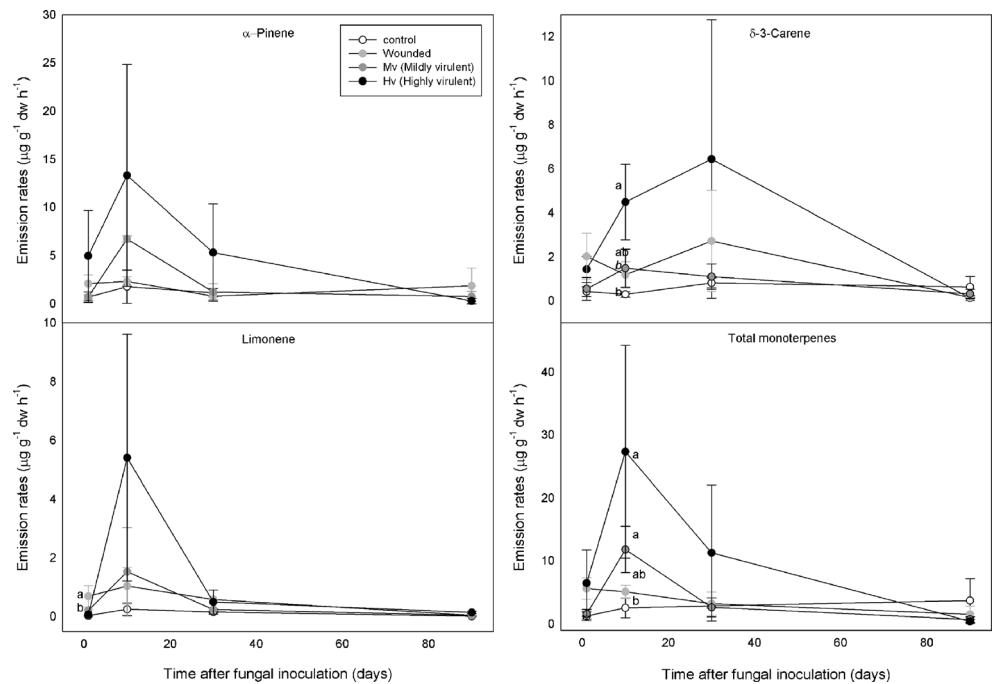


(Bridges 1987; De Alwis et al. 2009; Espinosa-garcia and Langenheim 1991; Kohzaki et al. 2009; Paine and Hanlon 1994). Herbicide application also can increase the concentration of terpinen-4-ol in *P. ponderosa* (Kidd and Reid 1979), a compound with remarkable biological activity on fungi (Kusumoto et al. 2014; Morcia et al. 2012; Nenoff et al. 1996) and bacteria (Kotan et al. 2007). Manool concentrations can increase in conifers under biotic attack (Hanari et al. 2002; Tomlin et al. 2000), and can inhibit growth of several canker agents (Yamamoto et al. 1997) and pathogenic bacteria (Ulubelen et al. 1994). In our study, the concentrations and proportions of two unidentified compounds, diterpenes 2 and 5, increased substantially in infected trees (Table 1, Fig. 2) and may play a role in cypress defense, thus warranting further efforts to identify them.

The concentrations and proportions of the minor monoterpenes increased in the infected treatments at the expense of the two main monoterpenes, α-pinene and δ-3-carene (their sum represented more than 90 % of the monoterpene fraction in the control), which significantly decreased in proportion to 50–70 % (Table 1, Fig. 3). The proportions PCA (Fig. 4) corroborates these observations, showing the main monoterpenes going in opposite direction to minor terpenes. Proportional changes also were observed in the diterpenes, where that of totarol, the main compound of the diterpene fraction, decreased from 50–60 % in the control to 30 % in infected treatments (Table 1, Fig. 2) primarily in favor of diterpene 2 and manool. These results, thus, suggest that infected cypresses invest more in minor compounds than in major ones. This strategy had been observed in *Picea abies*, *Abies grandis*, and *Pinus resinosa*, where their main monoterpenes (pinenes),

lowered proportions in infected trees in favor of minor monoterpenes such as sabinene and terpinolene (Klepzig et al. 1995; Raffa and Berryman 1982a; Zhao et al. 2010). Some tree terpenes (usually the main compounds) have low inhibiting effects (Kusumoto et al. 2014) or can even enhance the growth of some fungal pathogens (Bridges 1987; Cakir et al. 2004; Davis and Hofstetter 2011), because some pathogenic fungi have developed the ability to survive in the presence of the major compounds of their common hosts, detoxifying them or even exploiting them as carbon sources (Kusumoto et al. 2014; Wang et al. 2013). One plausible hypothesis accounting for our results is that a strong concentration and proportion increase of minor terpenes in infected cypresses would help to lower the success of *S. cardinale* infection or slow its growth considerably, thereby allowing the tree to react effectively, at least in resistant varieties.

The absence of differences between Mv and Hv suggests that *C. sempervirens* cannot distinguish between these two *S. cardinale* isolates. The short time period that this conifer and fungus have coexisted suggests that co-evolution or a capacity to elicit specific responses in their interactions is unlikely. Hv tended to elicit slightly (non-significantly) higher reactions compared to Mv, but probably due to the aggressiveness of the isolate and not to a specific reaction of the tree against it. Further study should compare the terpene reaction of *C. sempervirens* to different canker species or similar fungal pathogens to determine if the tree reaction elicited by *S. cardinale* is species-specific or just a general pathogen defense.

The main mechanism of reaction to *S. cardinale* infections in cypresses is based on formation of a necrophylactic

periderm, a quantitative (polygenic) trait that in resistant trees is able to compartmentalize and prevent fungal growth in bark tissues. Resistant and susceptible trees differ in the speed of reaction (how quickly they can build the barrier) and in the thickness (number of cell rows) of the barrier and its rate of suberization (Ponchet and Andreoli 1990). This mechanism is not specific against a particular ungus but is the same that is activated by cypresses as a consequence of a simple wound (without infection). This mechanism is disturbed by an invading fungus in infected trees. The production of inhibiting terpenes induced by infection in more resistant trees might affect the 'struggle' between host and pathogen, shifting this equilibrium by slowing fungal development and favoring the host to build an effective pathogen barrier.

The terpene compounds found in the phloem of *C. sempervirens* were consistent with those found in previous studies (Gallis et al. 2007; Piovetti et al. 1980, 1981). Concentrations also were within the ranges of those in similar studies of other conifers infected by fungal pathogens (Blodgett and Stanosz 1998; Raffa and Berryman 1982b; Viiri et al. 2001).

*Foliar Terpene Concentration* Terpene species and the foliar proportions in our study coincided with those in Schiller and Madar (1991), who reported that α-pinene and δ-3-carene were the most abundant terpenes. Mazari et al. (2010) also observed α-pinene as the main compound, but limonene was the second most abundant, and δ-3-carene was among the minor monoterpenes.

None of the compounds or tendencies for the infected treatments in our study, however, behaved similarly to those reported in Schiller and Madar (1991). The only trend in our study was a lower foliar concentration in Hv and Wounded than in the control cypresses (Table 2). No compound showed a consistent trend throughout the 90-day experiment. The inconsistencies between our study and that by Schiller and Madar (1991) suggest that leaves may not show a clear pattern of changes in terpene concentrations when infected by *S. cardinale*. The lack of differences among our treatments may have several explanations. The constitutive foliar chemotype of Agrimed is very different from those of the other resistant genotypes, and reaction patterns seemed to differ among the genotypes. The distance of the twig from the fungal infection, which varied from 3 to 21 cm, also was not correlated with foliar terpene concentration. The lower terpene concentrations in leaves may have been due to increased foliar emission. However, only a statistically significant relationship, between total monoterpene emission and total monoterpene concentration of day 10, was found, so our results do not provide enough support for this hypothesis. In addition, the inhibition of photosynthesis caused by *S. cardinale* may have affected terpene concentrations (Muthuchelian et al. 2005; Penuelas and Llusia 1999).

*Foliar Emission Rates* Foliar terpene emission rates of the control ranged between 2 and 4 $\mu g\ g^{-1}$ dw $h^{-1}$, similar to rates reported by Yatagai et al. (1995) and Yani et al. (1993) for the same species. The compounds detected also were similar to those in the previous two studies, but the monoterpene proportions were similar only to those in Yani et al. (1993). Yatagai et al. (1995) reported that limonene was responsible for 83 % of the emission blend, however, limonene represented only ca. 4 % of the emissions in the control in this current study (Table 3, Fig. 4).

The sampled leaves could represent only systemic responses to infection (twigs were up to 21 cm from the inoculated zone), but the infected plants usually displayed higher emissions than the control and sometimes the Wounded plants. These higher emissions were statistically significant, however, only at day 10 after inoculation (for δ-3-carene and total monoterpenes). Many other compounds showed a non-significant highest emission at day 10, possibly indicating that their maximum emission in response to *S. cardinale* infection occurs around this time. This change in volatile bouquet could be used by the vectors of cypress bark canker, such as *Phloeosinus aubei* (Covassi et al. 1975), *Megastigmus Watchli*, or *Orsillus maculatus* (Battisti et al. 1999; Zocca et al. 2008), or even parasitoids of these vectors (Adams and Six 2008; Boone et al. 2008; Sullivan and Berisford 2004).

In summary, all resistant genotypes of Italian cypress reacted strongly and similarly to *S. cardinale* infection by drastically increasing the phloem concentrations of several minor terpenes and moderately increasing the concentrations of major terpenes. This translated into moderate increases in total concentrations. Monoterpenes (especially the oxygenated monoterpenes, which increased quantitatively but also may be generated *de novo* in response to infection) and diterpenes were the most induced terpene classes in the infected trees, thus leading to a considerable proportional increase in minor monoterpenes and a consequent proportional decrease in the main monoterpenes. Such a strategy could help cypress defense, because some pathogens are adapted to the principal constituents of trees. Foliar concentrations did not show any clear trend apart from a concentration decrease in the infected treatments, which may have been due to a canker-induced inhibition of photosynthesis or a decrease due to increased emissions. Emission rates of foliar terpenes suggest that emission bouquets change under infection, opening the possibility of attracting *S. cardinale* vectors. The emission rates of foliar terpenes and several phloem proportions of oxygenated monoterpenes, terpinolene, and manool among others, reacted quite quickly, reaching their maximum proportions between days 1 and 10, while proportions of most phloem terpenes (α-thujene, α- pinene, sabinene, or totarol) continued to increase during infection, peaking around day 30 or 90. No clear differences were found between the fungal isolates for any tissue

examined, despite trends suggesting that a slightly stronger reaction was elicited by the more virulent fungal isolate (Hv).

This study is the first to describe the complex dynamics of the terpene reaction of *C. sempervirens* to *S. cardinale* in the early stages of infection. The results raise questions that warrant further research. Such studies should compare terpene and physiological reactions of *C. sempervirens* clones that are susceptible and resistant to bark canker, identify unknown induced compounds (e.g., diterpenes 2 and 5), and test Italian cypress terpenes against *S. cardinale* in experiments of growth inhibition and fungal biotransformation. In relation to indirect defenses, further research should study the emissions of cankered cypresses ca. 10 days after inoculation and test the attraction of several potential pathogen vectors to foliar terpene emissions.

## References

Adams AS, Six DL (2008) Detection of host habitat by parasitoids using cues associated with mycangial fungi of the mountain pine beetle, *Dendroctonus ponderosae*. Can Entomol 140:124–127

Bajpai VK, Rahman A, Kang SC (2007) Chemical composition and antifungal properties of the essential oil and crude extracts of *Metasequoia glyptostroboides* Miki ex Hu. Ind Crop Prod 26:28–35

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils: a review. Food Chem Toxicol 46:446–475

Battisti A, Rogues A, Colombari F, Frigimelica G, Guido M (1999) Efficient transmission of an introduced pathogen via an ancient insect-fungus association. Naturwissenschaften 86:479–483

Blodgett JT, Stanosz GR (1998) Monoterpene and phenolic compound concentrations in water-stressed red pine inoculated with *Sphaeropsis sapinea*. Phytopathology 88:245–251

Bonello P, Capretti P, Luchi N, Martini V, Michelozzi M (2008) Systemic effects of *Heterobasidion annosum* ss infection on severity of *Diplodia pinea* tip blight and terpenoid metabolism in Italian stone pine (*Pinus pinea*). Tree Physiol 28:1653–1660

Boone CK, Six DL, Zheng Y, Raffa KF (2008) Parasitoids and dipteran predators exploit volatiles from microbial symbionts to locate bark beetles. Environ Entomol 37:150–161

Boone CK, Aukema BH, Bohlmann J, Carroll AL, Raffa KF (2011) Efficacy of tree defense physiology varies with bark beetle population density: a basis for positive feedback in eruptive species. Can J For Res 41:1174–1188

Boulogne I, Petit P, Ozier-Lafontaine H, Desfontaines L, Loranger-Merciris G (2012) Insecticidal and antifungal chemicals produced by plants: a review. Environ Chem Lett 10:325–347

Bridges JR (1987) Effects of terpenoid compounds on growth of symbiotic fungi associated with the southern pine-beetle. Phytopathology 77:83–85

Cakir A, Kordali S, Zengin H, Izumi S, Hirata T (2004) Composition and antifungal activity of essential oils isolated from *Hypericum hyssopifolium* and *Hypericum heterophyllum*. Flavour Frag J 19:62–68

Covassi M, Intini M, Panconesi A (1975) Preliminary observations on the relationship between *Coryneum cardinale* Wag. and *Phloeosinus aubei* Perr. in Tuscany. Redia 56:159–166

Danti R, Panconesi A, Di Lonardo V, Della Rocca G, Raddi P (2006) 'Italico' and 'Mediterraneo': two *Seiridium cardinale* canker-resistant cypress cultivars of *Cupressus sempervirens*. Hortscience 41:1357–1359

Danti R, Di Lonardo V, Pecchioli A, Della Rocca G (2013) 'Le Crete 1' and 'Le Crete 2': two newly patented *Seiridium cardinale* canker-resistant cultivars of *Cupressus sempervirens*. Forest Pathol 43:204–210

Davis TS, Hofstetter RW (2011) Reciprocal interactions between the bark beetle-associated yeast *Ogataea pini* and host plant phytochemistry. Mycologia 103:1201–1207

De Alwis R, Fujita K, Ashitani T, Kuroda K (2009) Volatile and non-volatile monoterpenes produced by elicitor-stimulated *Cupressus lusitanica* cultured cells. J Plant Physiol 166:720–728

Della Rocca G, Eyre CA, Danti R, Garbelotto M (2011) Sequence and SSR analyses of the fungal pathogen *Seiridium cardinale* indicate California is the most likely source of the Cypress canker epidemic for the Mediterranean region. Phytopathology 101:1408–1417

Eberhardt TL, Han JS, Micales JA, Young RA (1994) Decay resistance in conifer seed cones - Role of resin acids as inhibitors of decomposition by white-rot fungi. Holzforschung 48:278–284

Espinosa-garcia FJ, Langenheim JH (1991) Effects of sabinene and gamma-terpinene from coastal redwood leaves acting singly or in mixtures on the growth of some of their fungus endophytes. Biochem Syst Ecol 19:643–650

Faldt J, Solheim H, Langstrom B, Borg-Karlson AK (2006) Influence of fungal infection and wounding on contents and enantiomeric compositions of monoterpenes in phloem of *Pinus sylvestris*. J Chem Ecol 32:1779–1795

Franceschi VR, Krokene P, Christiansen E, Krekling T (2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phtyol 167:353–376

Gallis AT, Doulis AG, Papageorgiou AC (2007) Variability of cortex terpene composition in *Cupressus sempervirens* L. provenances grown in Crete, Greece. Silvae Genet 56:294–299

Graniti A (1998) Cypress canker: a pandemic in progress. Annu Rev Phytopatol 36:91–114

Guenther AB, Zimmerman PR, Harley PC, Monson RK, Fall R (1993) Isoprene and monoterpene emission rate variability - model evaluations and sensitivity analyses. J Geophys Res-Atmos 98:12609–12617

Hammer KA, Carson CF, Riley TV (2003) Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. J Appl Microbiol 95:853–860

Hanari N, Yamamoto H, Kuroda K (2002) Comparison of terpenes in extracts from the resin and the bark of the resinous stem canker of *Chamaecyparis obtusa* and *Thujopsis dolabrata* var. hondae. J Wood Sci 48:56–63

Hudgins JW, Christiansen E, Franceschi VR (2004) Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. Tree Physiol 24:251–264

Hussain AI, Anwar F, Nigam PS, Sarker SD, Moore JE, Rao JR, Mazumdar A (2011) Antibacterial activity of some Lamiaceae essential oils using resazurin as an indicator of cell growth. LWT-Food Sci Technol 44:1199–1206

Jiao J, Fu Y-J, Zu Y-G, Luo M, Wang W, Zhang L, Li J (2012) Enzyme-assisted microwave hydro-distillation essential oil from *Fructus forsythia*, chemical constituents, and its antimicrobial and antioxidant activities. Food Chem 134:235–243

Kalemba D, Kunicka A (2003) Antibacterial and antifungal properties of essential oils. Curr Med Chem 10:813–829

Kidd F, Reid CPP (1979) Stimulation of resinosis and apparent inhibition of blue stain development in ponderosa pine by paraquat. Forest Sci 25:569–575

Klepzig KD, Kruger EL, Smalley EB, Raffa KF (1995) Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. J Chem Ecol 21:601–626

Kohzaki K, Gomi K, Yamasaki-Kokudo Y, Ozawa R, Takabayashi J, Akimitsu K (2009) Characterization of a sabinene synthase gene from rough lemon (Citrus jambhiri). J Plant Physiol 166:1700–1704

Kopper BJ, Illman BL, Kersten PJ, Klepzig KD, Raffa KF (2005) Effects of diterpene acids on components of a conifer bark beetle-fungal interaction: tolerance by Ips pini and sensitivity by its associate Ophiostoma ips. Environ Entomol 34:486–493

Kossuth SV, Barnard EL (1983) Monoterpene content of healthy sand pine and sand pine with root disease. For Sci 29:791–797

Kotan R, Kordali S, Cakir A (2007) Screening of antibacterial activities of twenty-one oxygenated monoterpenes. Z Naturforsch 62:507–513

Krokene P, Nagy NE, Krekling T (2008) Traumatic resin ducts and polyphenolic parenchyma cells in conifers. In: Shaller A (ed) Induced plant resistance to herbivory. Springer, Dordrecht, pp 147–169

Kumbhar PP, Dewang PM (2001) Eco-friendly pest management using monoterpenoids. I. Antifungal efficacy of thymol derivates. J Sci Ind Res India 60:645–648

Kusumoto N, Zhao T, Swedjemark G, Ashitani T, Takahashi K, Borg-Karlson A-K (2014) Antifungal properties of terpenoids in Picea abies against Heterobasidion parviporum. Forest Pathol 19:404–410

Leufvén A, Bergström G, Falsen E (1988) Oxygenated monoterpenes produced by yeasts, isolated from Ips typographus (Coleoptera: Scolytidae) and grown in phloem medium. J Chem Ecol 14:353–362

Madar Z, Gottlieb HE, Cojocaru M, Riov J, Solel Z, Sztejnberg A (1995a) Antifungal terpenoids produced by Cypress after Infection by Diplodia pinea f. sp. cupressi. Phytochemistry 38:351–354

Madar Z, Solel Z, Riov J, Sztejnberg A (1995b) Phytoalexin production by cypress in response to infection by Diplodia pinea f. sp. cupressi and its relation to water-stress. Physiol Mol Plant P 47:29–38

Marei GIK, Rasoul MAA, Abdelgaleil SAM (2012) Comparative antifungal activities and biochemical effects of monoterpenes on plant pathogenic fungi. Pestic Biochem Phys 103:56–61

Mazari K, Bendimerad N, Bekhechi C, Fernandez X (2010) Chemical composition and antimicrobial activity of essential oils isolated from Algerian Juniperus phoenicea L. and Cupressus sempervirens L. J Med Plants Res 4:959–964

Michelozzi M (1999) Defensive roles of terpenoid mixtures in conifers. Acta Bot Gallica 146:73–84

Morcia C, Malnati M, Terzi V (2012) In vitro antifungal activity of terpinen-4-ol, eugenol, carvone, 1,8-cineole (eucalyptol) and thymol against mycotoxigenic plant pathogens. Food Addit Contam A 29:415–422

Muthuchelian K, La Porta N, Bertamini M, Nedunchezhian N (2005) Cypress canker induced inhibition of photosynthesis in field grown cypress (Cupressus sempervirens L.) needles. Physiol Mol Plant P 67:33–39

Nenoff P, Haustein UF, Brandt W (1996) Antifungal activity of the essential oil of Melaleuca alternifolia (tea tree oil) against pathogenic fungi in vitro. Skin Pharmacol Phys 9:388–394

Paine TD, Hanlon CC (1994) Influence of oleoresin constituents from Pinus ponderosa and Pinus jeffreyi on growth of mycangial fungi from Dendroctonus ponderosae and Dendroctonus jeffreyi. J Chem Ecol 20:2551–2563

Panconesi A (1991) In: Panconesi A (ed) Il Cipresso. Proposte di Valorizzazione Ambientale e Produttiva nei Paesi Mediterranei della

Comunità Economica Europea. Consiglio Nazionale delle Richerche, Firenze, p 228

Panconesi A, Raddi P (1991) Agrimed No. 1 and Bolgheri: two new cypress varieties resistant to canker. Cellulosa e Carta 42:47–52

Penuelas J, Llusia J (1999) Seasonal emission of monoterpenes by the Mediterranean tree Quercus ilex in field conditions: relations with photosynthetic rates, temperature and volatility. Physiol Plantarum 105:641–647

Phillips MA, Croteau RB (1999) Resin-based defenses in conifers. Trends Plant Sci 4:184–190

Piovetti L, Gonzalez E, Diara A (1980) Diterpene composition of Cupressus dupreziana and Cupressus sempervirens. Phytochemistry 19:2772–2773

Piovetti L, Francisco C, Pauly G, Benchabane O, Bernarddagan C, Diara A (1981) Volatile constituents of Cupressus dupreziana and the sesquiterpenes of Cupressus sempervirens. Phytochemistry 20:1299–1302

Ponchet J, Andreoli C (1990) Compartmentalization and reaction in the host. In: Ponchet J (ed) Agriculture – AGRIMED research programme: progress in EEC research on cypress diseases. Report EUR 12493 EN. Commission of the European Communities, Brussels, pp 96–111

Pragadheesh VS, Saroj A, Yadav A, Chanotiya CS, Alam M, Samad A (2013) Chemical characterization and antifungal activity of Cinnamomum camphora essential oil. Ind Crop Prod 49:628–633

Raffa KF, Berryman AA (1982a) Accumulation of monoterpenes and associated volatiles following inoculation of grand fir with a fungus transmitted by the fir engraver, Scolytus ventralis (Coleoptera: Scolytidae). Can Entomol 114:797–810

Raffa KF, Berryman AA (1982b) Physiological differences between lodgepole pines resistant and susceptible to the mountain pine beetle and associated microorganisms. Environ Entomol 11:486–492

Raffa KF, Smalley EB (1995) Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. Oecologia 102:285–295

Ramos S, Rojas LB, Lucena ME, Meccia G, Usubillaga A (2011) Chemical composition and antibacterial activity of Origanum majorana L. essential oil from the venezuelan Andes. J Essent Oil Res 23:45–49

Ramsewak RS, Nair MG, Stommel M, Selanders L (2003) In vitro antagonistic activity of monoterpenes and their mixtures against 'toe nail fungus' pathogens. Phytoter Res 17:376–379

Schiller G, Madar Z (1991) Variation in foliage resin composition within the Italian Cypress (Cupressus sempervirens L) species complex and its relation to canker diseases. Eur J For Pathol 21:179–184

Siddhardha B, Kumar MV, Murty USN, Ramanjaneyulu GS, Prabhakar S (2011) Biotransformation of alpha-pinene to terpineol by resting cell suspension of Absidia corulea. Indian J Microbiol 52:292–294

Sullivan BT, Berisford CW (2004) Semiochemicals from fungal associates of bark beetles may mediate host location behavior of parasitoids. J Chem Ecol 30:703–717

Tan Q, Day DF (1998) Bioconversion of limonene to α-terpineol by immobilized Penicillium digitatum. Appl Microbiol Biot 49:96–101

Tomlin ES, Antonejevic E, Alfaro RI, Borden JH (2000) Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. Tree Physiol 20:1087–1095

Trapp S, Croteau R (2001) Defensive resin biosynthesis in conifers. Annu Rev Plant Biol 52:689–724

Ulubelen A, Topcu G, Eriş C, Sönmez U, Kartal M, Kurucu S, Bozok-Johansson C (1994) Terpenoids from Salvia sclarea. Phytochemistry 36:971–974

Viiri H, Annila E, Kitunen V, Niemela P (2001) Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus, Ceratocystis polonica. Trees-Struct Func 15:112–122

Wagener WW (1939) The canker of Cupressus induced by *Coryneum cardinale* n. sp. J Agric Res 58:1–46

Wang Y, Lim L, DiGuistini S, Robertson G, Bohlmann J, Breuil C (2013) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to Grosmannia clavigera, a bark beetle-associated fungal pathogen of pine trees. New Phytol 197:886–898

Xenopoulos SG (1990) Screening for resistance to cypress canker (*Seiridium cardinale*) in 3 greek provenances of *Cupressus sempervirens*. Eur J For Pathol 20:140–147

Yamamoto H, Asano N, Sawano C, Sone T, Gasha T, Ono Y (1997) Diterpenes isolated from the resin of the resinous stem canker of Japanese Cypress, *Chamaecyparis obtusa*. Mokuzai Gakkaishi 43: 558–565

Yani A, Pauly G, Faye M, Salin F, Gleizes M (1993) The effect of a long-term water-stress on the metabolism and emission of terpenes of the foliage of *Cupressus sempervirens*. Plant Cell Environ 16:975–981

Yatagai M, Ohira M, Ohira T, Nagai S (1995) Seasonal variations of terpene emission from trees and influence of temperature, light and contact stimulation on terpene emission. Chemosphere 30:1137–1149

Zamponi L, Michelozzi M, Capretti P (2007) Terpene response of *Picea abies* and *Abies alba* to infection with *Heterobasidion* sl. Forest Pathol 37:243–250

Zhao T, Krokene P, Björklund N, Långström B, Solheim H, Christiansen E, Borg-Karlson A-K (2010) The influence of *Ceratocystis polonica* inoculation and methyl jasmonate application on terpene chemistry of Norway spruce *Picea abies*. Phytochemistry 71:1332–1341

Zhou HE, Tao NG, Jia L (2014) Antifungal activity of citral, octanal and alpha-terpineol against *Geotrichum citri-aurantii*. Food Control 37: 277–283

Zocca A, Zanini C, Aimi A, Frigimelica G, La Porta N, Battisti A (2008) Spread of plant pathogens and insect vectors at the northern range margin of cypress in Italy. Acta Oecol 33:307–313

Zouari N, Fakhfakh N, Zouari S, Bougatef A, Karray A, Neffati M, Ayadi MA (2011) Chemical composition, angiotensin I-converting enzyme inhibitory, antioxidant and antimicrobial activities of essential oil of tunisian *Thymus algeriensis* Boiss. et Reut. (Lamiaceae). Food Bioprod Process 89:257–265