

Inducibility of chemical defences by two chewing insect herbivores in pine trees is specific to targeted plant tissue, particular herbivore and defensive trait

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However, few studies have attempted to jointly investigate whether the induction of plant defences is specific to a targeted plant tissue, plant species, herbivore identity, and defensive trait. Here we studied those factors contributing to the specificity of induced defensive responses in two economically important pine species against two chewing insect pest herbivores. Juvenile trees of *Pinus pinaster* and *P. radiata* were exposed to herbivory by two major pest threats, the large pine weevil *Hylobius abietis* (a bark-feeder) and the pine processionary caterpillar *Thaumetopoea pityocampa* (a folivore). We quantified in two tissues (stem and needles) the constitutive (control plants) and herbivore-induced concentrations of total polyphenolics, volatile and non-volatile resin, as well as the profile of mono- and sesquiterpenes. Stem chewing by the pine weevil increased concentrations of non-volatile resin, volatile monoterpenes, and (marginally) polyphenolics in stem tissues. Weevil feeding also increased the concentration of non-volatile resin and decreased polyphenolics in the needle tissues. Folivory by the caterpillar had no major effects on needle defensive chemistry, but a strong increase in the concentration of polyphenolics in the stem. Interestingly, we found similar patterns for all these above-reported effects in both pine species. These results offer convincing evidence that induced defences are highly specific and may vary depending on the targeted plant tissue, the insect herbivore causing the damage and the considered defensive compound.

1. Introduction

Because constitutive and induced plant defences are costly to produce and maintain, their concentration and distribution can vary considerably across plant tissues and within-plant parts differing in value, cost or risk of attack (Zangerl and Rutledge, 1996; Ohnmeiss and Baldwin, 2000). In particular, within-plant distribution of induced responses to herbivores may vary depending on the fitness value and the frequency of herbivore attack on each organ and/or tissue (Zangerl and Rutledge, 1996; Gutbrodt et al., 2011; Moreira et al., 2012). According to the Optimal Defence Theory, plants invest in high constitutive levels of defence and low inducibility for tissues that have high fitness value and are most fre-

quently attacked, and vice-versa (Zangerl and Rutledge, 1996; Ohnmeiss and Baldwin, 2000). On the other hand, there is also increasing evidence that plants responses to herbivores can be highly specific and rely on the recognition of the specific herbivore species causing damage (e.g. Mithöfer and Boland, 2008; Bingham and Agrawal, 2010; Halitschke et al., 2011; Karban, 2011; Bonaventure, 2012; Gutbrodt et al., 2012). Accordingly, these plant responses could differ among plant tissues or be restricted to particular tissues or plant parts in order to minimize costs of defence induction.

Over the past decade, it has become increasingly accepted that plant induced resistance to herbivores depends on plant and herbivore species-specific characteristics (e.g. Underwood, 1999; Agrawal, 2000; Mumm et al., 2004; Köpke et al., 2010; Halitschke et al., 2011; Carrillo-Gavilán et al., 2012). The biotic stimulus needed to elicit specific induced responses may include a direct recognition of the physical stimuli and specific molecular patterns of the

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enemies (denoted as herbivore-associated molecular patterns, Mithöfer and Boland, 2008). Moreover, this biotic stimulus may also include indirect clues such as the recognition of specific combinations of biogenic volatile compounds (reviewed by Kessler and Baldwin, 2002), and the independent and interactive effects of those exogenous triggering factors with damage-self recognition clues (damage-associated molecular patterns) from their own plant tissues after being damaged by the herbivores (Heil, 2009; Erb et al., 2012; Heil et al., 2012). The suite of triggering factors

elicited directly or indirectly by herbivore feeding could be shared to some extent within taxonomical insect groups or within herbivore feeding guilds. Plant responses to herbivory have been repeatedly shown, however, to vary depending on the insect diet breadth and insect feeding guild. It is well known, for example, that generalist and specialist herbivores can elicit different plant defensive responses (reviewed by Ali and Agrawal, 2012). On the other hand, herbivores from different feeding guilds vary in their salivary constituents, timing, intensity and pattern of damage, and may thus

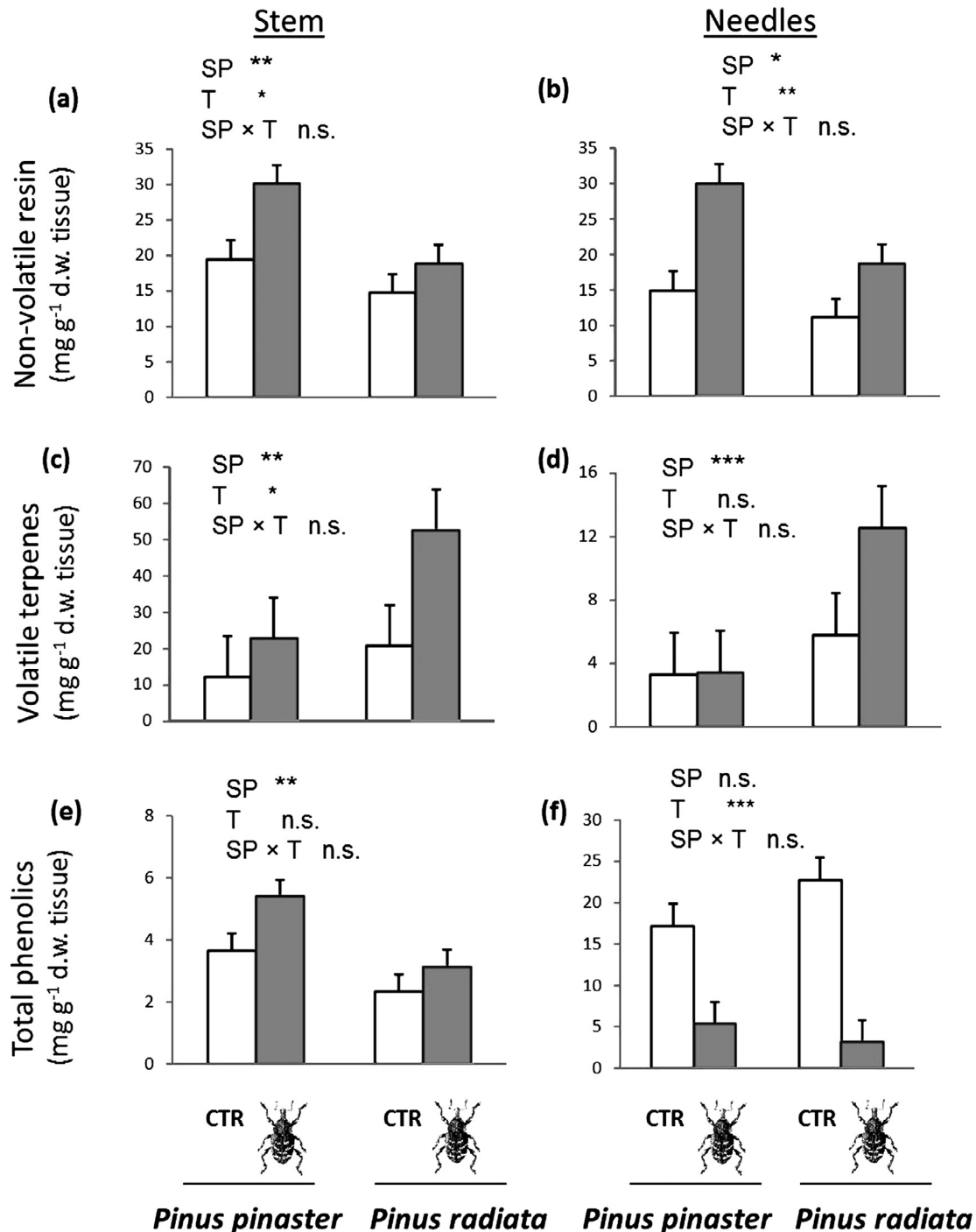


Fig. 1. Effects of the herbivory-induction by the large pine weevil *Hylobius abietis* (grey bars for the herbivore-treatment and white bars for the control) on the concentration of (a) non-volatile resin, (c) volatile terpenes and (e) total phenolics in the stem tissues; and (b) non-volatile resin, (d) volatile terpenes and (f) total phenolics in the needles of two pine species. Data are shown as LS means \pm s.e.m. $N = 10$. Asterisks indicate significant differences (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$) among pine species (SP), herbivore-induction treatments (T) and their interaction (SP \times T). n.s. = non-significant differences. F and P -values are shown in the Table 1.

provoke largely different plant induced reactions (Agrawal, 2000; Mithöfer and Boland, 2008). Moreover, there is increasing evidence that depending on their feeding behaviour some insect herbivores could actively suppress or disrupt effective immune plant responses (Musser et al., 2002; Bede et al., 2006; Zarate et al., 2007; Conales et al., 2012). This strategy is likely more widely spread than previously thought, and differences in the ability for disruptive damage signalling between closely related insect species or host plants may exist (e.g. Sarmiento et al., 2011; Verhage et al., 2011). Although there are a number of studies investigating the particular defensive responses in a diverse array of plant species (mostly non-woody model plants) and insect herbivores (see Mithöfer and Boland, 2008; Agrawal and Heil, 2012 and references therein), few have jointly tested whether the inducibility of plant chemical defences is specific to targeted plant tissues, particular herbivores, plant species, and defensive traits.

In this study, we used two economically important pine species to evaluate whether induced chemical responses elicited by two chewing insect herbivores are specific to a plant tissue, herbivore species, plant species, and/or vary depending on the defensive trait considered. To achieve these objectives, we conducted a greenhouse experiment with young pine trees that were exposed to herbivory by two chewing insects: a bark-feeder and a folivore (*Hylobius abietis* and *Thaumetopoea pityocampa*, respectively). After exposure, we analyzed the concentration of constitutive (control plants) and herbivore-induced chemical defences in two tissues with contrasting fitness value: stem and needles. We measured polyphenolics, and non-volatile and volatile resin as quantitative chemical defensive traits, and also analysed the profile of mono- and sesqui-terpenes in each plant tissue. Phenolic compounds are usually non-nutritious and unpalatable for herbivores and inhibit herbivore digestion by binding to consumed plant proteins (Salminen and Karonen, 2011). On the other hand, conifer resin – a complex, toxic mixture of terpenes segregated in specialized ducts – is one of the best known examples of chemical defence in conifer trees (Phillips and Croteau, 1999; Trapp and Croteau, 2001).

2. Results

2.1. Effects of herbivore-induction treatments and pine species on chemical defences in the stem and needles

Phloem feeding by the pine weevil significantly increased the concentration of non-volatile resin in the stem and in the needles five days after experimental herbivory (Fig. 1a, b). Feeding by the weevil also caused a marginally significant increase in polyphenolics in the phloem ($P = 0.082$; Fig. 1e), and a strong 5-fold decrease of polyphenolics in the needles ($P < 0.001$, Fig. 1f). Phloem feeding by the pine weevil significantly increased the concentration of total volatile terpenes (Fig. 1c) and monoterpenes (Fig. 2a) in the phloem 2.5-fold, but did not significantly affect that of sesquiterpenes (Fig. 2b). Such changes led to an increased molar fraction of monoterpenes in the oleoresin (Table 1a) after pine weevil feeding that raised from 80 to 95% (Table SM1). Phloem feeding by the pine weevil did not significantly affect the total concentration and relative contribution of major groups of monoterpenes and sesquiterpenes, and total volatile terpenes in the needles (Fig. 1d; Table 1b; Table SM1).

Defoliation by the processionary caterpillar did not significantly affect the concentration of non-volatile resin and volatile terpenes in either the stem or needles (Fig. 3a–d), nor did it change polyphenolic content in the needles (Fig. 3f), or the concentration of mono- and sesquiterpenes in the phloem and needles (Table 2). However,

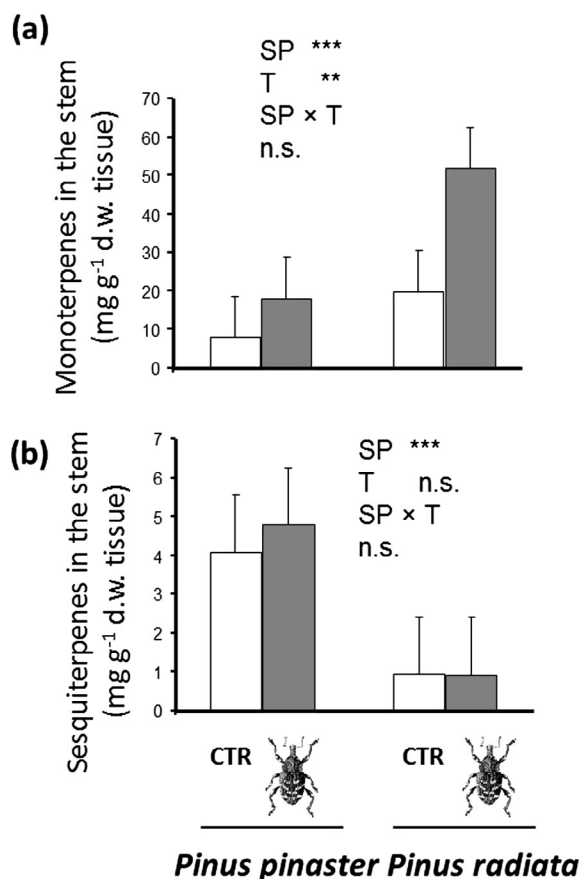


Fig. 2. Effects of the herbivore-induction by the large pine weevil *Hylobius abietis* (grey bars for the herbivore-treatment and white bars for the control) on the concentration of volatile (a) monoterpenes and (b) sesquiterpenes in the phloem of two pine species. Data are shown as LS means \pm s.e.m. $N = 10$. Asterisks indicate significant differences (*** $P < 0.001$, ** $P < 0.01$) among pine species (SP), herbivore-induction treatments (T) and their interaction (SP \times T). n.s. = non-significant differences. F and P -values are shown in the Table 1.

we found that polyphenolics in the phloem increased 5-fold in response to needle chewing by the caterpillar ($P < 0.001$, Fig. 3e).

All the studied major chemical traits significantly differed between the two pine species (Tables 1 and 2). We did not find, however, significant interactive effects between pine species and herbivore induction treatment for any of the defensive traits measured, i.e. induced responses to herbivore feeding were of similar magnitude and direction between pine species (Figs. 1 and 3; Tables 1 and 2).

2.2. Effects of herbivore-induction treatments and pine species on the profile of volatile terpenes in the stem and needles

Pine species differed in the concentration of the major groups of volatile terpenes and also that of many individual terpenes (Tables SM2–SM5), with *Pinus radiata* having greater concentration of almost all single terpenes than *Pinus pinaster* (Tables SM6–SM7). The monoterpenes β -pinene, α -pinene β -phellandrene, limonene, and the sesquiterpene trans-caryophyllene were the most abundant compounds in both species (Tables SM6–SM7).

Bark feeding by the pine weevil significantly increased the concentration of three individual monoterpenes in the phloem: limonene, β -phellandrene and β -pinene (Figs. 4 and 5a). The concentration of limonene increased 4- and 2-fold in weevil-induced *P. pinaster* and *P. radiata*, respectively (Fig. 4a). Concentration of β -phellandrene in the phloem was 4.0 and 1.3 times

Table 1 Summary of the mixed model for the concentration of chemical defences contained (a) in the phloem and (b) in the needles of pine trees showing the effects of pine species (*P. pinaster* and *P. radiata*), herbivory by the large pine weevil *Hylobius abietis*, a bark-feeder, and the corresponding interaction. Molar fraction of the lighter monoterpene fraction is also showed. Bold *P* values are significant.

	Pine species		Weevil induction		Species × weevil	
	$F_{(1,18)}$	<i>P</i>	$F_{(1,9)}$	<i>P</i>	$F_{(1,18)}$	<i>P</i>
(a) Phloem						
Total phenolics	10.73	0.004	3.82	0.082	0.71	0.409
Non-volatile resin	9.14	0.007	9.58	0.013	1.49	0.237
Σ Monoterpenes	18.38	<0.001	12.65	0.006	0.01	0.925
Σ Sesquiterpenes	31.61	<0.001	0.54	0.480	0.09	0.766
Total volatile terpenes	8.32	0.010	8.50	0.017	0.12	0.730
Monoterpenes % mol	38.14	<0.001	6.30	0.033	3.66	0.072
(b) Needles						
Total phenolics	0.53	0.477	117.84	<0.001	2.97	0.102
Non-volatile resin	7.68	0.013	16.41	0.003	1.86	0.190
Σ Monoterpenes	30.87	<0.001	3.45	0.096	0.28	0.601
Σ Sesquiterpenes	13.76	0.002	0.28	0.607	0.63	0.438
Total volatile terpenes	21.49	<0.001	2.81	0.128	0.56	0.462
Monoterpenes % mol	50.45	<0.001	2.30	0.164	1.20	0.288

greater in weevil-induced *P. pinaster* and *P. radiata* plants, respectively than in the corresponding control plants (Fig. 4b). Similarly, the concentration of β -pinene in the phloem was 2-fold greater in weevil-induced *P. pinaster* and *P. radiata* plants than in control plants (Fig. 5a). We did not find significant pine species × weevil interaction for these major changes in volatile terpenoid chemistry, suggesting similar patterns of induced response against the weevil in both pine species (Figs. 4 and 5a). We also found significant changes in the phloem concentration of minor terpenes after pine weevil feedings such as a 2-fold increase in bornyl acetate observed in *P. pinaster* and a significant decrease in the concentration of this terpene in *P. radiata* (Table SM6) (pine species × pine weevil effect $F_{1,18} = 7.97$; $P = 0.011$, Table SM2). We observed for *P. pinaster* (but not *P. radiata*) a significant reduction in the molar fraction of trans-caryophyllene (the dominant sesquiterpene) from 28% mol in control plants to 11% mol in weevil plants (pine species × pine weevil effect $F_{1,18} = 8.01$; $P = 0.011$).

Weevil feeding did not affect the mono and sesquiterpene concentration in the needles, except in the case of β -pinene. The concentration of β -pinene in the needles of weevil-induced plants was 2 times that of control plants for both pine species (Fig. 5b, Tables SM3, SM7). This magnitude of change was similar to that observed for β -pinene in the phloem (Fig. 5a).

A subsequent analysis showed that phloem wounding by the weevil did not affect the enantiomeric composition of α -pinene, β -pinene and limonene in *P. pinaster* (Appendix 2 in the Supplementary material).

Needle feeding by the pine processionary caterpillar induced a marginally significant 1.4- and 2-fold increase in the concentration of β -pinene in the needles of *P. pinaster* and *P. radiata*, respectively (Fig. 5b, Table SM5). Consequently, the molar fraction of β -pinene in the needles of both pine species raised from ca. 20% in control plants to 35% of total needle volatile terpenes in plants experiencing damage by the caterpillar ($F_{1,9} = 11.9$; $P = 0.007$). We also found a significant effect of caterpillar feeding on the concentration of limonene in the needles, but with this response varying in magnitude between pine species (pine species × caterpillar interaction: $F_{1,18} = 6.79$; $P = 0.018$, Table SM5). Caterpillar feeding induced a 2-fold increase in the concentration of limonene in the needles of *P. pinaster*, while causing a 50% reduction in *P. radiata* (Tables SM6, SM7). In *P. pinaster*, this effect raised the limonene molar fraction in the needles from 2.6% mol in the control plant to 5.4% mol in the caterpillar-induced plants. While those in *P. radiata* dropped from 20% to 8% mol.

Needle feeding by the caterpillar induced a significant 2-fold increase in the concentration of β -pinene in the phloem (Fig. 5a;

Table SM4). Similarly to that found in response to pine weevil, the concentration of bornyl acetate in the phloem of *P. pinaster* was 3.8-fold greater in caterpillar-induced plants than that in the control plants (significant pine species × caterpillar interaction, $F_{1,18} = 12.21$; $P = 0.003$; Tables SM4, SM6).

3. Discussion

We found strong changes in major groups of defensive chemicals in response to phloem chewing by the large pine weevil, but smaller undetectable effects after needle chewing by the pine processionary caterpillar. Just five days after exposure to the weevil we found significantly increased concentrations of non-volatile resin (diterpenoid fraction), volatile monoterpene fraction and a marginally greater concentration of polyphenolics in the stem tissues. Moreover, the weevil also caused a significant increase in the concentration of non-volatile resin, a marked decrease of polyphenolics and a marginally significant increase in the volatile monoterpenes in the needles. Likewise, the analysis of individual volatile terpenoids showed that the 2-fold increase in monoterpenes was not due to a generalized rise in their concentration, but due to a quite specific and marked increase in a small number of highly responsive monoterpenes (limonene, β -pinene and β -phellandrene). In contrast, chewing by the pine processionary caterpillar caused no major quantitative changes in defensive chemicals, except for a strong increase in the concentration of polyphenolics in the phloem and a marginally significant increase in the monoterpene fraction. Interestingly, we found similar induced response patterns to each herbivore across both pine species (*P. pinaster* and *P. radiata*). Overall, these results strongly evidence that defensive induced responses in young pine trees are specific to the targeted plant tissue, the insect herbivore that elicits the response, and the chemical compound under study, but that these responses are equivalent between two pine species.

Pine induced responses were more intense in the targeted tissues, even when signalling of damage was clearly systemic. This fact was evidenced by the existence of changes in the concentration of some compounds and chemical species in the foliage in response to phloem wounding by pine weevil, and also changes in the phloem after needle chewing by the caterpillar. Particularly, we found a significant increase (5-fold) in the concentration of total phenolics in the stem after needle chewing by caterpillars, suggesting a strong basipetal response to caterpillar feeding. The occurrence of systemic-induced resistance basipetally to the damage site has been increasingly reported in a diverse array of plant

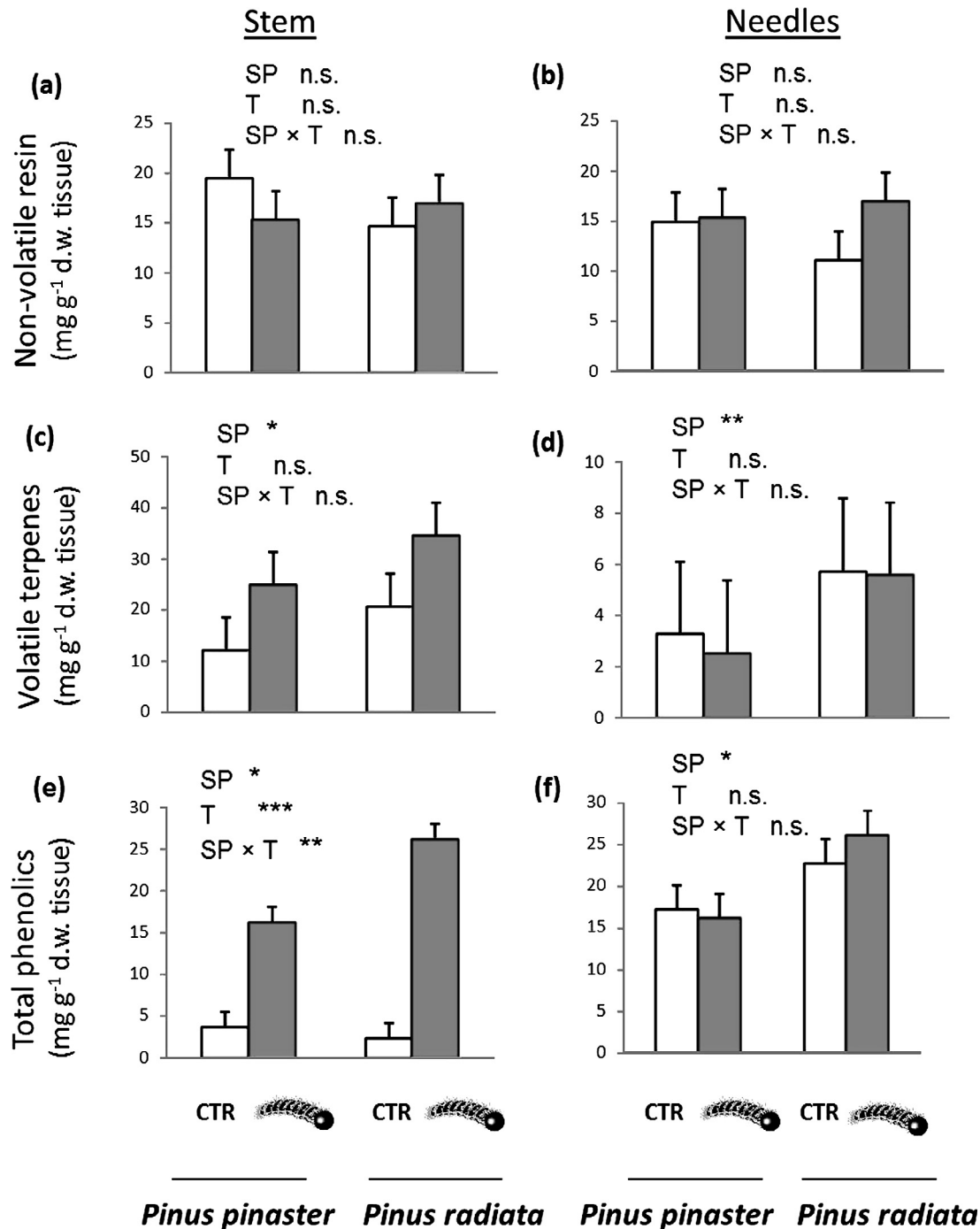


Fig. 3. Effects of the herbivory-induction by the pine processionary caterpillar *T. pityocampa* (grey bars for the herbivore-treatment and white bars for the control) on the concentration of (a) non-volatile resin, (c) volatile terpenes and (e) total phenolics in the stem tissues; and (b) non-volatile resin, (d) volatile terpenes and (f) total phenolics in the needles of two pine species. Data are shown as LS means \pm s.e.m. $N = 10$. Asterisks indicate significant differences (**** $P < 0.001$, *** $P < 0.01$, * $P < 0.05$) among pine species (SP), herbivore-induction treatments (T) and their interaction (SP \times T). n.s. = non-significant differences. F and P -values are shown in the Table 2.

species (e.g. Erb et al., 2009; Gutbrodt et al., 2011), indicating that signalling pathways involved in induced herbivore resistance may be multidirectional. These results have important implications for our understanding of tissue-specific induced responses associated to pine herbivore resistance. First, a number of researchers have reported that defences can be induced throughout a plant, even in unattacked tissues, producing systemic induced resistance in small-sized plants (e.g. Heil and Bostock, 2002; Heil and Silva-Bueno, 2007). However, because plant defences are expensive to produce and maintain the induction of tissue-specific resistance

traits may be considered as a cost-saving energy strategy that avoids redundant defensive responses (Sampedro et al., 2011a; Moreira et al., 2012). Pines may thus allocate more resources to induce specific defences in damaged tissues in detriment of undamaged tissues. Second, the induction of specific resistance traits is likely to be more precisely focused, so plants may deploy their defensive mechanisms more rapidly providing less time for enemies to attack them.

Pine induced responses were much lower for pine caterpillar than pine weevil. One of the possible explanations is that constitu-

Table 2
Summary of the mixed model for the concentration of chemical defences contained (a) in the phloem and (b) in the needles of pine trees showing the effects of pine species (*P. pinaster* and *P. radiata*), herbivory by the pine processionary caterpillar (*Thaumetopoea pytiocampa*), a folivore, and the corresponding interaction. Molar fraction of the lighter monoterpene fraction is also showed. Bold *P* values are significant.

	Pine species		Caterpillar induction		Species × caterpillar	
	<i>F</i> _(1,18)	<i>P</i>	<i>F</i> _(1,9)	<i>P</i>	<i>F</i> _(1,18)	<i>P</i>
(a) Phloem						
Total phenolics	5.23	0.034	36.12	<0.001	8.97	0.008
Non-volatileresin	0.30	0.588	0.11	0.742	1.28	0.273
Σ Monoterpenes	18.36	<0.001	3.73	0.086	0.03	0.866
Σ Sesquiterpenes	23.13	<0.001	0.10	0.762	0.00	0.978
Total volatile terpenes	8.28	0.010	3.07	0.114	0.04	0.845
Monoterpenes%mol	30.61	<0.001	0.88	0.372	1.62	0.219
(b) Needles						
Total phenolics	7.17	0.015	0.28	0.610	0.58	0.457
Non-volatileresin	0.14	0.713	1.31	0.282	0.88	0.362
Σ Monoterpenes	19.36	<0.001	0.68	0.430	0.28	0.606
Σ Sesquiterpenes	8.79	0.008	0.80	0.393	0.17	0.683
Total volatile terpenes	12.67	0.002	0.85	0.382	0.05	0.825
Monoterpenes%mol	22.45	<0.001	0.07	0.794	2.01	0.174

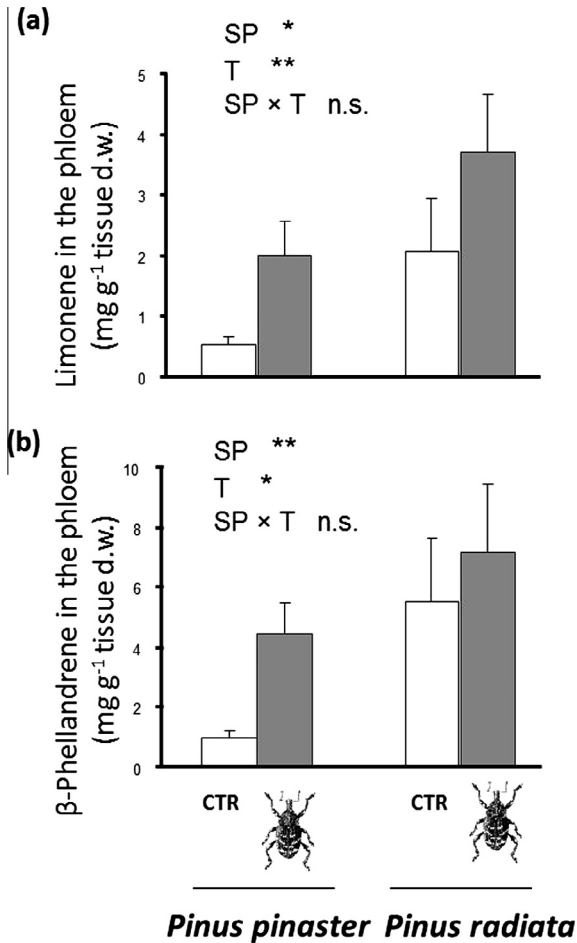


Fig. 4. Effects of the herbivory-induction by the large pine weevil (*Hylobius abietis*) (grey bars for the herbivore-treatment and white bars for the control) on the concentration of (a) limonene and (b) β-phellandrene in the phloem of two pine species. Data are shown as LS means ± s.e.m. *N* = 10. Asterisks indicate significant differences (***P* < 0.01, **P* < 0.05) among pine species (SP), herbivore-induction treatments (T) and their interaction (SP × T). n.s. = non-significant differences.

tive concentration of chemical defences in needles (targeted tissue for caterpillars) might be already very high, leaving only a small margin for induction, as suggested for needle volatile terpenoids by Sampedro et al. (2010). Another possible explanation would

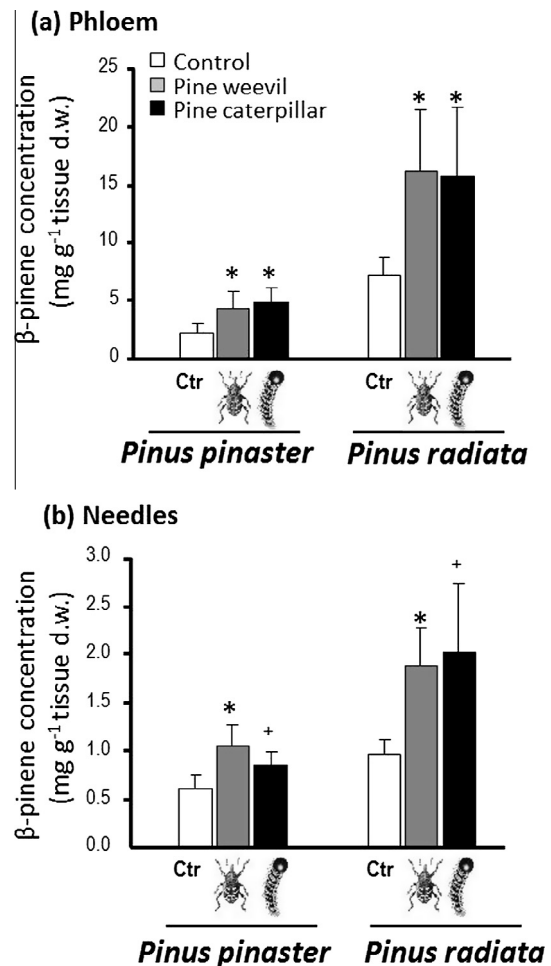


Fig. 5. Concentration of β-pinene in the phloem and needles of two pine tree species in control plants and after experimental herbivory in the stem by the large pine weevil (*Hylobius abietis*), a bark-feeder, and defoliation by the pine processionary caterpillar (*Thaumetopoea pytiocampa*), a folivore. Means ± s.e.m.; *N* = 10. The symbols * and + over the error bars indicate significant (*P* < 0.05) and marginally significant differences (*P* < 0.06), respectively, in comparison to the control plants.

be, as reported by some previous studies, that defoliating caterpillars are able to interfere and suppress the host's immune responses

of some plant species (Musser et al., 2002; Bede et al., 2006; Zarate et al., 2007; Consales et al., 2012). For instance, Consales et al. (2012) found that oral secretions by two lepidopteran herbivores (*Pieris brassicae* and *Spodoptera littoralis*) are able to suppress the wound-induced expression of defence genes in *Arabidopsis*, and in so doing, increase larval growth. Similarly, Musser et al. (2002) found that salivary components from the caterpillar *Helicoverpa zea* suppressed induced resistance in the tobacco plant *Nicotiana tabacum*. Such presumable lack of induced chemical responses against the processionary caterpillar (despite the high amount of damage usually inflicted by this caterpillar) deserves further attention under the context of interfering and inhibitory mechanisms through oral secretions in pine immune responses leading to suppress pine chemical defences. A third possible explanation would be that processionary caterpillar elicited other kinds of induced defensive responses rather than terpenoids and phenolics, such as defensive proteins (e.g. digestive and proteinase inhibitors or polyphenol oxidases), changes in the emission of volatile compounds for indirect defence or even delayed induced resistance (changes in following year needle morphology and chemistry). Although we found different plant induced responses among both chewing herbivores, we cannot strictly talk about a specific response to the insect species identity, as we have tested only an herbivore species per plant tissue. Specificity in the response of the herbivore identity should be tested with different herbivore species feeding in the same way on the same tissue (Agrawal, 2000).

Our results showed that the two studied pine species displayed similar induced responses after insect herbivory for most of the studied defensive chemicals, even in the case of individual volatile terpenes. Responses to insect herbivory were fairly similar in both pine species despite their disparate biogeographical and phylogenetical relationship, and no known congeners of pine weevil and caterpillar living in the range of *P. radiata*. Pine species responses were in the same direction (even not the same fold-changes for all compounds), with no major discrepancies in the responding compounds and tissues between them. This fact leads to speculate on a common evolutionary history of herbivore pressure. Moreover, we could also speculate about shared damage-self signaling (Heil, 2009), plant perception of herbivore damage (Bonaventure, 2012), herbivore associated molecular patterns (Mithöfer and Bolland, 2008) and possible common herbivore associate effectors and modulators of pine immunity between the two pine species. It could be also that response patterns are shared within feeding guilds. The existence of a common response pattern between pine species could be consistent with the idea that there is a generalized response pattern against all the weevil-stem feeders and another against all the caterpillar-needle folivores across continents (Europe and North America) which should be further investigated.

As we mentioned above, we found particular changes for three compounds in response to pine weevil feeding (limonene, β -pinene and β -phellandrene) and one in response to pine caterpillar feeding (β -pinene). Some previous studies have reported that both limonene and β -pinene are likely more herbivore-deterrent than other monoterpenes in conifer trees (e.g. Cook and Hain, 1988; Nordlander, 1990; Sadof and Grant, 1997; Latta et al., 2000; Mita et al., 2002; Thoss and Byers, 2006), and might be thus more inducible after herbivore attack (Holopainen et al., 2009; Heijari et al., 2011). For example, Nordlander (1990) found that limonene completely inhibited the attraction of two *Hylobius* species to α -pinene in field traps. Mita et al. (2002) found that *P. pinea* trees with high content of limonene were more resistant to the caterpillar *Marchalina hellenica* (Hemiptera: Margarodidae). In addition to limonene changes after herbivore-induction, we found a similar 2-fold increase in the concentration of β -pinene in the phloem and needles irrespective from the identity of the chewing insect, the pine species, and the targeted tissue. The role of this compound as a deter-

rent to herbivores in coniferous trees has been also previously reported (e.g. Litvak and Monson, 1998; Sampedro et al., 2010). For instance, large increases in the needle concentration of pinene in young pine trees after exogenous application of methyl jasmonate, a chemical elicitor of induced responses, have been reported in several conifer species (Holopainen et al., 2009; Sampedro et al., 2010; Zhao et al., 2010).

4. Conclusions

This study demonstrates that pine trees, irrespective of whether they have co-evolved or not with particular insect herbivores, are able to discriminate herbivory feeding patterns or molecular cues and respond against them with specific induced responses, which are mostly restricted to the targeted tissues, and depend on the defensive trait. These specific responses are probably differentially biosynthesized in order to reduce overlapping or redundant defence responses. Interestingly, responses of the two pine species were very similar despite the large biogeographical and phylogenetic distance separating them. Further studies should identify the signal and receptors of herbivore-associated molecular patterns to improve our understanding of the mechanisms by which conifers may recognize insect herbivores.

5. Experimental

5.1. Natural history

We studied two Mediterranean pine species largely used for forestry purposes in the Iberian Peninsula and the whole Mediterranean Basin: *P. pinaster* Ait., native to the Iberian Peninsula, and *P. radiata* D. Don., native to California and introduced to the Iberian Peninsula around 1840. Both pine species coexist in mixed forests in southern Europe, with overlapping distributions ranging from altitudes of 0 to 800 m in Northern Spain.

We used two chewing insects for the induction treatment: the large pine weevil, *H. abietis* L. (Coleoptera: Curculionidae) and the pine processionary caterpillar, *Thaumetopoea pityocampa* Denis and Schiff (Lepidoptera: Thaumetopoeidae) (hereafter pine weevil and pine caterpillar, respectively). The pine weevil is a bark-chewer, feeding on the bark, phloem and vascular cambium to the xylem of young conifers, especially pines, firs and spruces. It is widely distributed across Europe and northern Asia where it causes extensive damage and mortality (e.g. Wainhouse et al., 2005; Zasetal., 2011). The pine caterpillar is a pine needle folivore from the Mediterranean region of southern Europe and North Africa that causes severe defoliation to young and adult trees of several Mediterranean pine species, significant loss in tree growth and, in extreme infestations, tree death (e.g. Palacio et al., 2012). Both herbivore insects are two of the most economically important insect threats to pine forests in Europe.

5.2. Experimental design

We carried out a two-factorial greenhouse experiment with two pine species (*P. pinaster* and *P. radiata*), and three treatments of plant defence induction (control, pine weevil feeding and pine caterpillar feeding; hereafter herbivore-induction treatments) as the main factors. The experiment followed a randomized split-plot design replicated in 10 blocks, with herbivore-induction treatments as the whole factor and pine species as the split factor. In total, there were 60 pine seedlings.

5.3. Plant growth, greenhouse conditions and herbivore-induction treatments

In October 2008, pine seeds were individually sown in 2-L pots filled with a mixture of perlite and peat (1:1 v:v), fertilized with 12 g of a slow-release fertilizer (Multicote[®] N:P:K 15:15:15), and covered with a 1–2 cm layer of sterilized sand. To avoid interference from pathogens, seeds were treated with a fungicide before sowing (Fernide[®], Syngenta Agro, Spain). Pots were placed in a glass greenhouse with controlled light (minimum 12 h per day), and temperature (10 °C night, 25 °C day) and watered daily. Plants were grown at the Forestry Research Centre of Lourizan (Xunta de Galicia) greenhouse facilities.

One year after sowing, when plant height of *P. pinaster* and *P. radiata* plants were 41.2±2.4 cm and 62.7±4.0 cm respectively (mean±S.E.), we applied the herbivore-induction treatments (pine weevil and caterpillar, see Fig. SM1 in the Supplementary material). Adult pine weevils were collected in the field (San Xurxo de Sacos Forest, Galicia, Spain, 42.30 °N; 8.30 °W) during the summer of 2009 following the method described by Moreira et al. (2008), stored in culture chambers at 15 °C and fed with fresh pine twigs for a maximum of two weeks before the experiment started. Prior to initiating the weevil-induction treatment, pine weevils were food-deprived for 48 h in labeled Petri dishes with a moist filter paper (15 °C, dark) and then weighed. One specimen was placed on each pine seedling, allowed to feed for 5 days and then removed and weighed again. Damage inflicted by the weevil after the feeding period was evaluated independently in every 1/5 stem sections as the relative debarked area using a four-level scale (0=undamaged; 1=1–25% damaged; 2=26–50% damaged; 3=>50% damaged), and the sum of values for the 5 sections per seedling (i.e. 0–15 score) was considered to be the debarked area.

Sampling of pine processionary caterpillar was achieved by collecting entire caterpillar nests directly from infested trees at Arousa Island (Galicia, Spain, 42.33 °N; 8.51 °W) during the summer of 2009. Nests were carefully opened at the lab and 2nd-instar larvae randomly separated into groups of 10 caterpillars, starved for 12 h and weighed as above. One pre-weighed group of 10 caterpillars was added on needles of the top plant section and another on needles of the bottom plant section of each pine seedling. Caterpillars were allowed to feed on the needles for 6 days, and then removed, counted and weighed. Foliar damage caused by caterpillars after the feeding period was evaluated for the whole plant in a three-level scale: 0=undamaged needles, 1=less than 5 damaged needles, 2=more than 5 damaged needles (i.e. 0–2 score).

All plants within each induction treatments (control, pine weevil and caterpillar) were carefully covered with a nylon mesh to avoid herbivore escape or interference among treatments. No weevils or caterpillars died during the feeding period, and all plants were damaged. The extent of damage caused by weevils and caterpillars did not significantly differ between pine species (see Carrillo-Gavilán et al., 2012).

5.4. Sampling, measurements and chemical analyses

One week after initiating the herbivore-induction treatment, we measured plant height and stem basal diameter. Then, all pine juveniles were harvested by cutting the stem above ground, transported to the lab in ice coolers and immediately sampled for further chemical analyses and total aboveground biomass determination. A fresh 5-cm-long segment of the lowest part of the stem of each plant was collected, weighed, immediately frozen and preserved at –30 °C for analysis of non-volatile resin content. A fresh, 1.5-cm-long stem segment located midway along the stem, as well as a sample of needles (approximately 0.2 g randomly chosen from the whole pool of needles) were collected from each

plant, weighed, then frozen and preserved at –80 °C in cryogenic vials for volatile terpenoid analysis. In parallel, another fresh, 5-cm-long segment of the medium part of the stem and a sample of needles (approximately 2 g) was immediately weighed, oven-dried (45 °C to constant weight) and then manually ground in a mortar with liquid nitrogen for analyses of total phenolic compounds. We specifically targeted phloem tissue for the analyses of phenolics and volatile terpenes. Phloem was separated from the xylem by hand using a surgical knife.

Total phenolics in the phloem and needles were extracted and analyzed as described by Moreira et al. (2009). Briefly, phenolics were extracted from 300 mg of plant tissue with aqueous methanol (1:1 vol:vol) in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of the methanolic extract. Total phenolic content was determined colorimetrically by the Folin-Ciocalteu method in a Biorad 650 microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA, USA) at 740 nm, using tannic acid as standard, and concentrations were based on dry weights (d.w.).

Conifer resin is composed mainly of a volatile fluid fraction, monoterpenes (C₁₀) and sesquiterpenes (C₁₅), and a non-volatile fraction, diterpenes (C₂₀), which make resin thick and sticky. Non-volatile terpenoids provide an excellent physical and chemical barrier against herbivores (Phillips and Croteau, 1999). Volatile terpenoids (mono- and sesqui-terpenes) are known to have toxic effects or negatively affect the success of invading herbivores and pathogens (e.g. Schiebe et al., 2012). However, due to their volatile nature, they also have multiple ecological roles in plant–insect interactions (e.g. attracting herbivore predators Mummand Hilker, 2006), insect–insect interactions (e.g. co-factors for bark beetle aggregation, Erbilgin et al., 2003) and even plant–plant signalling (e.g. interplant priming, Heil and Silva Bueno, 2007). Thus, we performed a more detailed chromatographic analysis of the concentration of the volatile terpenoid fraction (mono- and sesquiterpenes) in the pine tissues.

Concentration of non-volatile resin in the stem (phloem+xylem) and needles was estimated gravimetrically (Moreira et al., 2013). About 5 g fresh weight of stem/needle material was transferred into pre-weighed borosilicate test tubes, resin was extracted with 3 mL of hexane (15 min at 20 °C in an ultrasonic bath and then for 24 h at room temperature), the extract was filtered (Whatman GFF, Whatman Int. Ltd, Maidstone, Kent, UK) into new pre-weighed test tubes, and the entire extraction step was then repeated again. The solvent in the tubes was evaporated to dryness and the mass of the non-volatile resin residue was determined at the nearest 0.0001 g and expressed as mg of non-volatile resin g⁻¹ stem d.w. This gravimetric determination of non-volatile resin was highly correlated with the concentration of the diterpenoid fraction ($r = 0.9214$; $P = 0.00002$), as quantified by gas chromatography in previous trials (Sampedro et al., 2011b).

Extraction and analysis of volatile terpenoids in the phloem and needles were performed following description by Sampedro et al. (2010). Briefly, needle and phloem samples were ground under liquid nitrogen in Teflon tubes and terpenes were extracted with ultrapure *n*-hexane in an ultrasonic bath at 25 °C using dodecane (Merck, #1.09658.0005; $M = 170.33 \text{ g mol}^{-1}$) as internal standard. The monoterpenes and sesquiterpenes in the extract were analysed at KTH (Stockholm, Sweden) by gas chromatography mass spectrometry in single ion monitoring mode (SIM: m/z 68, 69, 93, 121, 136, 161, 170, 204, 222, 272) used to make visible known terpenefragments. The instrument used was a HP7890A two-dimensional GC–MS (2DGC–MS, Agilent Technologies, CA, USA), where the first GC is equipped with a HP-5MS capillary column (30 m, ID 0.25 mm, film thickness 0.25 μm, Agilent Technologies, CA, USA). A volume of 1 μl of each of the sample was injected in splitless mode, using Helium as carrier gas. The oven temperature pro-

gram was set at 40 °C for 3 min, followed by a temperature rise of 4 °C min⁻¹ up to 235 °C and maintained at this final temperature for 18 min. The injector was set at 60 °C for 1 min, followed by a temperature rise of 10 °C min⁻¹ to 240 °C and isothermal for 1 min, splitless to column for 1.5 min. The identification of each present peak in the chromatogram was performed by comparing the retention times and mass spectra to known standards (all from Fluka, Chemie AG, Buchs, Switzerland) and to those in the NIST Mass Spectral Library included in G1701EAMSD Chem Station software (Agilent Technologies, CA, USA). Calibration curves for quantification were prepared with commercial standards of the most abundant compounds in the samples. Individual terpene concentration was expressed in mg g⁻¹ leaf dry weight (d.w.). A subset of samples were run on the second GC–MS equipped with a Cyclodex-B capillary column (30 m, ID 0.25 mm, film thickness 0.25 μm, Agilent Technologies, CA, USA) to confirm the correct identification of limonene and β-phellandrene which co-elute on the HP-5 column but separates on a chiral column for monoterpene enantiomeric analysis (see Appendix 2 in the Supplementary material). The HP-5 column peak area assigned to limonene was calculated on the *m/z* 68–93 ratio (*m/z* 68 present in limonene but not in β-phellandrene) and the rest of the peak was assigned to β-phellandrene. For these selected samples the chiral composition of limonene, β-pinene and α-pinene were also determined (see Appendix 2 in the Supplementary material). As it is known that pine weevil neurons may be more responsive to one enantiomer than the other (Wibe et al., 1998), it is of interest not to overlook the possibility that chiral analysis may be of importance.

5.5. Statistical analyses

The effects of each insect herbivore on the concentration of chemical defences in the stem and needles of each pine species were analysed with a mixed model for solving split-plot designs according to Littell et al. (2006) using the Proc Mixed procedure in SAS 9.2 (Cary, NC). For each herbivore species, the main effects of induction treatment (T), pine species (SP) and T × SP interaction were treated as fixed factors. The block (B) and B × T interaction effects were considered random factors in order to test for the herbivore-induction treatment using the appropriate error term (Littell et al., 2006). Independent analyses were performed for studying the effect of each insect herbivore. When needed, normality was achieved by log-transforming the raw data. We use least square means ± standard error of the mean (s.e.m.) as descriptive statistics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.phytochem.2013.05.008>.

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