No evidence of induced defence after defoliation in three pine species against an expanding pest, the pine processionary moth

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ABSTRACT

Trees may react against defoliation by producing new tissues with modified morphological and nutritive characteristics, in order to prevent future herbivore attacks. In this work, we analyse the capacity of three pine species, namely cluster (*Pinus pinaster*), black (*Pinus nigra*) and Scots (*Pinus sylvestris*) pine, naturally set along an altitudinal gradient (1350–2000 m a.s.l. in SE Spain), to produce an induced defence against defoliation by an expanding pest, the pine processionary moth (*Thaumetopoea pityocampa*). Pines were subjected to experimental defoliation, and the tree response was analysed by means of chemical analysis and bioassays with first-instar larvae of *T. pityocampa*. None of the pine species showed a notable change in chemistry after experimental defoliation, suggesting that chemical defences in pines represent constitutive rather than inducible defences. However, constitutive defences do not deter *T. pityocampa*, and larval survival did not vary depending of the previous damage suffered by trees. These results indicate that the three pine species analysed have a low capacity to respond after *T. pityocampa* attack, and thus a limited induced response against the herbivory caused by this specialist insect.

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1. Introduction

Plants have developed a wide array of defences against defoliators. These defences can broadly be divided into permanent constitutive defences and inducible defence that are elicited by herbivore damage (Agrawal and Karban, 1999; Mumm and Hilker, 2006; Mithöfer and Boland, 2012; Heil, 2010). Although most plants possess both types of defences, they cannot launch both strategies at the same time because they are costly (Bezemer and van VanDam, 2005; Mumm and Hilker, 2006; Mithöfer and Boland, 2012). Thus, constitutive and inducible defences represent alternative adaptive strategies, which evolve depending of the herbivory pattern (Karban et al., 1999).

The constitutive defence is optimal if the amount of biomass lost is large and the probability of herbivory is high (Ito and Sakai, 2009), that is, when herbivory attack is predictable and constant. This defence has the advantage of immediate effectiveness against herbivory, but has the drawback that plants cannot change when herbivores circumvent it or when herbivory varies over time (Karban and Baldwin, 1997; Ruiz et al., 2002). In contrast, if the

biomass loss is not so small but the probability of herbivory is low, the induced defence or no-defence is optimal (Ito and Sakai, 2009). The effectiveness of constitutive or inducible defences depend, thus, on the abiotic (climate, resource availability) and biotic (herbivores, competitors) framework in which the interaction takes place, but differences in such strategies among species are also genetically determined depending on the plant's phylogeny (Kempel et al., 2011). This means that in a situation of global change, with both abiotic and biotic conditions changing, herbivory rates included (Kienast et al., 1999; Ayres and Lombardero, 2000; Bale et al., 2002; de Sassi and Tylianakis, 2012), the plant defence strategies effective until now would no longer work in the same way.

Several theories seek to elucidate how different plant species engage one kind of defence or the other. The growth-rate or resource-availability hypothesis (Coley et al., 1985) states that slow-growing plant species, which typically evolved in resource-limited environments, are less able to replace lost tissue than fast-growing plant species from more productive and competitive environments, and should therefore invest in constitutive rather than induced resistance (Kempel et al., 2011). According to this theory, most conifers should have higher levels of constitutive than inducible defences. Conifers are an old and successful group that have colonised terrestrial systems to a greater extent than any other group of plants, and the success of the conifers

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derives in part from their highly evolved defence systems (Phillips and Croteau, 1999). Conifers display resistance against most generalist herbivorous insects, but some specialised insect species among bark beetles (Scolytidae), shoot and root weevils (Curculionidae), sawflies (Hymenoptera), caterpillars or budworms (Lepidopterae) are known to cause substantial damage in conifer forests (Dajoz, 2000). Conifers base their defence on resins and terpenes (Keeling and Bohlmann, 2006), which can be both constitutive and inducible against herbivore damage. For instance, Lewinsohn et al. (1991) found that species of Abies and Picea, with low to moderate levels of constitutive monoterpene cyclase activity, exhibited a sharp increase in cyclase activity after being wounded relative to unwounded controls, while Pinus species, with high levels of constitutive cyclase activity, did not significantly increase the level of cyclase activity after being wounded. Among conifers, pines in fact have the highest constitutive production of resin, which is stored in an intricate network of resin ducts (Trapp and Croteau, 2001).

The genus Pinus has been one of the most extensively used trees in afforestation worldwide, and particularly in Central and Southern Europe (Richardson et al., 2007). Apart from the areas originally covered by pines, forestry practices have considerably increased the cover of Pinus species, some of them exotic, either with economic (timber) or ecological (soil protection and erosion prevention) purposes (Richardson et al., 2007). In this framework, specific pests such as the pine processionary moth (Thaumetopoea pityocampa) may thrive (Battisti et al., 2015). The pine processionary moth was known in mild areas from both Mediterranean rims, affecting diverse Pinus species at different intensities depending on the pine species, the age and density of the plots, and the changes in weather conditions from one year to another (Netherer and Schopf, 2010). However, in recent decades, the distribution limit of the pine processionary moth has steadily ascended in elevation (upslope) and in latitude (polewards), invading areas rarely or never previously attacked (Hódar and Zamora, 2004; Battisti et al., 2005, 2015).

Many works explain and document the differential capacity of the pine processionary moth to develop by feeding on different pine species (Avtzis, 1986; Devkota and Schmidt, 1990; Hódar et al., 2002; Arnaldo and Torres, 2006; Stastny et al., 2006). In contrast, the information available on the different chemical response of these pine species against defoliation is scant (Battisti, 1988; Hódar et al., 2004; Achotegui-Castells et al., 2013; Jactel et al., 2015). This is a key question because the effects of the expanding forest pest will change not only depending on the susceptibility of the pine species that the moth finds in its expansion, but also on how these pine species react against this defoliator. The previous information in this respect indicates that only after severe and repeated defoliations is the pine processionary moth unable to develop on defoliated pines (Hódar et al., 2004), and that, after defoliation, changes in pine chemistry are slight (Hódar et al., 2004; Achotegui-Castells et al., 2013; Jactel et al., 2015). However, this evidence is based on Scots pine (Pinus sylvestris), which in reason of its distribution is not one of their main hosts, while is lacking for Aleppo (Pinus halepensis), black (Pinus nigra) or Cluster (Pinus pinaster) pines, species that are more frequently attacked.

In this work, our aim is to evaluate the capacity of three pine species, namely Cluster (P. pinaster), black (P. nigra) and Scots (P. sylvestris) pines, to produce an induced defence against defoliation by the pine processionary moth. These pine species differ in their degree of susceptibility to defoliation by the pine processionary moth (Jactel et al., 2015). The Cluster pine is the least susceptible of the three species, the Black pine is the main host for the pine processionary moth in W Europe, and Scots pine has been less common as a host until recent years, due to the higher elevations/latitudes inhabited by this pine (e.g. Montoya and Hernández, 1998; Hódar et al., 2003; Hódar and Zamora, 2004; Stastny et al., 2006). The distribution of these three pine species along an altitudinal gradient in Sierra Nevada mountains (SE Spain) allows us to work with them simultaneously under the same environmental conditions, an optimal approach to envisage the future outbreak dynamics in forests as a consequence of climatic change (Björkman et al., 2011; Fischer et al., 2011). The hypotheses we specifically wish to test are: (1) defoliation by pine processionary moth provokes changes in the chemical composition of pine needles; (2) these changes reduce the subsequent survival capacity of PPM larvae (i.e. these changes constitute a true induced defence); and (3) the induced response differs among pine species according to the initial degree of susceptibility to the defoliator (susceptibility: P. nigra ≈ P. sylvestris > P. pinaster). We test these hypotheses by a field experiment.

2. Methods and material

2.1. Study site

The study area was the Loma de Lanjarón, in Sierra Nevada Natural and National Park (Granada SE Spain). This area is composed by scattered patches of reforested pine trees, 35–45 years old, and some spontaneous regeneration areas, with younger individuals. We established three sites along the elevational gradient. The sites had similar orientation (south-westerly), slope, and bedrock (micaschists). Three pine species were present, differing in site according to their ecological requirements along this elevational and moisture gradient. That is, the Cluster pine P. pinaster was in the lowest site (Cortijo Quemado, 1350 m a.s.l.), black pine P. nigra in the intermediate site (Cruce de Tello, 1700 m), and Scots pine P. sylvestris in the highest site (Peña Caballera, 2050 m). All three species are native to the region, although they were extensively planted in the area for forestry purposes. The three sites had vigorous and healthy trees mostly from spontaneous regeneration, 7–15 years old and between 1.5 to 4 m high.

The climate in the area is Mediterranean, with hot, dry summers and wet, mild winters. Mean annual precipitation is $470 ± 50$ mm (±SE, 1988–2008; climatic data from a meteorological station at 1450 m a.s.l.). Snow falls during winter, usually persisting from November to March above 2000 m a.s.l. The mean annual temperature is $12.3 ± 0.4\,°C$ at 1650 m a.s.l. (State Meteorological Agency, period 1994–2008, Ministry of the Environment). Along the elevational gradient, temperature decreases around 0.5 °C and rainfall increases 27 mm every 100 m of increase in elevation. A more detailed description of climate and soil in the area can be found in Cuadros and Francia (1999).

2.2. Experimental setting

In September 2008 we labelled in each site 30–39 trees, belonging to the dominant species at the site (P. pinaster in Cortijo Quemado, P. nigra in Cruce de Tello, P. sylvestris in Peña Caballera). The choice of trees was made according to their similarity in height and appearance (2–4 m), and all them had no signs of defoliation by the pine processionary moth for at least the two previous winters. The number of trees per site varied because it depended of the availability of suitable trees for the experiment: 30 trees for P. pinaster and 39 for P. nigra and P. sylvestris. All trees in each site were within an area of ca. 1 ha. A sample of current-year needles was collected from each tree at the same time the pines were chosen. Needles were kept fresh with ice and quickly frozen at −20 °C until analysis.

In October 2008, we assigned one-third of the pines of each site to the control treatment, and the other two-thirds to the herbivory treatment. For this, we left untouched the control trees, while we infested the other pines with 3–6 colonies of pine processionary moth bearing 3rd-instar larvae, collected in the nearby trees. The number of colonies per tree varied according to both tree and colonies size, because the aim was to generate at least 90% defoliation in the tree herbivory treatment. That level of defoliation is possible because the late instars of the pine processionary larvae feed on all needle age classes, and may cause massive defoliation in the pine. However, because the colonies can die when the winter is cold, we assigned twice the number of trees to the defoliation treatment respect to control, in order to ensure trees enough with a high level of defoliation. We fixed colonies between 1.5 and 2.5 m in height within each tree. We checked the defoliating activity across the winter whenever the snow cover allowed access to the study sites.

In spring 2009, before the flush of new needles, we evaluated the degree of defoliation in infested trees. Winter defoliation developed well, so most of the initially selected trees were kept; as a consequence, the final number of defoliated trees almost doubled control ones. Four trees were disregarded because they had not produced enough current-year needles for an adequate sampling. We also discarded two control trees, one *P. nigra* and other *P. sylvestris*, showing symptoms of damage by other herbivores (ungulates) and by frost, respectively. Then, in September 2009, we collected again current-year needles from the trees, just as in September 2008.

2.3. Bioassay experiment

In September 2009, we fixed six egg batches of pine processionary moth per tree, in all pines. We fixed the egg batches to current-year needles with adhesive tape, and we checked larval hatching and development twice per week until larvae moulted to the second instar (see Hódar et al., 2002, 2004 for a similar procedure). Then, we collected the egg batches as well as the silk tent with larvae. In laboratory, we counted the number of hatched eggs in the batch and the number of larvae reaching the second instar in the tent, this giving us the percentage of success at this level of development. Therefore, the larval performance was estimated as percentage of hatched larvae reaching second instar. We restricted the analysis to larvae reaching the second-instar because the first instar is the larval phase most strictly dependent on food quality (Zalucki et al., 2002; Hódar et al., 2002, 2004).

2.4. Chemical analysis of needles

Mono- and sesquiterpenes were analysed following the procedure described by Kainulainen et al. (1992), with some modifications. The extraction was made for 500 mg of freshly thawed needles, clipped in small pieces (⩽1 mm) with scissors, and placed overnight in 4 ml of *n*-hexane at room temperature. The internal standard was 1-chlorooctane (0.1 ml/L). The extract was analysed in a high-resolution gas chromatograph Agilent 7890A (Agilent Technologies, USA) containing a ZB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) in apolar phase, coupled to a gas spectrometer Quattro micro GC (Waters Chromatography, USA). The terpenes were identified by comparison with known samples and the NIST/NBS library. Terpene amounts are expressed as mg g^{-1} of fresh needle weight.

The rest of the needle sample was cleaned and left to dry between 30 and 40 °C in the darkness. When dry, samples were milled and sieved (1 mm pore size), and then used for the analysis of N, C, total phenols, and condensed tannins. N and C were determined by combustion at 850 °C in a Leco TruSpec autoanalyzer, and the content expressed as a percentage of dry weight. We determined the total phenol content by the Folin–Ciocalteu method, and the results were expressed as mg of tannic acid equivalents per g of sample dry weight. For condensed tannins the proanthocyanidin assay was used, and the results were expressed as mg of quebracho equivalents per g of dry weight of sample (Waterman and Mole, 1994).

2.5. Statistical analyses

Prior to statistical analyses, we checked all the response variables to meet the requirements of normality and homoscedasticity. Equality of residual variance across treatments was tested in all cases, but significant deviations were not found. When necessary, normality was achieved by transforming the original variables. We used log, squared root or arcsine transformations, according to the nature of data (Quinn and Keough, 2002).

We sought to determine both the chemical differences between species, as well as the effect of defoliation treatments on the chemical characteristics of trees. To that end, we set two different blocks of statistical analyses. In the first block we analysed the 2008 dataset, in order to verify the absence of initial differences between pines before performing the defoliation treatments. Our response variables were N, C, C/N, tannins, phenols, monoterpenes, sesquiterpenes, total terpenes, and a total of 15 individual terpenes. We fitted for these variables a model as a function of pine species and treatment (control vs. defoliated), as well as the interaction. In the second block we analysed the effect of defoliation treatments, once these were carried out, by using the 2009 dataset. Firstly, we performed a principal component analysis (PCA) to reduce the number of variables and to detect structure in the relationships between chemical variables. In this analysis we used all individual chemical variables, excluding the summations variables like total terpenes, sesquiterpenes, monoterpenes and C/N. The PCA was carried out with the raw data for better interpretation of results. The score coordinates of the PCA variables were subjected to two-way ANOVAs (pine species, treatment, and the interaction), retaining the axis with eigenvalue >1 and with cumulative variance >60%. Finally, the most correlated variables with each of the selected axes were also analysed with two-way ANOVAs to observe the individual behaviour of each of them.

Larval performance, measured as the percentage of larvae reaching the 2nd instar on a tree basis, was also analysed with a linear model, as a function of pine species and treatment.

Comparisons between treatment levels within pine species were performed after ANOVAs, both for chemical differences in 2009 and for bioassay, by using Tukey HSD test.

All analyses were run on R environment, version 3.0.1 (R Development Core Team, 2013).

3. Results

3.1. Initial differences of experimental pines

The initial analysis of chemical features in the pines evidenced pronounced differences between species, whereas control and defoliated trees did not show significant differences before the application of the treatment for any of the variables analysed, with the exception of N content. Overall, the pines subject to defoliation registered a higher initial N content than control (Online Resource 1); however, these differences vanished when control was compared with each species of defoliated trees using the Tukey HSD test, and thus we considered adequate the tree assignment to groups for the experiment.

Nitrogen, carbon, C/N ratio, phenols, sesquiterpenes, and total terpenes, as well as a total of nine of the fifteen individual terpenes

reviewed, show marked differences between species, notably the higher content of sesquiterpenes in *P. sylvestris* than in *P. nigra* or *P. pinaster* (Table 1). Apart from N, there were no initial differences neither between treatment not in the species × treatment interaction.

3.2. Effect of defoliation treatments

The PCA gathered 62% of the variance by the first three PCs (those with eigenvalue >1, Table 2). PC1 was linked to monoterpenes (α-Pinene, Ocimene and β-Myrcene) and some sesquiterpene like Elemene. PC2 was mainly linked to sesquiterpenes (β-Caryophyllene and α-Caryophyllene) and PC3 to phenols, tannins and C/N ratio (Table 2). The ANOVA analysis on the PCA scores (Table 3) revealed that only PC3 showed significant differences for treatment ($F_{1,96}$ = 3.45, P = 0.03) and species ($F_{2,96}$ = 47.09, P < 0.001).

The analysis of the variables selected by PCA revealed scant differences between species and treatments after the experimental defoliation (Table 4). In agreement to the PCA results, only the variables linked to PC3, i.e. %N, %C, phenols, and tannins proved significant differences between species, while treatment was significant for %N and tannins (Table 4 and Fig. 1; see Online Resource 2 for all the variables). Nevertheless, differences between treatments within species were always non-significant (Fig. 1).

3.3. Results of the bioassay

In agreement with the scant differences shown by the pine characteristics, the bioassay revealed only minor differences between treatments. Larval survival followed the pattern *P. sylvestris* ≈ *P. nigra* > *P. pinaster*, although differences between species were not significant ($F_{2,48}$ = 1.7415, P = 0.1861). Defoliation had a null effect on larval survival in *P. pinaster*, while *P. sylvestris* registered a slightly higher larval survival rate in defoliated pines, and *P. nigra* a higher survival rate in control (Fig. 2), but again the differences were not significant, whether within species (Tukey HSD between 0.1969 and 0.1493, P always >0.16) or overall ($F_{1,48}$ = 0.0110, P = 0.9168).

4. Discussion

Our results provide evidence that defoliation by the pine processionary moth does not promote chemical changes in pines that could be identified as an induced defence, since the moth performs equally well when feeding on defoliated pines or control. This was true at least for young-juvenile trees after a single and complete whole-tree defoliation. Pines keep rather constant their levels of defensive compounds, and the pattern is the same for the three pine species, irrespective of their susceptibility to defoliation by the pine processionary moth.

4.1. No evidence of induced defence after defoliation

The changes in needle quality after defoliation were in general meagre. Since the experimental defoliation was severe (all the infested trees registered above 90% defoliation), this lack of response seems surprising, although it is not unique in comparison to previous similar experiments (Hódar et al., 2004; Palacio et al., 2012). Apart from the slight increase in N content and tannins after defoliation, the other variables showed minor or null variations (Fig. 1 and Table 4). In fact, the between-species changes (Table 1), or the simple variability between individuals proved far greater than the expected impact of defoliation.

Table 1
Results of the two-way ANOVA for the main chemical variables analysed of the 2008 dataset, depending on pine species (*Pinus pinaster*, *P. nigra* and *P. sylvestris*), treatment (control vs defoliated), and the interaction between the two factors. Figures are *F* values, in bold when significant (***P < 0.001, **P < 0.01, *P < 0.05). See Online Resource 1 for the results of this analysis for all chemical variables and the initial values of the variables in 2008.

	R^2	Species	Treatment	S × T
d.f.		2	1	2
%N	0.48	**43.88*****	**4.58***	0.00
%C	0.33	**23.42*****	0.25	1.79
C/N	0.53	**55.61*****	**4.63***	0.08
Phenols	0.12	**6.85*****	0.17	0.05
Tannins	0.06	2.32	0.96	0.53
Monoterpenes	0.03	0.63	1.51	0.60
Sesquiterpenes	0.34	**25.79*****	0.35	0.26
Total terpenes	0.12	6.01	1.87	0.33

Table 2
Results of the PCA for all chemical variables analysed in all experimental pines. The PCA was performed with untransformed data of 2009, after the application for the defoliation treatment. Figures in bold indicate the highest correlations of variables with the PCs.

Variables	PC1	PC2	PC3
%N	−0.01	0.06	0.62
%C	0.12	0.24	0.63
Phenols	0.11	0.00	**0.85**
Tannins	0.39	−0.23	**0.68**
Tricyclene	0.63	**−0.72**	−0.02
α-Pinene	**0.87**	−0.27	−0.05
Camphene	0.64	**−0.72**	−0.01
β-Pinene	0.37	−0.02	−0.21
β-Myrcene	**0.73**	−0.03	0.01
ᴅ-Limonene	0.27	0.42	0.30
β-Fellandrene	0.43	0.11	−0.26
Ocimene	**0.77**	−0.21	0.06
Linalool	0.35	0.47	−0.17
β-Caryophyllene	0.38	**0.87**	−0.04
α-Caryophyllene	0.40	**0.86**	−0.04
Germacrene-D	0.53	0.61	−0.05
Elemene	**0.81**	−0.16	−0.20
Cadinol	0.63	0.36	−0.03
Eigenvalues	5.02	3.70	2.26
% Variance explained	28	21	13

Table 3
Results of the three-way ANOVAs on the scores obtained from the PCA analysis. Figures are exact *F* values, in bold when significant (P < 0.05).

Variables	R^2	Species	Treatment	Species × Treatment
d.f.		2	1	2
PC1	0.02	0.29	0.01	0.97
PC2	0.03	1.23	0.11	0.28
PC3	0.51	**47.09**	**3.45**	1.14

Defoliated pines in general show higher N content and lower C/N ratio (Smits and Larsson, 1999; Nykänen and Koricheva, 2004; Hódar et al., 2004), which could offer better quality for larval feeding. However, phenols and tannins also increase, which are known to be digestion inhibitors (Waterman and Mole, 1994). Interestingly, these three variables link together in the PC3. In a previous work on a near study site (Hódar et al., 2002) the differences in larval survival between the same three pine species studied here were attributed to the N content in needles, while no significant differences were found in phenols and tannins, but in this case only pines that were not previously defoliated were included. By contrast, in Schopf and Avtzis (1987), variations in

Table 4
Results of the two-way ANOVA for the main chemical variables most correlated with each of the selected PCA axis. Figures are F values, in bold when significant (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). See Online Resource 2 for the initial values of the variables in 2009 and for the results of this analysis.

| Variables		R^2	Species	Treatment	S × T
PCA axis	d.f.		2	1	2
PC1	α-Pinene	0.01	0.22	0.03	0.50
PC1	β-Myrcene	0.02	0.23	0.33	0.47
PC1	Ocimene	0.02	0.49	0.39	0.22
PC1	Elemene	0.03	1.24	0.08	0.46
PC2	Tricyclene	0.02	0.24	0.13	0.52
PC2	Camphene	0.02	0.27	0.08	0.58
PC2	β-Caryophyllene	0.03	1.60	0.00	0.23
PC2	α-Caryophyllene	0.04	1.75	0.00	0.26
PC3	%N	0.65	**85.98*****	**6.97***	0.12
PC3	%C	0.55	**56.11*****	0.42	3.31
PC3	Phenols	0.24	**14.84*****	1.03	0.16
PC3	Tannins	0.15	**5.84****	**3.86***	0.53

larval survival between different pine species were related to the quantity of phenols and silica in the needles. Thus, the role of phenolics for conifer defence remains ambiguous, because their effect against herbivores can be positive, neutral, or negative (see Table 1 in Mumm and Hilker, 2006 for a review). Although N, phenols, and tannins increased and C/N decreased after defoliation, this tendency was not always significant (Fig. 1 and Table 4; see Online Resource Table 2 for details). The increase in N content after defoliation is usually considered a plant reaction to boost photosynthetic activity and, consequently, recover the tissues and reserves lost by defoliation (Palacio et al., 2012); therefore it cannot be interpreted as an induced defence since a higher N content and a lower C/N ratio would in fact be positive for herbivores. Only the

faint increase in phenols and tannins resembles a true induced defence against defoliation (Roitto et al., 2009), but of limited effectiveness.

One of the most clear-cut results in our experiment was the lack of response in terpenes, which differed only slightly even between species. Some studies have found the expected increase of defensive compounds (Wallin and Raffa, 1999), but a lack of change or even a reduction in terpenes after herbivory have also been reported (Vourc'h et al., 2003). In a recent study with two varieties of Scots pine, Achotegui-Castells et al. (2013) reported lower amounts of terpenes in trees defoliated by the pine processionary moth compared to control (and in defoliated branches compared to undefoliated ones within the same tree), although they suggest a higher production of monoterpenes coupled to a higher emission due to the herbivore activity. This case is not totally equivalent to our experiment, since Achotegui-Castells et al. (2013) refers to needle composition and emissions at the end of winter, the period of maximum consumption by the late-instar caterpillars of the pine processionary moth, while our results refer to the end of summer, when first-instar larvae consumed needles produced after a whole-tree defoliation. Nevertheless, the pattern is similar: terpenes in general registered lower amounts in defoliated pines, contrary to expectations of an induced defence, and the individual terpenes that increased after defoliation were invariably sesquiterpenes in very low amounts. This suggests that terpenes are much more constitutive than inducible (Lewinsohn et al., 1991; Muzika, 1993; Haukioja et al., 1998), and thus their role as a true induced defence would be doubtful (Mumm and Hilker, 2006).

4.2. Defoliated pines do not significantly reduce larval survival

The results of the bioassay revealed no difference in larval survival between control and defoliated trees for any of the species
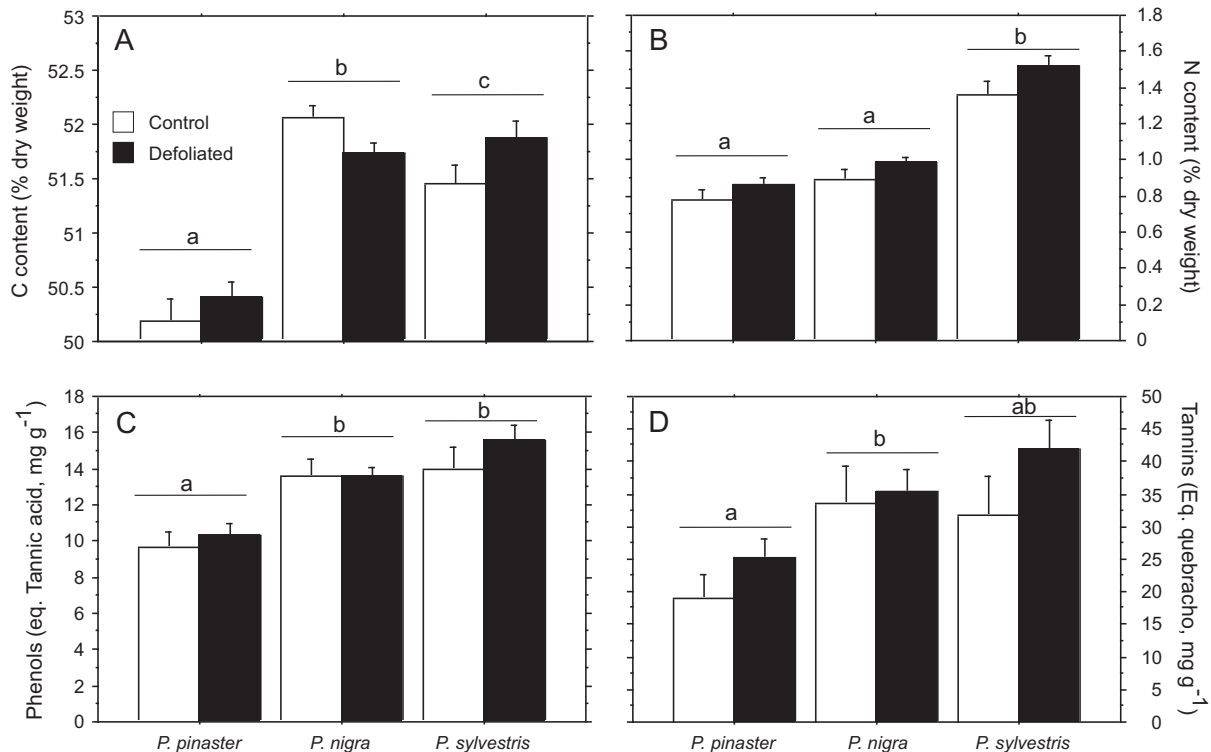
Fig. 1. Needle content in total carbon (A), nitrogen (B), phenols (C) and tannins (D), (*bars* represent mean + SE) in the three pine species analysed, after applying defoliation treatments (2009 dataset). Carbon and nitrogen and are expressed as percent of dry weight, phenols and tannins as equivalents of tannic acid and quebracho, respectively. Different letters mean significant differences between species irrespective of treatment (LS means, Tukey HSD test, $P < 0.05$). Differences between treatments within species were always non-significant.
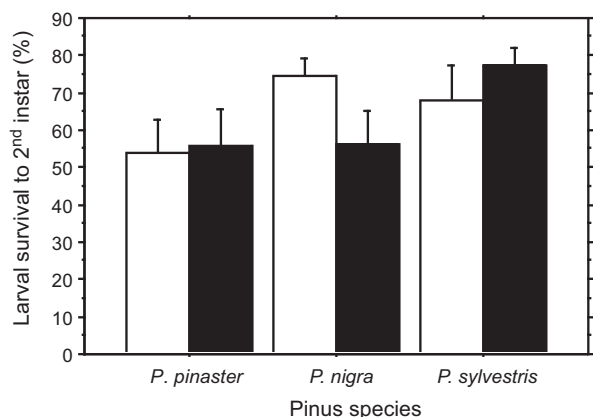
Fig. 2. Larval survival (until 2nd instar, *bars* represent mean + SE) of pine processionary larvae feeding on control (white bars) and defoliated pines (black bars). Differences between treatments within species (LS means, Tukey HSD test, $P < 0.05$) were always non-significant.

considered (Fig. 2). Even between pine species differences were scant: larval survival in defoliated *P. nigra* was slightly (not significantly) lower than in control ones, while those in defoliated and control *P. pinaster* and *P. sylvestris* trees were very similar (Fig. 2). In a previous study only with *P. sylvestris* in a nearby area, larval survival varied from 65% in previously undefoliated pines to 53% in defoliated ones (Hódar et al., 2004), similar to the figures reported here. However, this work also showed survival falling to 32% when the trees suffered defoliation in two consecutive winters (Hódar et al., 2004).

The three pine species analysed showed the same pattern of chemical variation after defoliation, and changes are too meagre (Table 4) to produce an effect on larval survival (Fig. 2). Overall, the effect of a single event of defoliation on the chemistry of the pines (and subsequent larval survival) was equally negligible for the three pine species, suggesting that the pines are unable to generate an induced defence to prevent later defoliations after a previous attack (see Watt et al., 1991; Clark et al., 2010 for similar cases).

Two questions emerge from these statements: why chemical compounds usually acknowledged as defence against defoliators do not seem to work against a main one, and why pines do not develop specific defences against this main defoliator. The first one probably is due to the character of the pine processionary moth as a specialised herbivore (Ali and Agrawal, 2012; Mithöfer and Boland, 2012), which is able not only to circumvent the basal (constitutive) defences of pines, but even use as cues for host recognition (Keeling and Bohlmann, 2006). The second one is dependent on the selective pressure that pine processionary moth represents for Mediterranean pines. Some recent works suggest that the main controllers of the pine processionary moth are climate and parasitoids (Zovi et al., 2008; Pimentel et al., 2011; Hódar et al., 2012), ascribing plant response a limited or irrelevant role in this sense. Furthermore, the defoliation by the pine processionary moth rarely kills trees, and the long-term effects of pine processionary attack on trees (growth and reproduction reductions) are not as bad as suggested until now (Palacio et al., 2012; Linares et al., 2014; but see Jacquet et al., 2012, 2013). With these premises, and even recognising the role of the pine processionary moth as a main defoliator for pines, the development of a specific defence would not be advantageous. Pines are attacked by many different herbivores, and the susceptibility of the different herbivores might require different chemical defences, compromising the capacity of trees to develop induced defences against a single one (Iason et al., 2011; Henery, 2011). Thus, pines maintain a high

level of constitutive, generalistic defences against a wide array of defoliators, but present a rather limited capacity for induced defences. This generalistic defence does not deter specialist herbivores such as the pine processionary moth, being unable to prevent for episodic defoliations.

5. Conclusions

The lack of induced response and stable levels of constitutive defence of the three pine species against the pine processionary moth represent the logical framework in the present conditions: the pine processionary moth can eventually cause severe defoliations, but they are episodic and not reiterated in time (Hódar et al., 2012), pines compensate after defoliation (Palacio et al., 2012; Linares et al., 2014), and pines have many other herbivores to avoid or react (Iason et al., 2011). Furthermore, this lack of response is not dependent of the initial susceptibility of the pine species (Jactel et al., 2015). Constitutive defences are an optimal strategy to counter predictable attacks by herbivores (Karban and Baldwin, 1997), which represent the "average" situation, but not to cope with a scenario where extreme episodes are becoming more and more frequent. However, these circumstances can emerge in the future if the frequency and/or intensity of the processionary outbreaks rises as a biotic response to global warming (Bidart-Bouzat and Imeh-Nathaniel, 2008; Björkman et al., 2011).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2015.07.022.

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