STRONG INDUCTION OF MINOR TERPENES IN ITALIAN CYPRESS, *Cupressus sempervirens,* IN RESPONSE TO INFECTION BY THE FUNGUS *Seiridium cardinale*

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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*.

Key Words – VOCs, cypress bark canker, sabinene, manool, oxygenated monoterpenes, *de novo*.

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, mainly terpenes and phenols (Franceschi et al. 2005; Phillips and Croteau 1999), to face 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 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-yr 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. 1981; Piovetti et al. 1980; 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; Madar et al. 1995b). 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

146 spacing and belonging to four genotypes patented by IPSP-CNR for their resistance to
147 cypress bark canker: Italico, Bolgheri, Agrimed and Mediterraneo (16 trees of each
148 genotype) (Danti et al. 2006; Panconesi and Raddi 1991). Cypresses were watered
149 twice a week during the first month after planting. Soil was a clayey reclaimed alluvial.
150 The climate is moderately continental, with hot summers and cold winters with sporadic
151 snowfall. The average rainfall is 815 mm yr^{-1} distributed on 80 rainy days with a peak in
152 autumn. The yearly average annual temperature is 13.8 °C. The coldest month is
153 January with an average minimum of 0 °C, and the warmest month is July with an
154 average maximum temperature of 30 °C.
155
156 *Experimental Design.* To monitor tree reactions against fungal infection, we applied
157 four treatments to the cypresses: 1) control (no damage); 2) mildly virulent (Mv, wound
158 + inoculation with a moderately aggressive *S. cardinale* isolate (ref. submitted)); 3)
159 highly virulent (Hv, wound + infection with a more aggressive *S. cardinale* isolate); and
160 4) Wounded (wound only, without inoculation). Trees were inoculated following a
161 standard procedure (Danti et al. 2006, Danti et al. 2013), which consists of removing a
162 disc of bark from the stem with a sterile cork borer of 4 mm diam and filling the wound
163 with a plug of the same size of malt extract agar (MEA). This plug was taken from the
164 margin of a colony of the fungus grown on MEA 2% in the dark for 15 days at 25 °C.
165 The inoculation site was covered with wet cotton wool and wrapped with Parafilm®.
166
167 Tissue samples were collected from 26 April to 25 July 2012, 1, 10, 30, and 90
168 d after applying the above treatments. The sampling method was destructive, so trees
169 were used only once to avoid any effects from the wounds. Each treatment, for each
170 sampling date, had four replicates (four treatments × four time points × four replicates =
171 64). Within the treatments, each of the four replicates contained each of the four tree
172 genotypes.
173

174 *Field sampling. Tissue Sampling.* Three types of samples were collected from each
175 tree: *i*) phloem removed from a segment of the inoculated stem containing the infected
176 tissues (samples were taken from a height of ca. 80 cm); *ii*) foliar tissue from the
177 closest branch to the inoculation point and; *iii*) foliar volatile organic compound (VOC)
178 emission, from the same branch where foliar tissue was taken. Emissions were
179 sampled first to avoid tree reactions to wounding. All sampled tissues were stored in
180 liquid nitrogen in the field and then at -20 ºC in the laboratory.
181
182 *VOC Sampling.* Twigs immediately above the inoculation point (3.5-21 cm) were
183 sampled to analyze VOC emissions. The selected twigs were wrapped first with Teflon
184 ribbon a few days before the sampling to minimize effects of mechanical manipulation
185 and alteration of the emissions.
186
187 The VOC emissions were sampled from 09:00 to 15:00 h (solar time) using the
188 conifer chamber (a 230 cm^3 cuvette) of the LiCor 6400 Portable Photosynthesis
189 System (Li-Cor Inc, Lincoln, NE, USA). The twig was carefully inserted into the
190 chamber, placing its closure on the Teflon ribbon. Air flow rate inside the conifer
191 chamber was set to 600 µmol s^{-1}. The chamber was allowed to stabilize for 15 min, as
192 monitored by environmental and physiological parameters such as temperature,
193 photosynthetic active radiance (PAR), photosynthesis, and stomatal conductance.
194 When the twig had physiologically stabilized, we placed one end of a metallic VOC trap
195 (Markes International Inc. Wilmington, DE, USA), filled with 115 mg of Tenax and 230
196 mg of Unicarb, in the chamber to collect the VOCs exhausted from the twig chamber. A
197 QMAX pump (Supelco, Bellefonte, PA, USA) attached to the other end of the metallic
198 trap pulled the air from the conifer chamber. A Defender 510 fluxometer (Bios
199 International Corporation, Butler, NJ, USA) was placed between the QMAX and the
200 VOC trap to control the air flux. Sampling time was 5 min, with an absorption flux of ca.
201 7 ml s^{-1}. The sampled VOC traps were stored in the field in a 4 ºC portable refrigerator

until transferred to a -20 ºC 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 ºC. 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 ºC for 2 min, increased at 15 ºC min^{-1} to 150 ºC and maintained for 5 min, increased at 30 ºC min^{-1} to 250 ºC and maintained for 5 min, and finally increased at 30 ºC min^{-1} to 280 ºC 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

230 of the most abundant compounds found in the samples. Four monoterpenes (α-pinene,
231 sabinene, limonene, and γ-terpinene), three sesquiterpenes (caryophyllene,
232 caryophyllene oxide, and cedrol), two diterpenes (phytol and totarol), and one non-
233 terpene internal standard (dodecane) were used (Fluka Chemie AG, Buchs,
234 Switzerland). All terpene calibration curves were highly significant ($r^2 \geq 0.99$) for the
235 relationship between signal strength and terpene concentration. The most abundant
236 terpenes exhibited similar sensitivities (differences <5%). Terpenes identified only by
237 published spectra that were considered important for the experiment were later verified
238 with standards: α-thujene (Chemos GmbH, Regenstauf, Germany) terpinolene,
239 terpinen-4-ol, sabinene hydrate, camphor, α-terpineol (Fluka Chemie AG, Buchs,
240 Switzerland), and manool (Sequoia Research Products Limited, Pangbourne, United
241 Kingdom).
242
243 *Terpene Emission Rates.* The terpene emissions collected by the VOC traps were
244 released with an automatic sample processor (TD Autosampler, Series 2 Ultra, Markes
245 International Inc. Wilmington, DE, USA) and desorbed using an injector (Unity, Series 2,
246 Markes International Inc. Wilmington, DE, USA) in the GC/MS described above. A full-
247 scan method was used for the chromatographic analyses. The desorbed sample was
248 retained in a cryotrap at -20 ºC. The split was 1:10. The sample was redesorbed at 250
249 ºC for 10 min, injected into the column with a transfer line at 250 ºC, and submitted to
250 the same chromatographic process described above for the analysis of terpene
251 concentrations.
252 No diterpenes were used as standards for the analyses of emission rates
253 because they are not volatile at ambient temperature. The terpene emission rates were
254 expressed in μg g^{-1} (dry weight (dw)) h^{-1}. Even though the days of sampling were
255 similar (sunny and warm), the terpene emission rates were standardized at 30 ºC using
256 an algorithm for terpene-storing species (Guenther et al. 1993):
257

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

where E represents the emission rates in μg g^{-1} (dw) h^{-1} of monoterpenes at temperature T (in degrees Kelvin, K), E_s is the emission factor in μg g^{-1} (dw) h^{-1} at standard temperature T_s (303 K), and β 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).

286 *Qualitative Differences among Treatments.* Six terpenes appeared exclusively in the
287 infected treatments (Mv and Hv) 30 and 90 days after inoculation. These six *de novo*
288 terpenes were found in all four cypress genotypes. Four of these were oxygenated
289 monoterpenes: oxygenated monoterpene *de novo 1* (detected in 15 of 16 samples of
290 Mv and Hv at days 30 and 90, 0.093±0.02 mg g^{-1}, mean±SE), sabinene hydrate (16/16;
291 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
292 mg g^{-1}). The monoterpene *de novo 2* (14/16; 0.11±0.04 mg g^{-1}) and the diterpene *de*
293 *novo* 3 (6/16; 5.4±1.7 mg g^{-1}) could not be identified. No differences in concentration
294 were detected between treatment or time for the *de novo* compounds (REML,
295 fixed=treatment, random=genotype, paired Tukey's *post-hoc* test, $P < 0.05$). Thymyl
296 methyl ether (another oxygenated monoterpene) did not appear in the control but was
297 detected in some of the Wounded samples and in all infected treatments from day 10
298 to day 90, reaching a mean concentration of 2.9±1.2 mg g^{-1} in Hv at day 30 (Table 1).
299
300 *Quantitative Differences among Treatments.* Total concentrations were lower in the
301 infected treatments than in the control at days 1 and 10 but increased substantially
302 after day 30 (Table 1). Total terpenes were nearly 4-fold higher in the infected
303 treatments compared to control at day 30, and reached a maximum of 140 mg g^{-1} at
304 day 90 (Table 1). This increase in total terpenes was due partly to increased
305 concentrations of some of the most abundant compounds (α-pinene, diterpene 1) but
306 also to the strong increases in concentrations of several minor compounds. These
307 changes led to a decrease in the proportions of the main compounds. α-Thujene was
308 among the most induced compounds in the infected treatments (up to a 57-fold
309 increase relative to the control), and presented differences from day 10, with
310 concentrations and proportions rising steadily until day 90. Next in order of retention
311 time was sabinene, whose concentrations (60-fold increase) had begun to differentiate
312 by day 10 and whose proportions peaked between days 10-30, and then dropped
313 slightly by day 90 (Fig. 1). Terpinolene concentrations (18-fold increase) had higher

314 proportions in the infected treatments throughout the experiment, reaching maximum
315 proportion at day 1. Terpinen-4-ol (622-fold increase) retained a high concentration and
316 proportional difference between treatments from days 10 to 90. Diterpene 2 was the
317 most induced diterpene (164-fold increase) and increased its concentration steadily
318 from day 1 to day 90 (Fig. 2). Diterpene 5 (43-fold), diterpene 6 (42-fold), and manool
319 (11-fold) increased in concentration and proportions from day 10 to 90. Limonene (12-
320 fold) and α-terpinene (15-fold) also notably increased, but the concentrations were
321 significantly higher than the control only at day 90. Oxygenated monoterpenes (the
322 sum of terpinen-4-ol, thymyl methyl ether, and bornyl acetate) were the most induced
323 terpene class, with up to 1063-fold higher concentrations in the infected treatments
324 than in the control (Fig. 1).
325
326 At day 1 post inoculation, total terpenes tended to decrease relative to control,
327 as did all terpene classes (mono-, sesqui-, and diterpenes), despite the lack of
328 statistical differences among treatments. Only cedrol exhibited differences, with Mv
329 higher than Wounded and Hv (REML, fixed=treatment, random=genotype, paired
330 Tukey's *post-hoc* test, $P < 0.05$) (Table 1). δ-3-Carene had a higher proportion in
331 Wounded than in all other treatments, and terpinolene, the minor monoterpenes (sum
332 of all monoterpenes except α-pinene and δ-3-carene), and diterpene 2 had higher
333 proportions in the infected treatments than in the control or Wounded (Table 1, Figs. 2-
334 3).
335
336 Terpene concentrations decreased significantly at day 10 in both infected
337 treatments relative to control for total terpenes and all terpene classes, except the
338 oxygenated monoterpenes, that increased 75-fold. α-Pinene, α-fenchene, β-pinene, β-
339 myrcene, δ-3-carene, total monoterpenes, all sesquiterpenes (including total
340 sesquiterpenes), the majority of diterpenes (including total diterpenes), and total
341 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).

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

453 fungal-infected cypresses discovered by Madar et al. (1995a) using thin layer
454 chromatography (TLC).
455
456 The scarce information that is available for the role of sabinene hydrate in tree
457 defense and fungal inhibition (Ramos et al. 2011; Tomlin et al. 2000) suggests that this
458 compound might have moderate defensive and antifungal activity. The role of camphor
459 (Kotan et al. 2007; Marei et al. 2012; Pragadheesh et al. 2013; Ramsewak et al. 2003)
460 is ambiguous, being inhibitory for some fungi but not for others, suggesting slight fungal
461 toxicity. α-Terpineol, however, is a powerful fungal inhibitor (Cakir et al. 2004; Hammer
462 et al. 2003; Kossuth and Barnard 1983; Kotan et al. 2007; Kusumoto et al. 2014; Zhou
463 et al. 2014) Thymyl methyl ether is among the least inhibitive chemical structures of
464 thymol to several fungi (Kumbhar and Dewang 2001).
465
466 The only *de novo* terpenes known to be produced by Italian cypress in response
467 to a fungal pathogen are the oxygenated sesquiterpenes cupressutropolone A and B,
468 produced under infection by *Diplodia pinea,* another canker-causing fungus (Madar et
469 al. 1995a). These two sesquiterpenes are considered *C. sempervirens* phytoalexins,
470 because they cause strong or total inhibition of mycelial growth and spore germination
471 for *S. cardinale* and other cypress pathogens (Madar et al. 1995a).
472 The *de novo* compounds we found could, thus, likely be antifungal phytoalexins
473 because *i*) sabinene hydrate, camphor, and α-terpineol appeared exclusively in the
474 infected treatments, *ii*) they are oxygenated monoterpenes, *iii*) their antifungal activity
475 has been reported in literature (especially α-terpineol), and *iv*) the report by Madar et al.
476 (1995a). The possibility that these *de novo* compounds (especially α-terpineol and
477 camphor) are a product or a biotransformation of the infecting fungal pathogen,
478 however, cannot be discarded (Kusumoto et al. 2014; Leufvén et al. 1988; Siddhardha
479 et al. 2011; Tan and Day 1998). Furthermore, any terpene concentration found in the
480 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 1982b; 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 et al. 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 (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. 2013; 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,

537 suggest that infected cypresses invest more in minor compounds than in major ones.
538 This strategy had been observed in *Picea abies*, *Abies grandis*, and *Pinus resinosa*,
539 where their main monoterpenes (pinenes), lowered proportions in infected trees in
540 favor of minor monoterpenes such as sabinene and terpinolene (Klepzig et al. 1995;
541 Raffa and Berryman 1982b; Zhao et al. 2010). Some tree terpenes (usually the main
542 compounds) have low inhibiting effects (Kusumoto et al. 2014) or can even enhance
543 the growth of some fungal pathogens (Bridges 1987; Cakir et al. 2004; Davis and
544 Hofstetter 2011), because some pathogenic fungi have developed the ability to survive
545 in the presence of the major compounds of their common hosts, detoxifying them or
546 even exploiting them as carbon sources (Kusumoto et al. 2014; Wang et al. 2013). One
547 plausible hypothesis accounting for our results is that a strong concentration and
548 proportion increase of minor terpenes in infected cypresses would help to lower the
549 success of *S. cardinale* infection or slow its growth considerably, thereby allowing the
550 tree to react effectively, at least in resistant varieties.
551
552 The absence of differences between Mv and Hv suggests that *C. sempervirens*
553 cannot distinguish between these two *S. cardinale* isolates. The short time period that
554 this conifer and fungus have coexisted suggests that co-evolution or a capacity to elicit
555 specific responses in their interactions is unlikely. Hv tended to elicit slightly (non-
556 significantly) higher reactions compared to Mv, but probably due to the aggressiveness
557 of the isolate and not to a specific reaction of the tree against it. Further study should
558 compare the terpene reaction of *C. sempervirens* to different canker species or similar
559 fungal pathogens to determine if the tree reaction elicited by *S. cardinale* is species-
560 specific or just a general pathogen defense.
561
562 The main mechanism of reaction to *S. cardinale* infections in cypresses is
563 based on formation of a necrophylactic periderm, a quantitative (polygenic) trait that in
564 resistant trees is able to compartmentalize and prevent fungal growth in bark tissues.

565 Resistant and susceptible trees differ in the speed of reaction (how quickly they can
566 build the barrier) and in the thickness (number of cell rows) of the barrier and its rate of
567 suberization (Ponchet and Andreoli 1990). This mechanism is not specific against a
568 particular fungus but is the same that is activated by cypresses as a consequence of a
569 simple wound (without infection). This mechanism is disturbed by an invading fungus in
570 infected trees. The production of inhibiting terpenes induced by infection in more
571 resistant trees might affect the 'struggle' between host and pathogen, shifting this
572 equilibrium by slowing fungal development and favoring the host to build an effective
573 pathogen barrier.
574
575 The terpene compounds found in the phloem of *C. sempervirens* were
576 consistent with those found in previous studies (Gallis et al. 2007; Piovetti et al. 1981;
577 Piovetti et al. 1980). Concentrations also were within the ranges of those in similar
578 studies of other conifers infected by fungal pathogens (Blodgett and Stanosz 1998;
579 Raffa and Berryman 1982a; Viiri et al. 2001).
580
581 *Foliar Terpene Concentration.* Terpene species and the foliar proportions in our study
582 coincided with those in Schiller and Madar (1991), who reported that α-pinene and δ-3-
583 carene were the most abundant terpenes. Mazari et al. (2010) also observed α-pinene
584 as the main compound, but limonene was the second most abundant, and δ-3-carene
585 was among the minor monoterpenes.
586
587 None of the compounds or tendencies for the infected treatments in our study,
588 however, behaved similarly to those reported in Schiller and Madar (1991). The only
589 trend in our study was a lower foliar concentration in Hv and Wounded than in the
590 control cypresses (Table 2). No compound showed a consistent trend throughout the
591 90-day experiment. The inconsistencies between our study and that by Schiller and
592 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 μg 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. (1995). Yatagai et al. (1993) 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*

621 *aubei* (Covassi et al. 1975), *Megastigmus Watchli*, or *Orsillus maculatus* (Battisti et al.
622 1999; Zocca et al. 2008), or even parasitoids of these vectors (Adams and Six 2008;
623 Boone et al. 2008; Sullivan and Berisford 2004).
624
625 In summary, all resistant genotypes of Italian cypress reacted strongly and similarly to
626 *S. cardinale* infection by drastically increasing the phloem concentrations of several
627 minor terpenes and moderately increasing the concentrations of major terpenes. This
628 translated into moderate increases in total concentrations. Monoterpenes (especially
629 the oxygenated monoterpenes, which increased quantitatively but also may be
630 generated *de novo* in response to infection) and diterpenes were the most induced
631 terpene classes in the infected trees, thus leading to a considerable proportional
632 increase in minor monoterpenes and a consequent proportional decrease in the main
633 monoterpenes. Such a strategy could help cypress defense, because some pathogens
634 are adapted to the principal constituents of trees. Foliar concentrations did not show
635 any clear trend apart from a concentration decrease in the infected treatments, which
636 may have been due to a canker-induced inhibition of photosynthesis or a decrease due
637 to increased emissions. Emission rates of foliar terpenes suggest that emission
638 bouquets change under infection, opening the possibility of attracting *S. cardinale*
639 vectors. The emission rates of foliar terpenes and several phloem proportions of
640 oxygenated monoterpenes, terpinolene, and manool among others, reacted quite
641 quickly, reaching their maximum proportions between days 1 and 10, while proportions
642 of most phloem terpenes (α-thujene, α- pinene, sabinene, or totarol) continued to
643 increase during infection, peaking around day 30 or 90. No clear differences were
644 found between the fungal isolates for any tissue examined, despite trends suggesting
645 that a slightly stronger reaction was elicited by the more virulent fungal isolate (Hv).
646
647 This study is the first to describe the complex dynamics of the terpene reaction
648 of *C. sempervirens* to *S. cardinale* in the early stages of infection. The results raise

649 questions that warrant further research. Such studies should compare terpene and
650 physiological reactions of *C. sempervirens* clones that are susceptible and resistant to
651 bark canker, identify unknown induced compounds (e.g., diterpenes 2 and 5), and test
652 Italian cypress terpenes against *S. cardinale* in experiments of growth inhibition and
653 fungal biotransformation. In relation to indirect defenses, further research should study
654 the emissions of cankered cypresses ca. 10 days after inoculation and test the
655 attraction of several potential pathogen vectors to foliar terpene emissions.
656
657
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Figure captions

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, $P < 0.05$)

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*)

Fig. 3 Mean phloem concentrations (±SE) and mean proportions (±SE) of minor monoterpenes (those <5% of total monoterpenes (MT): all except α-pinene at ca. 70% and δ-3-carene at ca. 20%). 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*)

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 ( )

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, $P < 0.05$)

Figures

Fig. 1

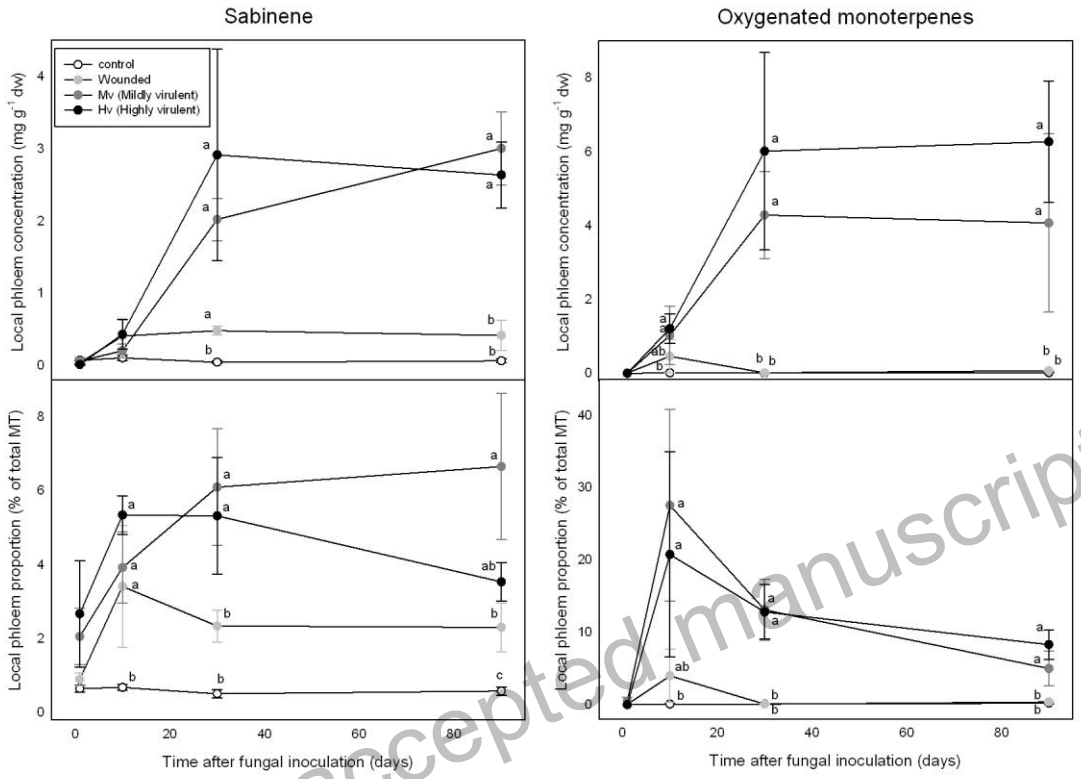
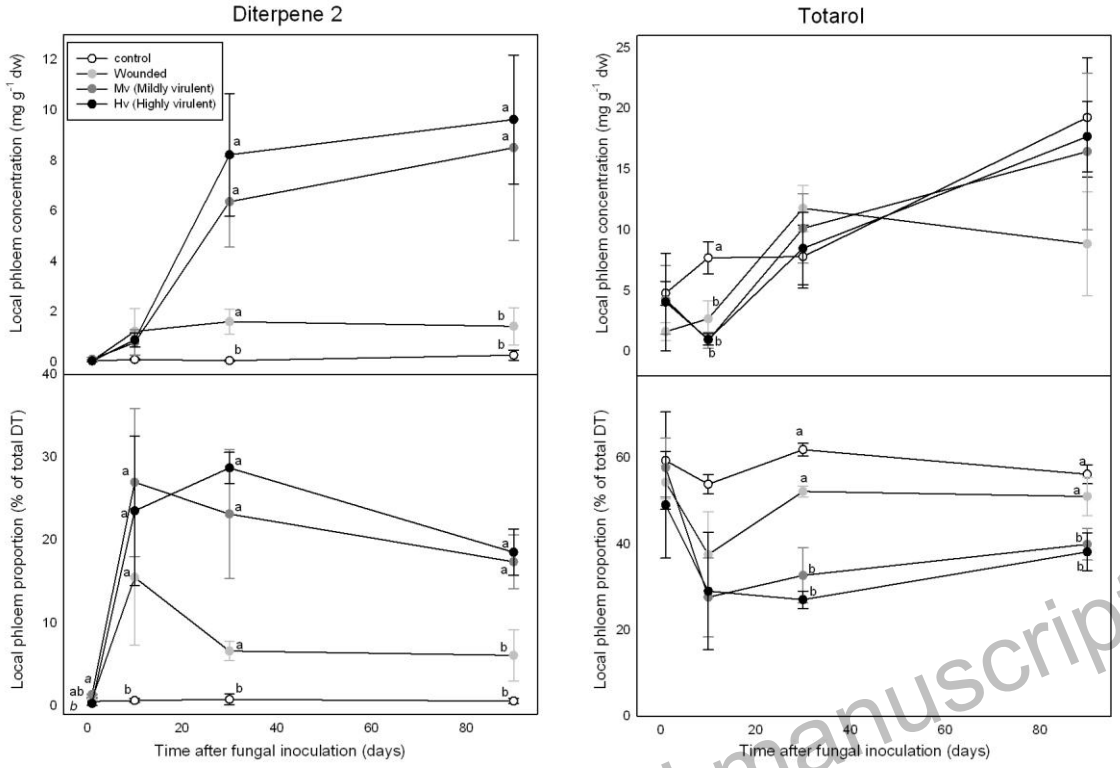


Fig. 2



1010 **Fig. 3**

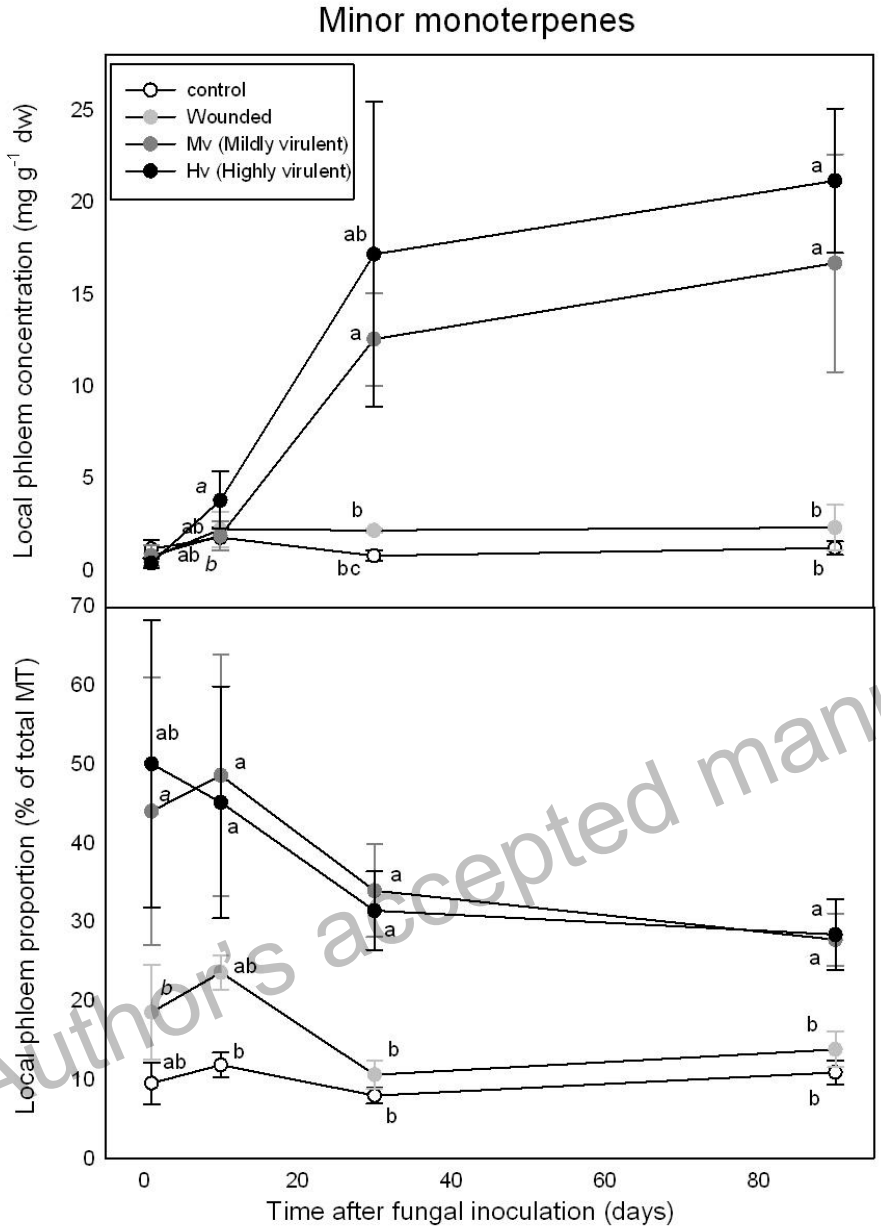


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Fig. 4

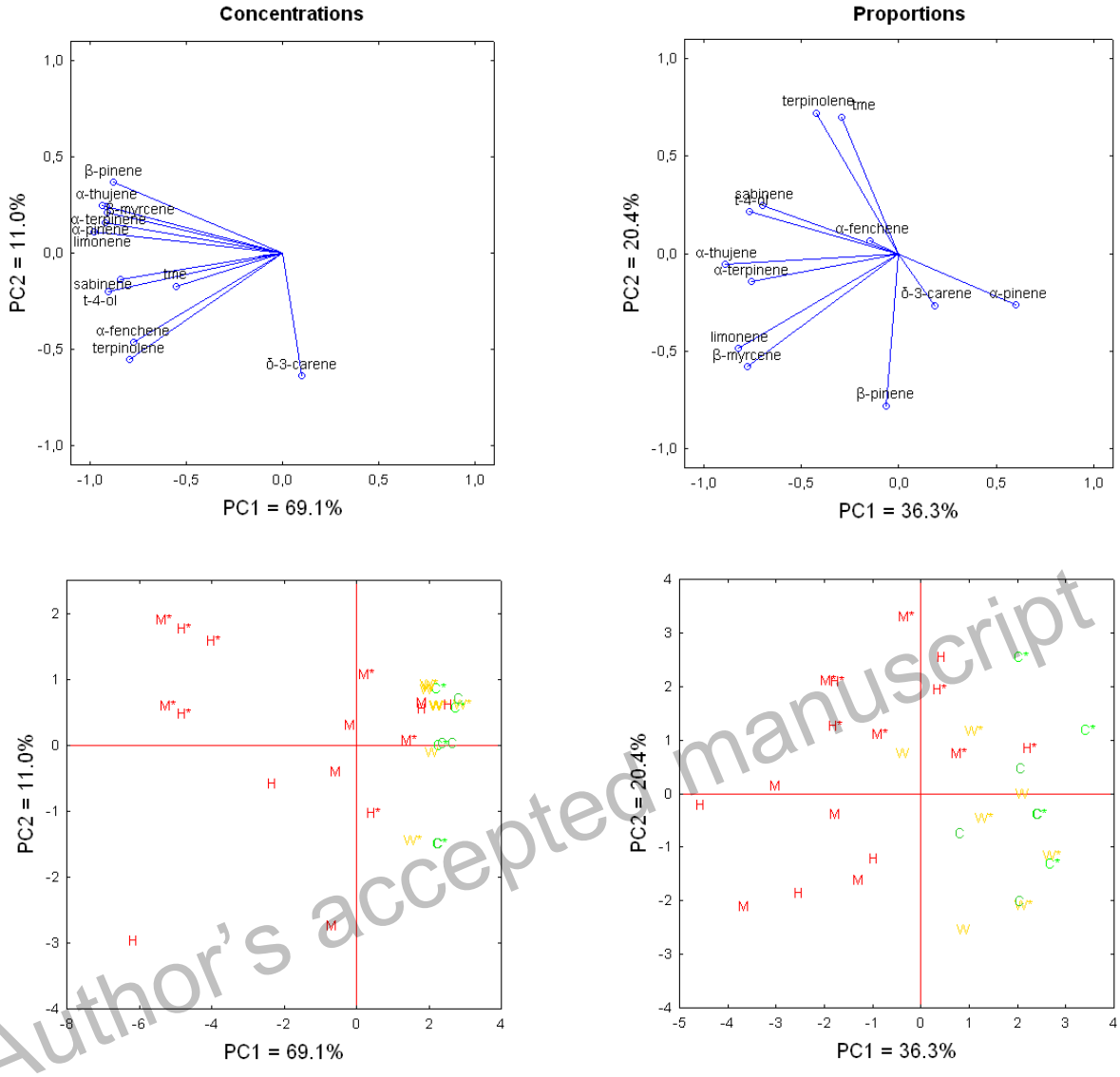


Fig. 5

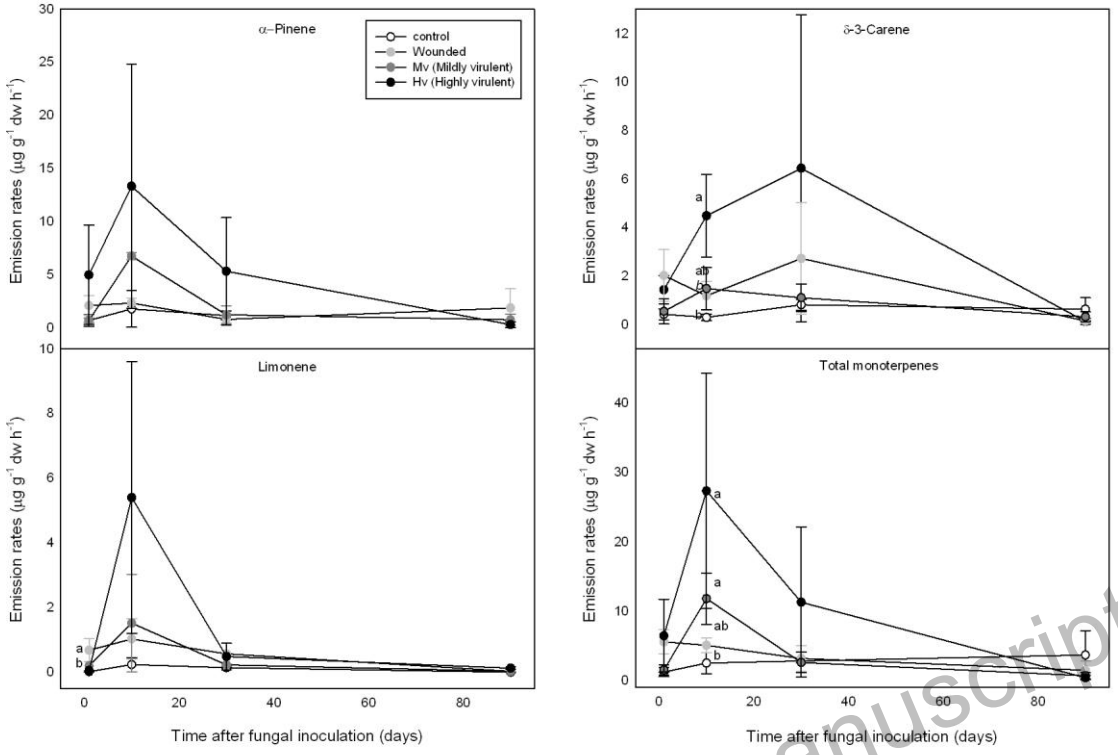
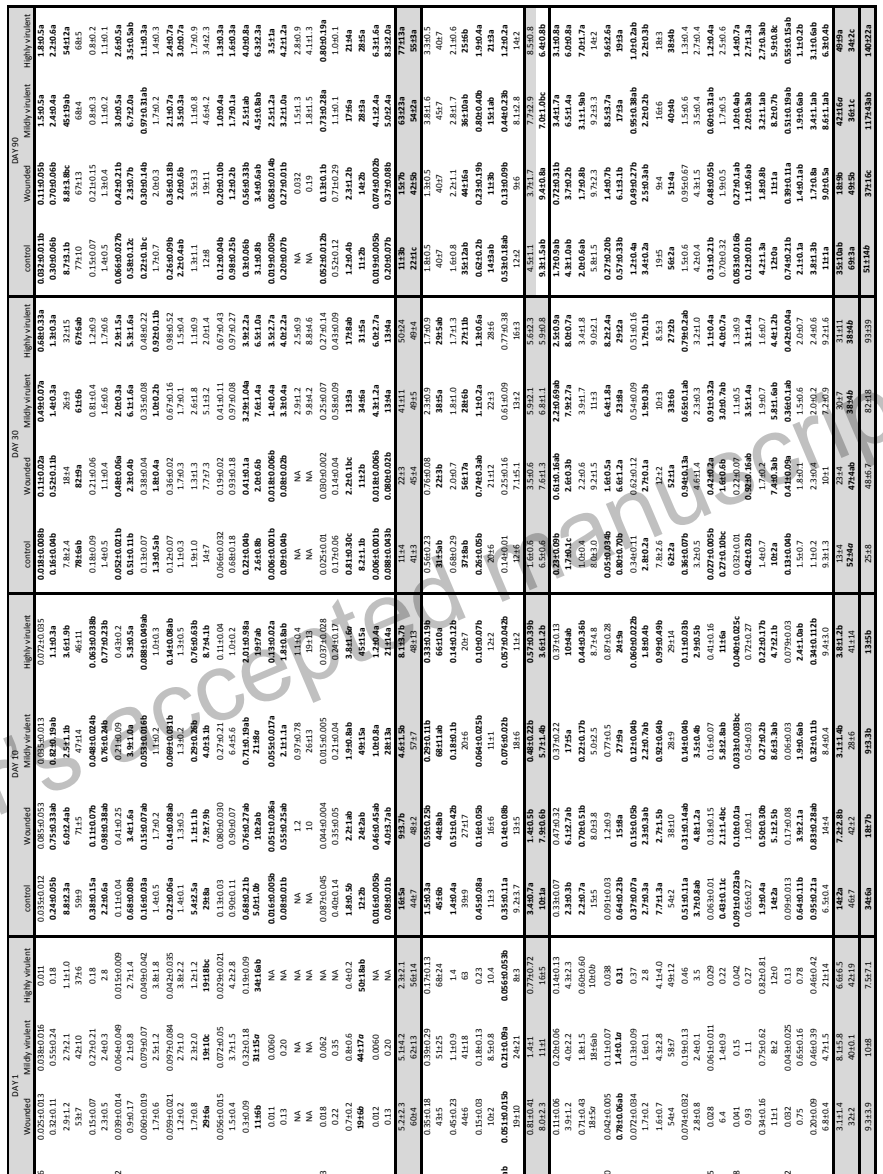


Table captions

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.*



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*)

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

		DAY 1				DAY 10				DAY 30				DAY 90			
Name	RT(min)	control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	Highly virulent	control	Wounded	Mildly virulent	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	0.071±0.035	0.081±0.015	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	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	0.29±0.23	0.31±0.27	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	7.82	12±5	8.7±3.9	11±4	12±5	1.5±7	7.8±3.0	13±5	6.2±4.1	16±8	16±5	23±8	19±8	19±5	17±6	24±2	17±8
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	0.23±0.09	0.19±0.012	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	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	1.1±0.9	1.1±0.8	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	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	0.24±0.10	0.23±0.04	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	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	0.36±0.12	0.29±0.05	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	8.77	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	8.8±3.7	5.7±0.8	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	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	0.46±0.17	0.31±0.05	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	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	0.039±0.025	0.051±0.031	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	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	0.38±0.14	0.28±0.04	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	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	0.019±0.004	0.011±0.007	0.013±0.001	0.018±0.011	0.013±0.004	0.015±0.003	0.034±0.024	0.014±0.013
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	0.074±0.032	0.049±0.014	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	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	0.93±0.39	0.68±0.10	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		21±9	15±6	18±7	19±8	26±9	14±5	22±7	9±4	29±11	25±3	37±14	29±12	30±5	23±7	33±3	18±10
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**	0.25±0.03	0.28±0.093	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	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	0.19±0.10	0.14±0.02	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	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**	0.43±0.21	0.43±0.12	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	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	1.3±0.7	1.3±0.4	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	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**	2.2±0.9	3.0±0.3	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	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**	0.078±0.032	0.11±0.02	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	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	0.69±0.32	0.45±0.05	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		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**	5.1±1.9	5.6±0.7	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		26±10	18±7	22±8	24±9	36±12	20±7	29±9	11±5	34±12	31±3	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, $P < 0.05$)

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.*

	RT	Day 1				Day 10			
Name	(min)	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±0.65*a***	**0.22±0.15*b***	**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±0.6*b***	**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±0.16*b***	1.0	1.8	**1.7±1.2*a***
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
	RT	Day 30				Day 90			
Name	(min)	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*)