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Article

The Differential Effects of the Blue-Stain Fungus *Leptographium qinlingensis* on Monoterpenes and Sesquiterpenes in the Stem of Chinese White Pine (*Pinus armandi*) Saplings

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Abstract: When conifers such as Chinese white pine (*Pinus armandi* Fr.) are attacked by insects or pathogens, they respond by increasing their content of monoterpenes and sesquiterpenes. In this study, we determined the effects of the blue-stain fungus *Leptographium qinlingensis* Tang and Chen on monoterpenes and sesquiterpenes in the phloem and xylem of the stem of *P. armandi* saplings. We found that the total monoterpene concentrations in the phloem and xylem of the stem and the total sesquiterpene concentrations in the xylem of the stem were significantly higher in *L. qinlingensis*-inoculated saplings than in control (mechanically wounded) saplings or untreated saplings. Additionally, the proportions of β-pinene in the xylem of the stem and limonene + β-phellandrene in the phloem and xylem of the stem were significantly higher in *L. qinlingensis*-inoculated saplings than in both control and untreated saplings. The proportions of individual sesquiterpenes in the phloem and xylem of the stem were significantly greater in *L. qinlingensis*-inoculated saplings than in untreated saplings. Based on the results of this study, we suggest that increases in total monoterpene and sesquiterpene concentrations, as well as increases in the concentrations of β-pinene and limonene + β-phellandrene, may play an important defensive role against blue-stain fungus *L. qinlingensis* inoculation.

Keywords: *Pinus armandi*; *Leptographium qinlingensis*; monoterpenes; sesquiterpenes

1. Introduction

When conifers are attacked by pests and pathogens, they develop physical and chemical defenses to protect their tissues [1,2]. The major chemical defenses of conifers against insects and pathogens involve terpenoid-based oleoresins and phenolics [3]. Terpenoids are a family of complex carbon compounds and include monoterpenoids (C10), sesquiterpenoids (C15), and diterpenoids (C20) [4–7]. The quantitative and qualitative terpenoid composition of the oleoresin and volatile constituents can significantly change the response of the conifers to insect and fungal attack [6–9]. Previous reports have described model systems in conifers that are well suited for investigating the anatomical, chemical, biochemical, molecular and genomic characteristics of conifer terpenoid defenses [10–13].

The Chinese white pine (*Pinus armandi* Fr.) is a coniferous species that is native to China. It is found in the Qinling and Bashan Mountains [14]. *Pinus armandi* plays an important ecological role by protecting the soil and resisting erosion and is an important element of regional socioeconomic development [15,16]. Since 1954, however, the blue-stain fungus *Leptographium qinlingensis* Tang and Chen, a symbiotic fungus carried by the Chinese white pine beetle (*Dendroctonus armandi* Tsai and Li), has become a severe threat to *P. armandi* in the Qinling Mountains. The fungus is particularly deadly to *P. armandi* when the tree reaches the age of 30 years [15–17]. Three toxins that are biosynthesised by *L. qinlingensis*, 6-methoxymethyleugenin, maculosin and cerevisterol, are phytotoxic to *P. armandi* seedlings [18].

Previous reports have described increases in terpenoids in conifer species, which serve as a means of increasing resistance to the blue-stain fungus [12,19–25]. For example, increases in monoterpenes in *Pinus resinosa* (Ait.) and *P. banksiana* (Lamb.) occur as defences against the blue-stain fungi *Ophiostoma ips* (Rumbold Nannf.) and *O. nigrocarpa* (R.W. Davidson) de Hoog [19]. Additionally, increases in terpenes in *Picea abies* (Karst.) have been found to promote the resistance of this species to the blue-stain fungus *Ceratocystis polonica* (Siem.) [12,20,21]. In addition, increasing monoterpenes in *Pinus sylvestris* (L.) facilitate resistance against the blue-stain fungi *Tomicus piniperda* (L.) and *T. minor* (Hart.) [22]; Moreover, increases in monoterpenes in *Pinus contorta* (Dougl. Ex Loud) and *P. banksiana* (Lamb.) facilitate resistance against the blue-stain fungi *Leptographium longiclavatum* sp. nov., *Grosmannia clavigera* (Robinsin-Jeffery and Davidson) Zipfel, de Beer and *Ophiostoma montium* (Rumbold) von Arx [23–25].

Conifers have evolved sophisticated constitutive and inducible defence mechanisms that reside in both bark and sapwood. Constitutive defences include resin-filled ducts in both bark and sapwood [26,27]. Fungi that grow more readily in the phloem stimulate primarily phloem defences, and those that grow more readily in the sapwood stimulate primarily sapwood defences [1]. Accordingly, we investigated the constitutive and induced chemical responses of *P. armandi* saplings against *L. qinlingensis* and mechanical wounding. The main objective of this study is to study changes in volatile compounds from a comparative perspective and to quantify the time sequence followed by monoterpenes and sesquiterpenes in the phloem and xylem in *P. armandi* saplings following blue-stain fungus

(*L. qinlingensis*) inoculation and mechanical wounding. The study will serve to contribute to the understanding of the ability of this host's defence system to overcome inoculation with this fungus as well to the understanding of the different roles of the phloem and xylem in resisting fungal growth.

2. Materials and Methods

2.1. Plant Material

P. armandi saplings were raised in a nursery at the College of Forestry, Northwest A & F University, P. R. China for 2 years under natural light and environmental conditions. The saplings were grown until they reached a height of 40–50 cm. The seeds from which the saplings were grown were collected from the Hoaditang Experimental Forest Farm, Qingling Forest Ecological Station, southern slope of the Qinling Mountains in middle Ningshan county, Shaanxi province, China (E 108°24′–108°29′, N 33°18′–33°28′). Upon reaching the desired height, the saplings were moved to a greenhouse which provided constant conditions of 25 °C of day/night temperature, 50% of humidity and 8 h of photoperiod. The saplings were watered daily and fertilised weekly up to 8 weeks. After the application of the experimental treatments, fertilisation was stopped to exclude any nutrient–defence interactions [28].

2.2. Culture of L. qinlingensis

The blue-stain fungus *L. qinlingensis* was isolated from *P. armandi* sapwood phloem that had been attacked by the China white pine beetle (*D. armandi*) in the Qinling Mountains, China. The fungus was inoculated aseptically onto 25 mL plates, with each plate containing 25 mL·H_2O with malt extract powder (2%) and agar powder (1.5%). The plate cultures were incubated at 24 °C for 5 days in a Mould incubator of MXJ-250B-Z (WanTong Precision Instrument Co., Ltd., Wuhan, China) before use [15,18].

2.3. L. qinlingensis Treatments

L. qinlingensis inoculation into *P. armandi* stems was modified from the methods in Vincent *et al.* (2002) [29] and Erbilgin and Colgan (2012) [25]. Ten saplings were inoculated twice and the point of inoculation was alternate to each other. Holes were drilled with a 1 cm cork borer at 4 cm above the soil line. A 5 mm plug of *L. qinlingensis* on colonised malt extract agar was placed in each hole. As a control for the inoculation effect, five saplings were mechanically wounded and another five normal saplings without any treatment were used. The mechanically wounded seedlings were prepared using the same method as fungal inoculation except that the holes were left empty. All holes that were drilled for fungal inoculation and mechanical wounding were wrapped with cling wrap and sealed with duct tape to avoid contamination. The experiment was replicated three times with new sapling and *L. qinlingensis* for each replicate.

2.4. Tissue Harvesting

Stem of saplings were harvested at 4 days, 8 days and 30 days intervals to extract monoterpenes and sesquiterpenes. After 30 days, some inoculated saplings showed the symptoms of wilt phenomenon. Therefore, harvesting was done only up to 30 days after treatments. Fungi treated six saplings and

three mechanically wounded, untreated saplings were harvested in each time point. To see the success of inoculation, lesions of a treated stem sample was flame sterilized and placed on malt extract agar and three successful inoculated saplings were selected.

Tissue harvesting was performed according to the method of Miller *et al.* (2005) [8]. To harvest stem tissues, saplings were cut above the sites of *L. qinlingensis* inoculation or mechanical wounding and below the branches of the crown. In addition, for comparative purposes, defoliated and untreated saplings were cut below the branches of the crown. The phloem and xylem in the *P. armandi* saplings were cut longitudinally with a razor blade and stored at −80 °C.

2.5. Analysis of Monoterpene and Sesquiterpenes

The extraction of monoterpenes and sesquiterpenes was performed using methods modified from those in Erbilgin and Colgan (2012) [25]. All tissues were ground in liquid nitrogen using a mortar and pestle. One hundred milligrams of ground tissues were added to 500 microlitres of dichloromethane (CH_2Cl_2) (Sigma-Aldrich, St. Louis, MO, USA) with 0.1% tridecane (Sigma-Aldrich) as a surrogate standard in a 1.5 mL microcentrifuge tube. The above mixture was vortexed for 30 s, placed in an ultrasonic bath for 15 min, and then centrifuged at 0 °C and 14,000 rpm for 15 min. The extract was pipetted into a gas chromatography vial and stored at −80 °C. The mixture was then extracted for a second time using the same method. The second extract was added to the first and stored at −80°C prior to chemical analysis.

Extracts (1 μL) were analysed using a GCMS-QP2010 Ultra device (Shimadzu Corporation, Kyoto, Japan) equipped with an RXI-5ms column (0.25 μm × 0.25 μm × 30 m), helium carrier gas flow at 1 $mL \cdot min^{-1}$, and a split ratio of 10:1 (for both the phloem and xylem). The temperature was set at 50 °C for 2 min, was then increased to 160 °C by 5 $°C \cdot min^{-1}$ and was then further increased to 250 °C by 15 $°C \cdot min^{-1}$ (15 min hold).

The identification of monoterpenes and sesquiterpenes was based on a comparison of retention times and mass spectra with authentic standards or with mass spectra in the Wiley or National Institute of Standards and Technology libraries, NIST08 mass spectral database library, http://webbook.nist.gov/chemistry/. Previous studies by Martin *et al.* (2002) [6], Zeneli *et al.* (2006) [12] and Arrabal *et al.* (2012) [30] have described monoterpenes and sesquiterpenes in conifers. Terpene concentrations were calculated by integration of peak area and normalization against peak areas of Tridecane standards. Mean terpenoid concentration in $mg \times g^{-1}$ dry weight and standard errors were calculated.

2.6. Statistical Analysis

Total monoterpene and sesquiterpene concentrations and their interactions in the phloem and xylem were tested using General Linear Model (GLM) with Tukey's test in SPSS 18.0 (IBM SPSS Statistics, Chicago, IL, USA). When the interaction was not significant, a one-way analysis of variance (ANOVA) has been used to examine the differences between the treated and the untreated saplings. Total monoterpene and sesquiterpene concentrations in the phloem and xylem were transformed for analysis. Total monoterpene and sesquiterpene concentrations in the phloem and xylem after treatment were tested using ANOVA to examine the differences between time points in individual treatments.

The monoterpene and sesquiterpene composition of all tissues was analysed by examining individual monoterpenes and sesquiterpenes as a proportion of total concentration. Individual monoterpenes (α-pinene, β-myrcene, β-pinene, 3-carene, bornyl acetate, camphene, and limonene + β-phellandrene) and sesquiterpenes (α-copaene, α-cubebene, α-humulene, α-muurolene, β-caryophyllene, δ-cadinene, germacrene, germacrene D-4-ol, and longifolene) were tested, as they represented more than 90% of the total monoterpene and sesquiterpene concentrations in the phloem and xylem (Supplementary Material and Data Deposit). We conducted GLM of the individual monoterpenes and sesquiterpenes and their interaction in the phloem and xylem to examine the differences among treatments at each time point. When the interaction was not significant, a one-way analysis of variance (ANOVA) for individual monoterpenes and sesquiterpenes were used. The individual monoterpenes and sesquiterpenes in the phloem and xylem were analyzed without transformations of the data. ANOVA was carried out to calculate the differences of monoterpene and sesquiterpene among the time points in the individual treatments.

Finally, we also examined the proportional change in monoterpenes and sesquiterpenes in the phloem and xylem relative to the corresponding in untreated saplings (Δ = [induced − untreated]/untreated). *T*-tests were used to compare the differences between the phloem and xylem for monoterpenes and sesquiterpenes in the same treated saplings at all time points after treatment. Individual monoterpenes (α-pinene, β-pinene, bornyl acetate, and limonene + β-phellandrene) and sesquiterpenes (α-muurolene, δ-cadinene, germacrene, and longifolene) were assessed in terms of the proportional difference in concentrations from untreated saplings.

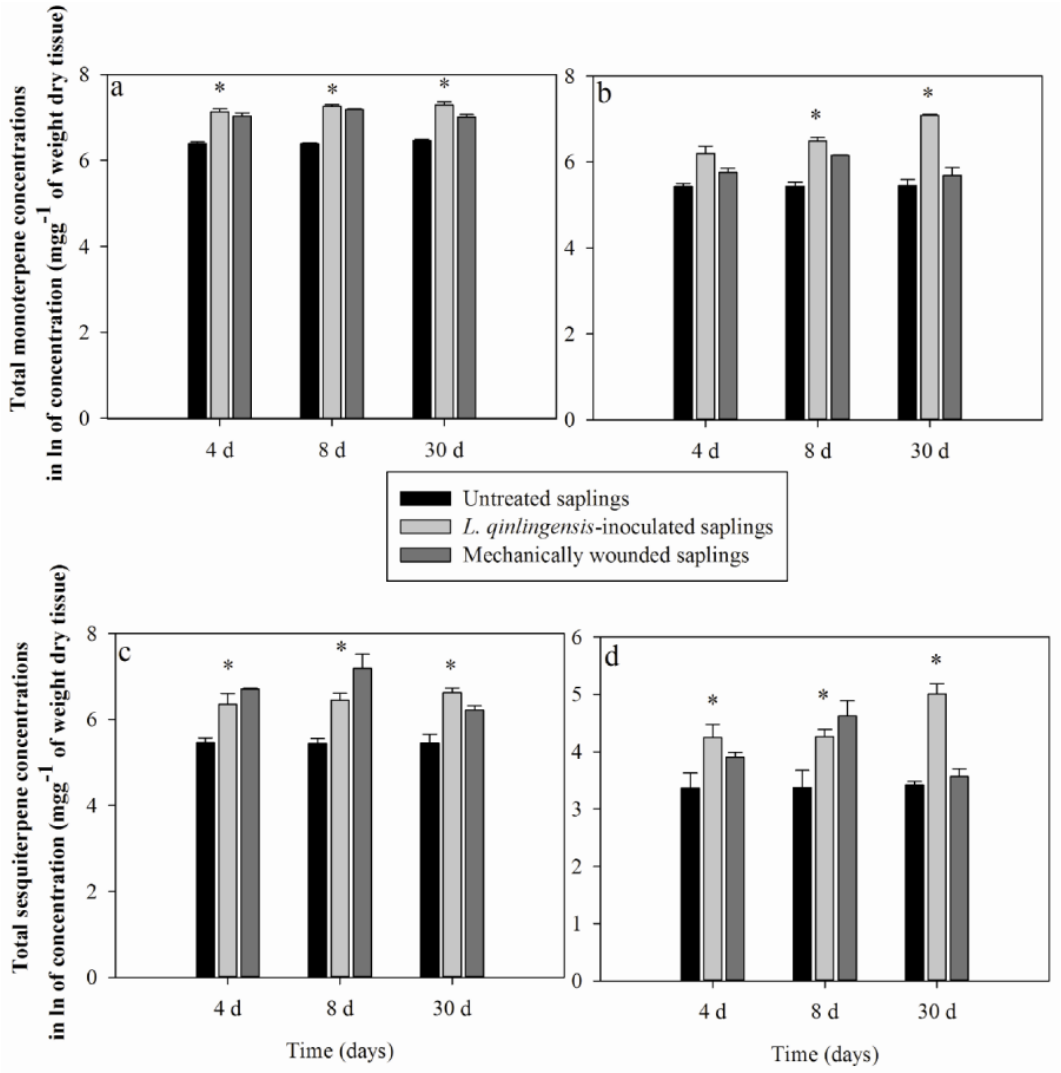
All statistics were calculated with SPSS 18.0. All plots were made with SigmaPlot 12.0 (Systat Software Inc, San Jose, CA, USA).

3. Results

3.1. Effect of L. qinlingensis and Mechanical Wounding on Monoterpenes in the Xylem of the Stem of P. armandi Saplings

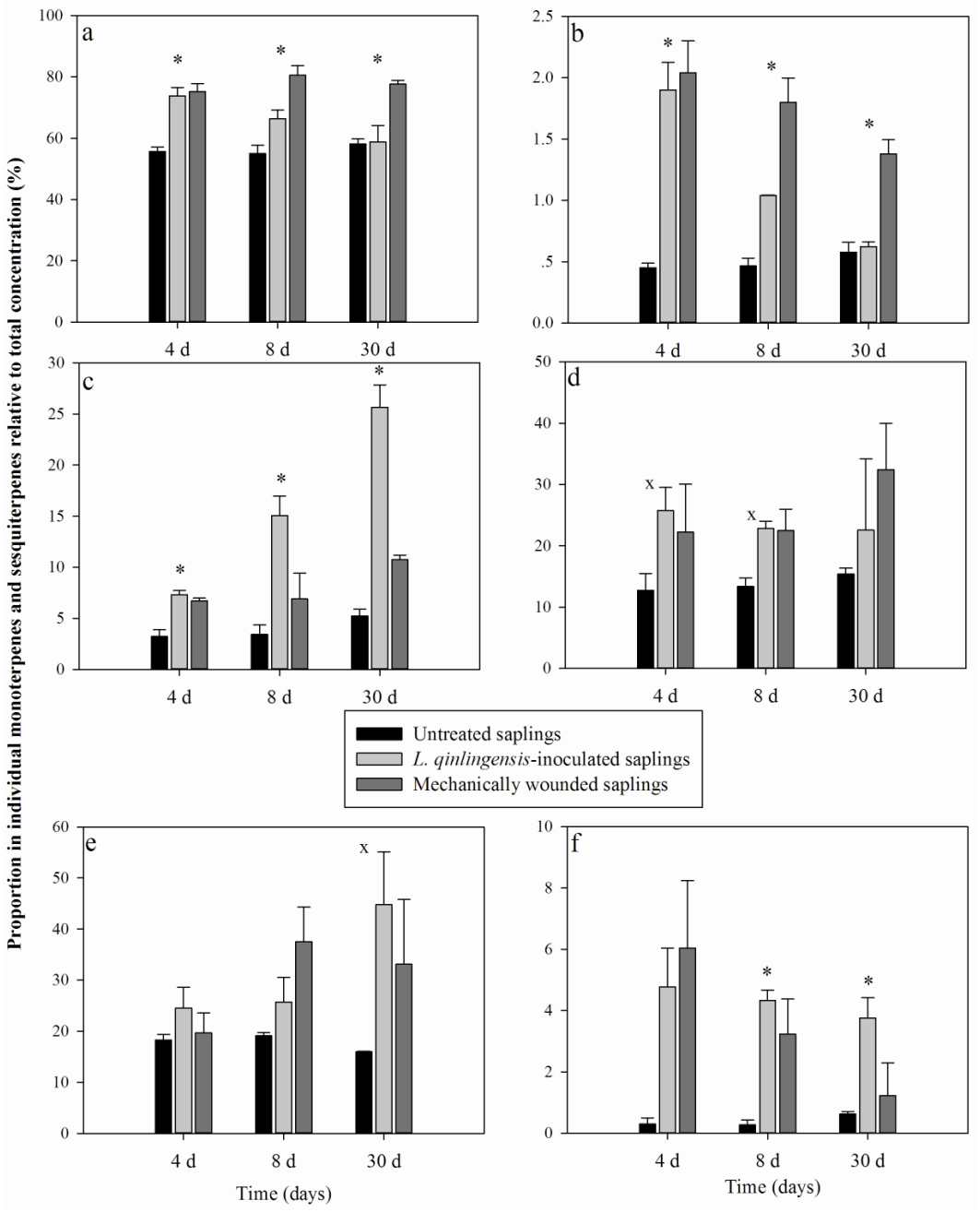
The total monoterpene concentrations in the xylem differed significantly among treatments at 8 days ($F = 64.484$, $df = 2$, $p = 0.001$) and 30 days ($F = 37.829$, $df = 2$, $p = 0.003$) after treatments (Figure 1b). A Tukey's test showed that the total monoterpene concentrations in the xylem were significantly higher only in *L. qinlingensis*-inoculated saplings than in control saplings or untreated saplings at both 8 days and 30 days after treatment (Table 1). Comparisons of total monoterpene concentrations among time points after treatment in individual treatments showed that the total monoterpene concentrations in the xylem differed significantly among time points after treatment in *L. qinlingensis*-inoculated saplings ($F = 9.98$, $df = 2$, $p = 0.018$), whereas the corresponding total concentrations did not differ significantly among time points after treatment in control and untreated saplings. A Tukey's test showed that the highest monoterpene concentrations in *L. qinlingensis*-inoculated saplings occurred at 30 days after treatment (Table 1).

Figure 1. The total monoterpene and sesquiterpene concentrations (μg·g^{-1} of dry weight tissue) in the phloem and xylem of the stem of *P. armandi* saplings differ among treatments at each time point. Date were transformed (ln) for analysis; **a**, **c** = phloem; **b**, **d** = xylem; * indicates that there was a significant difference ($p < 0.05$) among treatments using ANOVA at each time point. Each bar represents the mean total ± standard error of three saplings.



The proportions of α-pinene, β-pinene, β-myrcene, 3-carene, bornyl acetate, camphene and limonene + β-phellandrene relative total concentrations in the xylem were analysed to know the difference among treatment at time points after treatments (Supplementary Data Table S1). The results showed that the proportions of α-pinene, bornyl acetate and limonene + β-phellandrene were significantly different among treatments at all time points after treatments (Figure 2a–c). A Tukey's test indicated that the proportion of limonene + β-phellandrene was significantly higher in *L. qinlingensis*-inoculated saplings than in both control and untreated saplings at both 8 days and 30 days after treatment (Table 2). In contrast, the proportion of α-pinene and bornyl acetate was significantly higher in control saplings than in *L. qinlingensis*-inoculated or untreated saplings at both 8 days and 30 days after treatment (Table 2). For other individual monoterpenes, there was no difference among treatments or between treated and untreated saplings at any time point ($p > 0.05$, results not shown).

Figure 2. The proportional individual monoterpene and sesquiterpene concentrations (%) in the xylem of the stem of *P. armandi* saplings relative to total concentrations differ among treatments at each time point. **a** = α-pinene; **b** = bornyl acetate; **c** = limonene + β-phellandrene; **d** = longifolene; **e** = germacrene; **f** = δ-cadinene; * indicates that there was a significant difference ($p < 0.05$) between treatments using ANOVA at each time point and x indicates that there was a significant difference ($p < 0.05$) among *L. qinlingensis*-inoculated saplings and untreated saplings using ANOVA at each time point; Each bar represents the mean total ± standard error of three saplings.



In addition, the proportion of bornyl acetate and α-pinene in the xylem differed significantly among time points after treatment in *L. qinlingensis*-inoculated saplings. A Tukey's test indicated that the highest proportions of limonene + β-phellandrene in *L. qinlingensis*-inoculated occurred at 30 days after

treatment, whereas the proportion of bornyl acetate in *L. qinlingensis*-inoculated was highest at 4 days after treatments (Table 2).

3.2. Effect of L. qinlingensis and Mechanical Wounding on Monoterpenes in Phloem of Stem in P. armandi Saplings

In the phloem, total monoterpene concentrations differed significantly among treatments at 4 days ($F = 18.668$, $df = 2$, $p = 0.003$), 8 days ($F = 344.992$, $df = 2$, $p < 0.001$) and 30 days ($F = 51.178$, $df = 2$, $p = 0.001$) after treatment (Figure 1a). A Tukey's test indicated that the total monoterpene concentrations in the phloem were significantly higher only in *L. qinlingensis*-inoculated saplings compared to the control saplings or untreated saplings at 30 days after treatment. In addition, the total monoterpene concentrations in the phloem were not significantly different among time points after any treatment (Table 1).

Table 1. Tukey's tests showed significant differences results of comparisons of total monoterpenes and sesquiterpenes in the phloem and xylem of the stem among treatments at each time point and among time points in individual treatments.

Time Points (days)	Tissue/Terpenoids/Treatments											
	Phloem						Xylem					
	Total Monoterpenes			Total Sesquiterpenes			Total Monoterpenes			Total Sesquiterpenes		
	UT	LG	MC	UT	LG	MC	UT	LG	MC	UT	LG	MC
4	bA	aA	aA	bA	aA	aAB	aA	aB	aA	bA	aA	abAB
8	bA	aA	aA	bA	aA	aA	cA	aB	bA	bA	abA	aA
30	cA	aA	bA	bA	aA	aB	bA	aA	bA	cA	aA	bcB

UT = Untreated saplings, LG = *L. qinlingensis*-inoculated saplings, MC = Mechanically wounded saplings. Lower-case letters in a row show results of significant differences between treatments and capital letters in a column show results of significant differences between the time points. The same letters within each column (capital letters) and each row (lower-case letters) indicates no statistically significant differences.

The proportions of α-pinene, β-myrcene, β-pinene, bornyl acetate, camphene and limonene + β-phellandrene relative to concentrations in the phloem were analysed to ascertain the difference among treatments at different time points after treatments (Supplementary Data Table S2). This result showed that the proportions of α-pinene, β-pinene and limonene + β-phellandrene were significantly different among treatments at various time points (Figure 3a–c). For other individual monoterpenes, there were no differences among treatments or between treated and untreated saplings at any time point ($p > 0.05$, results not shown). The proportions of β-pinene were significantly higher in *L. qinlingensis*-inoculated saplings than in both control and untreated saplings at 30 days after treatment, whereas the proportions of limonene + β-phellandrene was significantly higher only in *L. qinlingensis*-inoculated saplings compared to the untreated saplings (Table 3). In contrast, the proportion of α-pinene was significantly higher in control saplings than in *L. qinlingensis*-inoculated or untreated saplings at both 8 days and 30 days after treatment (Table 3). Comparisons of these compounds among time points after treatment in individual treatments showed that both the proportions of α-pinene and β-pinene were higher at 30 days after treatment in *L. qinlingensis*-inoculated saplings, whereas the

corresponding proportions of α-pinene and β-pinene were highest at 8 days after treatment in control saplings (Table 3).

Figure 3. The proportional individual monoterpene and sesquiterpene concentrations (%) in the phloem of the stem of *P. armandi* saplings relative to total concentrations differ among treatments at each time point. **a** = α-pinene; **b** = β-pinene; **c** = limonene + β-phellandrene; **d** = longifolene; **e** = α-muurolene; **f** = δ-cadinene; * indicates that there was a significant difference ($p < 0.05$) between treatments using ANOVA at each time point and x indicates that there was a significant difference ($p < 0.05$) among *L. qinlingensis*-inoculated saplings and untreated saplings using ANOVA at each time point; Each bar represents the mean total ± standard error of three saplings.

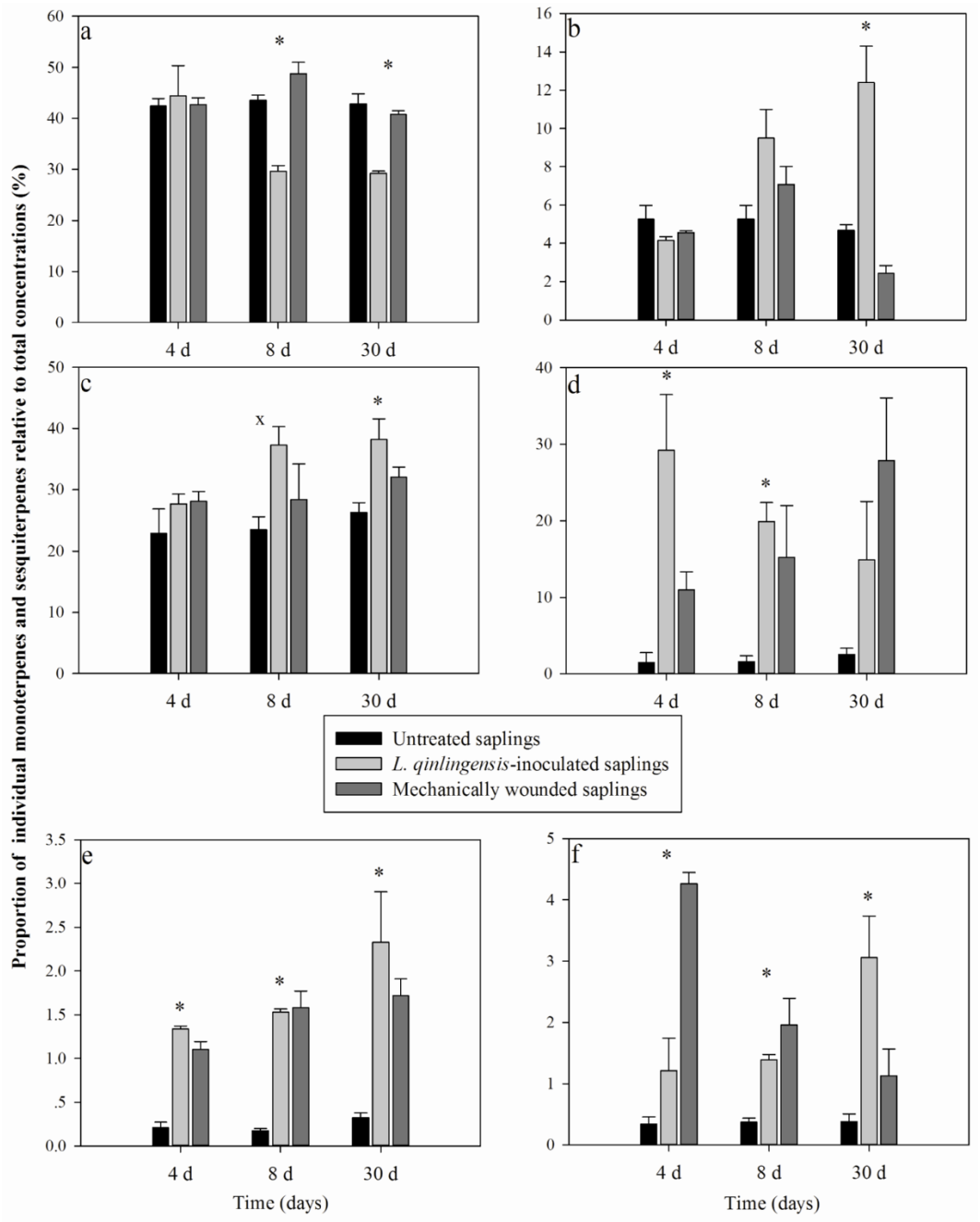


Table 2. Tukey's tests showed significant differences among comparisons of individual monoterpenes and sesquiterpenes in the xylem of the stem among treatments at each time point and across different time points in individual treatments.

Time Points (days)	Terpenoids/Treatments											
	α-Pinene			Bornyl Acetate			limonene + β-Phellandrene			δ-Cadinene		
	UT	LG	MC	UT	LG	MC	UT	LG	MC	UT	LG	MC
4	bA	aA	aA	bA	aA	aA	bA	aC	aA	aA	aA	aA
8	cA	bcA	aA	bA	bAB	aA	cA	aBC	bcA	bA	aA	abA
30	bA	bA	aA	bA	bB	aA	cA	aA	bcA	bA	aA	abA

UT = Untreated saplings, LG = *L. qinlingensis*-inoculated saplings, MC = Mechanically wounded saplings. Lower-case letters in a row show results of significant differences between treatments and capital letters in a column show results of significant differences between the time points. The same letters within each column (capital letters) and each row (lower-case letters) indicates no statistically significant differences.

3.3. Differential Effects of L. qinlingensis and Mechanical Wounding on Monoterpenes between Phloem and Xylem of Stem in P. armandi Saplings

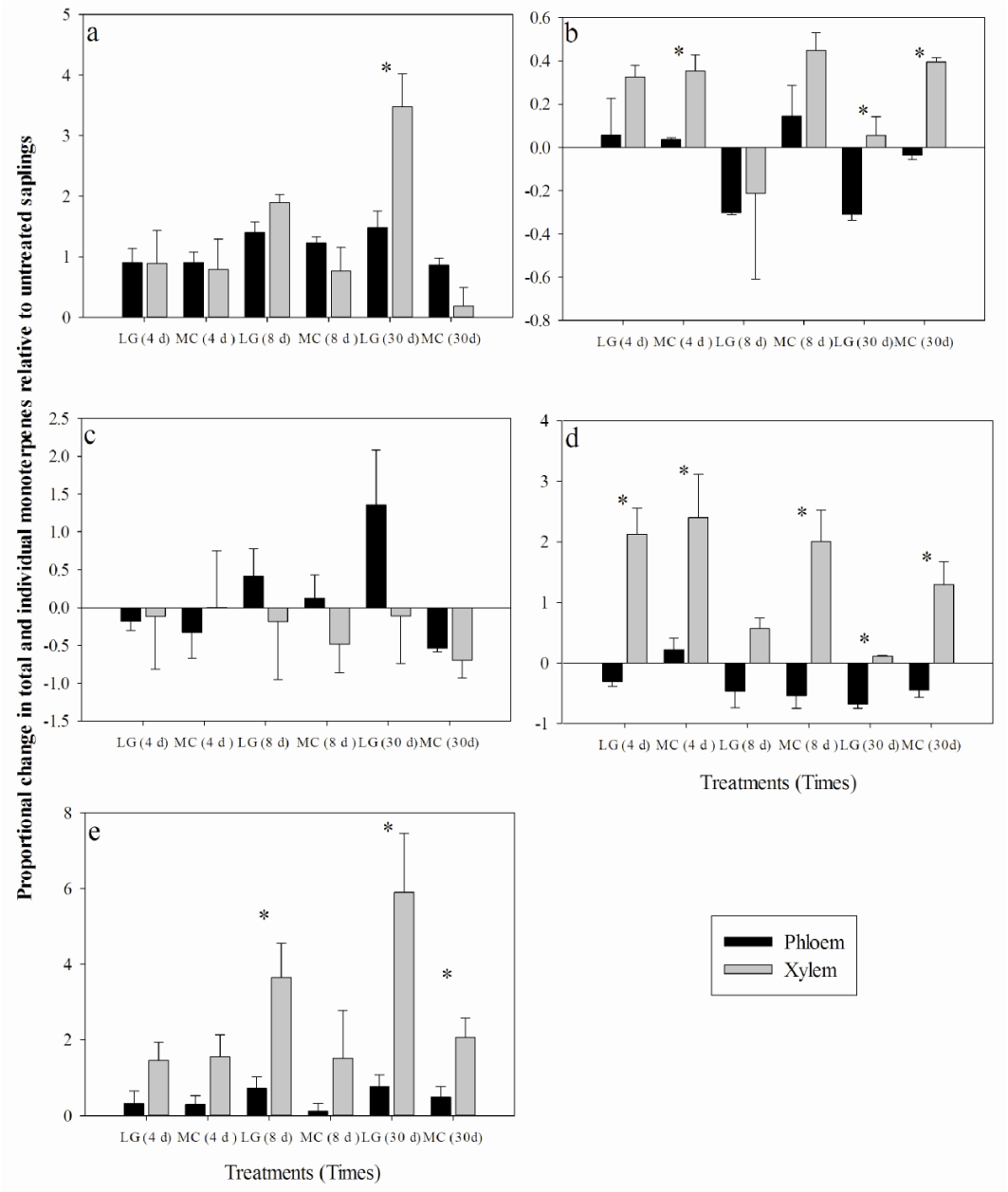
An examination of the proportional change of the total monoterpene concentrations in the same treated saplings relative to the corresponding total monoterpene concentrations in untreated saplings showed that *L. qinlingensis*-inoculated saplings showed significant differences between the xylem and phloem at 30 days ($t = -3.290$, $p = 0.030$) after treatment, with a greater increase in the xylem than in the phloem (Figure 4a). The proportional change in control saplings did not differ significantly between the phloem and xylem at any time point after treatment (Figure 4a). The proportional change in individual monoterpenes (α-pinene, β-pinene, bornyl acetate, limonene + β-phellandrene) relative to corresponding individual monoterpenes in untreated saplings was also examined. The proportional change in limonene + β-phellandrene differed significantly between the xylem and phloem at 8 days ($t = -3.763$, $p = 0.030$) and 30 days ($t = -3.231$, $p = 0.030$) after treatment for *L. qinlingensis*-inoculated saplings and at 30 days ($t = -2.705$, $p = 0.050$) for control saplings, with a greater increase in the xylem than in the phloem (Figure 4e). The proportional changes in α-pinene and bornyl acetate in *L. qinlingensis*-inoculated saplings showed significant differences between the xylem and phloem at 30 days ($t = -3.970$, $p = 0.017$) after treatment for α-pinene and at both 4 days ($t = -5.515$, $p = 0.005$) and 30 days ($t = -0.957$, $p = 0.003$) after treatment for bornyl acetate). These compounds increased in the xylem compared with control saplings but decreased in the phloem (Figure 4b,d). In the control saplings, significant differences were found between the xylem and phloem at both 4 days ($t = -3.261$, $p = 0.047$) and 30 days ($t = -14.787$, $p < 0.003$) after treatment for α-pinene and at all time points for bornyl acetate. These compounds increased more only in the xylem than in the phloem at 4 days after treatment (Figure 4b,d). The proportional changes in β-pinene in *L. qinlingensis*-inoculated and control saplings did not differ significantly between the phloem and xylem after treatment (Figure 4c).

Table 3. Tukey's tests showed significant differences across comparisons of individual monoterpenes and sesquiterpenes in the phloem of the stem among treatments at each time point and at different time points in individual treatments.

Time Points (days)	Terpenoids/Treatments																	
	α-Pinene			β-Pinene			limonene + β-Phellandrene			α-Muurolene			δ-Cadinene			Longifolene		
	UT	LG	MC	UT	LG	MC	UT	LG	MC	UT	LG	MC	UT	LG	MC	UT	LG	MC
4	aA	aB	aB	aA	aB	aAB	aA	aA	aA	bA	aA	aA	cA	bcA	aA	bA	aA	abA
8	aA	bB	aA	aA	aAB	aA	aA	aA	aA	bA	aA	aA	bA	abA	aB	bA	aA	abA
30	aA	bA	aB	bA	aA	bB	bA	aA	abA	abA	aA	aA	bA	aA	abB	aA	aA	aA

UT = Untreated saplings, LG = *L. qinlingensis*-inoculated saplings, MC = Mechanically wounded saplings. Lower-case letters in a row show results of significant differences between treatments and capital letters in a column show results of significant differences between the time points. The same letters within each column (capital letters) and each row (lower-case letters) indicates no statistically significant differences.

Figure 4. The change in total and individual monoterpenes between the phloem and xylem of the stem in treated saplings relative to the corresponding total and individual monoterpenes in untreated saplings at all time points after treatment. LG = *L. qinlingensis*-inoculated saplings; MC = Mechanically wounded saplings; **a** = total monoterpene concentrations; **b** = α-pinene; **c** = β-pinene; **d** = bornyl acetate; **e** = limonene + β-phellandrene; * indicates that there was a significant difference ($p < 0.05$) between the phloem and xylem of the stem in the same treated saplings based on a Student *t*-test at the time points; Each bar represents the mean total ± standard error of three saplings.



3.4. Effect of L. qinlingensis and Mechanically Wounding on Sesquiterpenes in the Xylem of the Stem in P. armandi Saplings

The total sesquiterpene concentrations in the xylem differed significantly among treatments at 4 days ($F = 6.969$, $df = 2$, $p = 0.027$), 8 days ($F = 11.981$, $df = 2$, $p = 0.020$) and 30 days ($F = 33.309$, $df = 2$, $p = 0.001$) after treatment (Figure 1d). The total sesquiterpene concentrations were significantly higher only in *L. qinlingensis*-inoculated saplings than in both control and untreated saplings at 30 days after treatment (Table 1). Comparisons of the total sesquiterpene concentrations among time points in individual treatment showed that the total sesquiterpene concentrations significantly differed only among the time points in control saplings ($F = 11.981$, $df = 2$, $p = 0.020$). The highest total sesquiterpene concentration in control saplings was recorded at 8 days after treatments (Table 1).

The proportions of α-copaene, α-cubebene, α-humulene, α-muurolene, β-caryophyllene, δ-cadinene, germacrene, germacrene D-4-ol, and longifolene relative total concentrations in the xylem were analysed in different treatments at different time points after treatments (Supplementary Data Table S3). The results showed that the proportions of δ-cadinene were significantly differed among treatments at 8 days ($F = 6.932$, $df = 2$, $p = 0.036$) and 30 days ($F = 5.242$, $df = 2$, $p = 0.048$) after treatment (Figure 2f), whereas the proportions of germacrene and longifolene have not exhibited significant differences among treatments at any time point. However, comparisons of the proportion of germacrene and longifolene between the treated and untreated saplings at each time point showed that the proportions of longifolene was significantly higher in *L. qinlingensis*-inoculated than in untreated saplings at 4 days ($F = 7.743$, $df = 2$, $p = 0.05$) and 8 days ($F = 18.302$, $df = 2$, $p = 0.023$) after treatment (Figure 2e,d), whereas the proportions of germacrene was significantly higher only in *L. qinlingensis*-inoculated saplings compared to untreated saplings at 30 days ($F = 7.777$, $df = 2$, $p = 0.049$) (Figure 2e). For other individual sesquiterpenes, there were no significant differences among treatments or between treated and untreated saplings at any time point ($p > 0.05$, results not shown). A Tukey's test showed that the proportions of δ-cadinene was significantly higher in *L. qinlingensis*-inoculated saplings than in untreated saplings but did not differ significantly from the control saplings (Table 2).

3.5. Effect of L. qinlingensis and Mechanical Wounding on Sesquiterpenes in Phloem of Stem in P. armandi Saplings

The total sesquiterpene concentrations in the phloem differed significantly among treatments at all time points after treatment (4 days ($F = 35.488$, $df = 2$, $p = 0.001$), 8 days ($F = 12.922$, $df = 2$, $p = 0.011$), 30 days ($F = 33.309$, $df = 2$, $p = 0.025$)) (Figure 1c). The total sesquiterpene concentration was significantly higher in treated saplings compared to untreated saplings but did not differ between treated saplings (Table 1). Only the total monoterpene concentrations in the phloem differed significantly at different time points after treatment in control saplings (Table 1).

The proportions of α-copaene, α-humulene, α-muurolene, β-caryophyllene, δ-cadinene, germacrene, and longifolene relative to the phloem were analysed for difference among treatment at time points after treatments (Supplementary Data Table S4). The results showed that the proportions of α-muurolene, δ-cadinene, and longifolene differed significantly among treatments at various time points (Figure 3d–e). For other individual sesquiterpenes, there were no differences among treatments or

between treated and untreated saplings at any time point ($p > 0.05$, results not shown). The proportions of α-muurolene and only longifolene were significantly higher in *L. qinlingensis*-inoculated saplings than in untreated sapling at both 4 days and 8 days after treatment (Table 3), whereas the proportion of δ-cadinene was significantly higher in control saplings than in *L. qinlingensis*-inoculated or untreated saplings at both 4 days and 30 days after treatment (Table 3).

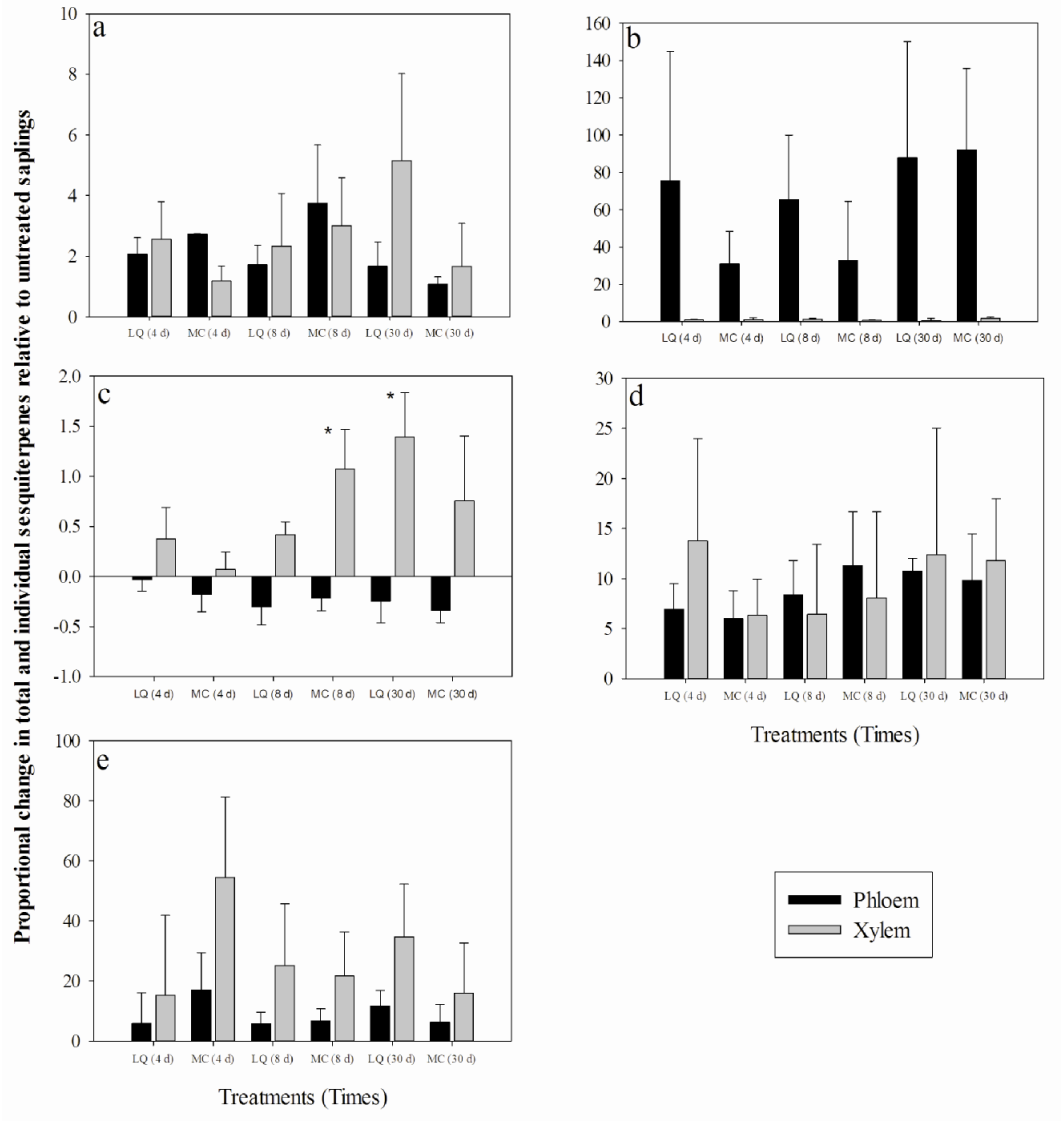
3.6. Differential Effects of L. qinlingensis and Mechanical Wounding on Sesquiterpenes between Phloem and Xylem of Stem in P. armandi Saplings

The proportional change in total sesquiterpene concentrations in the same treated sapling relative to the corresponding total sesquiterpene in untreated saplings at all time points after treatment did not differ significantly between the phloem and xylem in *L. qinlingensis*-inoculated or control saplings at any time after treatment (Figure 5a). In addition, the proportion of change in individual sesquiterpenes (α-muurolene, δ-cadinene, germacrene, and longifolene) relative to the corresponding time in untreated saplings was examined. The proportions of changes of α-muurolene and longifolene in *L. qinlingensis*-inoculated and control saplings did not differ significantly between the phloem and xylem at any time point after treatment. The proportions of change in germacrene differed significantly between the phloem and xylem at 30 days ($t = -3.335$, $p = 0.029$) after treatment for *L. qinlingensis*-inoculated saplings and at 8 days ($t = -1.291$, $p = 0.035$) for control saplings, with a greater increase in the xylem than in the phloem (Figure 5c).

4. Discussion

This is the first study in which changes in monoterpene and sesquiterpene chemical defence compounds have been compared in the phloem and xylem in *P. armandi* saplings after mechanical wounding and inoculation with the blue-stain fungus *L. qinlingensis*. In our experiment, total monoterpene concentrations in the phloem and xylem in *P. armandi* saplings after inoculation of the blue-stain fungus *L. qinlingensis* were significantly higher than in both control and untreated saplings. The results are in accordance with several previous reports in conifers [19–25,31], where total monoterpene concentrations increased significantly after the inoculation of blue-stain fungi. However, the comparison of the increases in total monoterpene concentrations found in this study with the results of previous studies may be influenced by differences in experimental design, such as differences in the treatment type, the age of the tree, and the sampling time points [21,31]. *P. abies* trees have been found to increase total sesquiterpene concentrations as a response to attack by the blue-stain fungus *C. polonica* [12]. However, in our experiment, the total sesquiterpene concentrations in the xylem after inoculation of the blue-stain fungus *L. qinlingensis* were only significantly higher in the treated *P. armandi* saplings than in the untreated or control saplings, and the total sesquiterpene concentrations in the phloem did not show a significant difference compared with the control saplings. Monoterpenes prevent the growth of pathogenic fungi [20,32,33]. Thus, a change in any of these compounds may be important for the defence of the tree against fungi. Thus, in this study, the increased total monoterpene and sesquiterpene concentrations represented a component of resistance to the inoculation of *L. qinlingensis*, suggesting that they may function in defence against this pathogen.

Figure 5. The change in total and individual sesquiterpenes between the phloem and xylem of the stem in the same treated saplings relative to the corresponding total and individual monoterpenes in the untreated saplings at all time points after treatment. LG = *L. qinlingensis*-inoculated saplings; MC = Mechanically wounded saplings; **a** = total sesquiterpene concentrations; **b** = longifolene; **c** = germacrene; **d** = α-muurolene; **e** = δ-cadinene; * indicates that there was a significant difference ($p < 0.05$) between the phloem and xylem of the stem in the same treated saplings based on a Student *t*-test at the various time points; Each bar represents the mean total ± standard error of three saplings.



The relative amounts of both β-pinene and limonene in conifers such as *P. sylvestris* and *P. abies* trees were found to increase after the inoculation of blue-stain fungi (*Leptographium wingfieldii* Morelet or *Ophiostoma canum* (Müch) Syd. & P. Syd. and *C. polonica*) compared with control trees, whereas the relative amounts of α-pinene and 3-carene were unchanged, decreasing, or otherwise altered [22,31]. Additionally, increased bornyl acetate concentrations in the resin of *Pinus contorta* trees were associated with Armillaria root disease (*Armillaria mellea* Karst.) [34]. In the present study, the proportion of β-pinene in the phloem was significantly higher in *L. qinlingensis*-inoculated saplings than in control

and untreated saplings, whereas the proportion of limonene + β-phellandrene in the phloem was significantly higher in *L. qinlingensis*-inoculated saplings than in untreated saplings but did not differ significantly from that found in control saplings. The proportion of limonene + β-phellandrene in the xylem was significantly higher in *L. qinlingensis*-inoculated saplings than in control and untreated saplings, but the proportions of α-pinene and bornyl acetate were significantly higher in control saplings than in *L. qinlingensis*-inoculated saplings. Moreover, monoterpenes (α-pinene, β-pinene, myrcene, terpinolene, sabinene, Δ-3-carene or limonene) have been shown to increase in response to fungal inoculation [20,35–37]. Limonene and β-pinene, produced in the defence response of *P. abies*, have been found to be the most potential inhibitors to the growth of the blue-stain fungus *Ceratocystis polonica* [21]. Similarly, our data suggest that β-pinene and limonene + β-phellandrene may play a critical role in inhibiting the spread of the blue-stain fungus *L. qinlingenesis*. Thus, research is required to understand the effects of these monoterpenes on the growth of the blue-stain fungus *L. qinlingensis*.

In addition, *Pinus massoniana* (Lamb.) contains the sesquiterpene α-muurolene, which repels the pinewood nematode, *Bursaphelenchus xylophilus* [38]. The sesquiterpenes (β-caryophyllene, α-muurolene, γ-muurolene, germacrene, bicyclogermacrene, δ-cadinene) in the phloem of *P. abies* trees were found to be significantly higher in fungus-inoculated trees than in unwounded and mechanically wounded trees [20]. In the current study, however, the proportions of individual sesquiterpenes (δ-cadinene, α-muurolene, longifolene, and germacrene) in the phloem and xylem were significantly greater in *L. qinlingensis*-inoculated saplings than in untreated saplings but did not differ significantly from the control saplings, whereas the proportion of δ-cadinene was significantly higher in the control saplings than in the *L. qinlingensis*-inoculated saplings. Thus, these sesquiterpenes may play a less obvious role in resistance to the inoculation of blue-stain fungus *L. qinlingensis.*

Subsequent to its development in the inner xylem of the host tree, *L. qinlingensis* overcomes the host's resistance, destroys the wood cells, blocks the resin ducts, disrupts the metabolic system and produces blue stains in the wood, resulting in the disruption of nutrient and water metabolism and the rapid death of the host tree [15,17]. In our study, total and individual monoterpene concentrations (α-pinene, β-myrcene, limonene + β-phellandrene) in *L. qinlingensis*-inoculated saplings increased significantly more in the xylem than in the phloem. Thus, we suggest that the xylem in *P. armandi* may show differential increases in monoterpenes to defend against the growth of the blue-stain fungus *L. qinlingensis* compared with the corresponding changes in monoterpenes in the phloem.

5. Conclusions

In this study, we performed experiments in which *P. armandi* saplings were inoculated with the blue-stain fungus *L. qinlingensis* to examine changes in total and individual monoterpenes and sesquiterpenes in the host sapling. We found that the total concentrations of monoterpenes in the phloem and xylem were significantly higher in *L. qinlingensis*-inoculated saplings than in control saplings or untreated at 30 days after treatment. Furthermore, the total monoterpene concentrations in the phloem and xylem *L. qinlingensis*-inoculated saplings were highest at 30 days after treatment. Moreover, the total concentration of sesquiterpenes in the xylem, but not in the phloem, was significantly higher in *L. qinlingensis*-inoculated saplings than in both control and untreated saplings at 30 days after treatment.

In addition, the proportions of limonene + β-phellandrene in the xylem and of β-pinene in the phloem were significantly higher in *L. qinlingensis*-inoculated saplings than in both control and untreated saplings at 30 days after treatment. In contrast, the proportions of α-pinene and bornyl acetate in the xylem were significantly higher in control saplings than in *L. qinlingensis*-inoculated or untreated saplings at 30 days after treatment. The proportions of δ-cadinene, α-muurolene, longifolene, and germacrene in the phloem and xylem increased significantly in *L. qinlingensis*-inoculated saplings compared with untreated saplings but did not differ significantly from the corresponding proportions in the control saplings.

Finally, the changes in the total and individual monoterpenes and sesquiterpenes between the phloem and xylem in the same treated saplings at the sampled time points, compared with the concentration in the untreated saplings, showed that the total monoterpene concentration in *L. qinlingensis*-inoculated saplings increased significantly more in the xylem than in the phloem at 30 days after treatment, whereas the total sesquiterpene concentration did not differ significantly between the phloem and xylem. In addition, the proportions of individual monoterpenes (α-pinene, bornyl acetate, and limonene + β-phellandrene) in *L. qinlingensis*-inoculated saplings increased significantly in the xylem relative to the phloem, whereas β-pinene differed significantly between the phloem and xylem. The proportions of germacrene in *L. qinlingensis*-inoculated saplings increased significantly in the xylem relative to the phloem at 30 days after treatment, whereas other individual sesquiterpenes did not differ significantly between the xylem and the phloem at any time point after treatment.

This research will improve the understanding of the changes in the defences of *P. armandi* saplings again the blue-stain fungus *L. qinlingensis* and provide a theoretical basis for and new ideas about the prevention and control of insect pests and pathogens in *P. armandi.*

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Author Contributions

Thanh Pham and Hui Chen conceived and designed the experiments; Thanh Pham and Lulu Dai performed the experiments; Thanh Pham and Jiamin Yu analyzed the data; Ranran Zhang and Thi Quynh Trang Vu contributed reagents/materials/analysis tools; Thanh Pham wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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