

# Early resistance of alien and native pines against two native generalist insect herbivores: no support for the natural enemy hypothesis

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## Summary

1. The natural enemy hypothesis (NEH) predicts that alien plant species might receive less pressure from natural enemies than do related coexisting native plants. However, most studies to date are based on pairs of native and alien species, and the results remain inconclusive. The level of attack by native generalist herbivores can vary considerably between plant species, depending on defensive traits and strategies. Plant defences include preformed constitutive and induced defences that are activated as plastic responses to herbivore attack. However, the efficacy of induced defences could be altered when alien species entering an area are exposed to native enemies.

2. We tested the NEH for several closely related alien and native pines to Europe by examining early anti-herbivore resistance to damage by two generalist native insect herbivores (*Hylobius abietis* and *Thaumetopoea pityocampa*); the differences in constitutive and inducible chemical defences (i.e. non-volatile resin and total phenolics in the stem and needles); and whether consumption preferences shift after induced defences have been triggered by real herbivory.

3. We did not find alien pines to be less damaged by two generalist herbivores than native pines were. The constitutive concentration of chemical defences significantly differed among pine species. The concentration of constitutive total phenolics in the stem was greater in native than in alien pines. The opposite trend was found for constitutive total phenolics in the needles. The concentration of chemical defences (non-volatile resin and total phenolics) in the stem significantly increased after herbivory by *H. abietis*. Moreover, the induction of total phenolics by *H. abietis* damage was significantly greater in native pine species than in alien pines. On the other hand, only concentrations of non-volatile resin in needles significantly increased after herbivory by *T. pityocampa*, but without significant differences in inducibility between alien and native pines. In cafeteria bioassays, *H. abietis* consumed the twigs from alien more than those from native species irrespective of prior exposure to the insect. Meanwhile, no differences among range origin were found in the *T. pityocampa* cafeteria bioassays.

4. Overall, we found no support for the NEH in alien pines to Europe. This suggests that alien pines, in regions where they coexist with native congeners, may be controlled by native generalist herbivores, this being one reason that invasion by alien pines is not frequent in Europe.

**Key-words:** condensed tannins, *Hylobius abietis*, induced defences, non-volatile resin, plant invasions, phenolics, plant–herbivore interactions, *Thaumetopoea pityocampa*

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## Introduction

The success of alien plants in their new range depends partially on the interaction with the native biota, particularly with herbivores that could damage establishing seedlings (Maron & Vilà 2001; Levine, Adler & Yelenik 2004). The impact of native herbivores on alien plants is determined by the interplay between their ability to recognize the alien plant as a food resource (Carpenter & Cappuccino 2005), the effectiveness of the alien plant defences against herbivores in the new range (Stowe *et al.* 2000) and the ability of herbivores to overcome plant defences of the alien (Rauscher 2001). All these mechanisms might influence the success or failure of an alien plant to invade a new area.

The natural enemies hypothesis (NEH) predicts that alien plant species are successful invaders in the new range because of a lack of specialist natural enemies, which were left behind in their native range (Maron & Vilà 2001). Several studies have shown how introduced plant populations in a new area are less damaged by native phytophages and pathogens than are populations in the native range (DeWalt, Denslow & Ickes 2004; Vilà, Maron & Marco 2004; Rogers & Siemann 2005). Nevertheless, there are contradictory results on whether native herbivores cause more damage to native than to alien plant species (Chun, van Kleunen & Dawson 2010). The level of damage inflicted against alien species and its influence on plant fitness is not always lower than against coexisting native plants, even when the species are taxonomically related (Agrawal & Kotanen 2003). The assumption of less damage in alien species in a new range has been attributed to freedom from specialist herbivores. However, it is not always possible to attribute damage to a particular herbivore species, and generalist herbivores would be the first to have the ability to choose between host species. A recent meta-analysis comparing pairwise native and alien trees noted that the majority of native insect species that colonize alien trees are generalist herbivores (Bertheau *et al.* 2010). In addition, reduced resistance of alien plants leading to rapid colonization by native generalist herbivores was found to occur when congener native plant species live in the introduced range (e.g. Goßner *et al.* 2009). However, most studies have compared alien–native species pairs, and, as suggested by Chun, van Kleunen & Dawson (2010), more than two species should be considered for testing-related alien and native species.

The level of attack by herbivores can vary considerably between plant species, depending on their defensive strategies (Agrawal *et al.* 2005). Plant chemical defences include constitutive defences, which are permanently expressed irrespective of the plant exposure to natural enemies, and induced defences that enhance the basal defence capacity as a result of plastic responses to natural enemy attack (Karban & Myers 1989). Induced strategies, rather than constitutive, are expected to be favoured when herbivore pressure varies spatially and temporally, and initial attacks are reliable cues of further attacks (Karban 2011). Closely related species may differ in their defensive strategy, depending on the biotic and abiotic environment where they have evolved (Orians & Ward

2010). Production of effective induced defences requires the plant to quickly recognize the herbivore damage as a potential risk, signalling the danger across tissues and subsequently triggering defences (Heil 2010). There is emerging evidence that induced responses to herbivory can show high specificity regarding both the damaging herbivore (i.e. specificity of elicitation) and the efficacy of the induced responses (i.e. specificity of the effect) (Agrawal 2011). The efficacy of the induced defensive strategies could thus be notably altered when alien plants are exposed to new enemies in the introduced area. Native plants sharing an evolutionary association with native generalist enemies may have evolved a greater inducible defence against such enemies than alien plants have (Joshi & Vrieling 2005). Recognition mismatch by the alien plant could hinder induced responses and, consequently, reduce the fitness of the alien plant. Although constitutive as well as induced defences can contribute to effective resistance to herbivory, the interplay between the two defensive strategies has been rarely considered when explaining invasiveness of alien species (Orians & Ward 2010).

Pines (*Pinus* spp.) constitute a classic model in the study of traits associated with plant-invasion potential (i.e. invasiveness) (Richardson 2006). Pine invasiveness has been associated with small seed mass, short juvenile period, short interval between large seed crops and fast relative early growth rate (Rejmánek & Richardson 1996; Grotkopp, Rejmánek & Rost 2002). However, there is pronounced geographical variation in invasion success within species. For example, the North American *Pinus radiata* is invasive in many regions of the Southern Hemisphere (Lavery & Mead 1998), while in Europe, little evidence of invasion has been reported (Carrillo-Gavilán & Vilà 2010). Differences in invasiveness among biogeographical regions have been suggested to be related to different propagule pressure (Pyšek & Jarošík 2005), but might also depend on other factors such as allelopathy (Hierro & Callaway 2003) or differences in the interaction with natural enemies (Richardson & Higgins 1998). For example, in Europe, *P. radiata* is attacked by many generalist pests that also damage native pine species (Lombardero, Vázquez-Mejuto & Ayres 2008), while in the Southern Hemisphere native pines – and hence their associated specialist and generalist herbivores – are absent. In addition, as survival at early stages is key for future fitness of pine trees, early resistance of pine seedlings is a likely determinant for invasiveness.

In this study, we investigated the early resistance to generalist herbivores in seedlings of alien and native pine species to Europe within the context of the predictions of the NEH. The specific objectives were as follows: (i) to compare the damage caused by two generalist native insect herbivores in alien and native pine species, (ii) to evaluate quantitative differences between alien and native pine species in constitutive chemical defences and in those expressed after exposure to herbivory by those insects and (iii) to assess whether feeding preferences on alien and native pines shift after defences have been induced. We expected that: (i) generalist herbivores would cause lower damage to alien than to native pine species as predicted by the NEH; (ii) alien plants exposed to generalist

native herbivores would induce weaker defences than would native pines because a lack of shared evolutionary history and finally (iii) herbivory by native herbivores would decrease further herbivore consumption more in native than in alien pines.

## Materials and methods

To test the above-mentioned objectives, we measured the whole-plant damage caused by two insect generalist herbivores to living seedlings, evaluated the differences in constitutive and inducible chemical defences after the treatments of exposure to herbivores and assessed through cafeteria bioassays whether feeding preferences of both insects shifted after plant defences have been induced by exposure to the herbivores.

### STUDY SPECIES

Nine pine species belonging to the *Pinus* clade (Eckert & Hall 2006), which are broadly planted world-wide, were used for this study: *Pinus canariensis* C. Sm. (CAN), *Pinus halepensis* Mill. (HAL), *Pinus pinea* L. (PIN), *Pinus pinaster* Ait. (PTR) and *Pinus sylvestris* L. (SYL) are native to Europe; while *Pinus coulteri* D. Don (COU), *P. radiata* D. Don (RAD), *Pinus roxburghii* Sarg. (ROX) and *Pinus sabiniana* Douglas ex D. Don (SAB) are alien to Europe (DAISIE 2009). Seeds were purchased in the following nurseries: Intersemillas, Spain (<http://www.intersemillas.es>); Sheffield's Seed Co., USA (<http://www.sheffields.com>); and Les Semences Du Puy, France (<http://www.semencesdupuy.com>). For more information on the native distribution and seed geographical provenances of *Pinus* species assayed, see Table 1 of Appendix S1 (Supporting information).

The herbivores assayed were the large pine weevil, *Hyllobius abietis* L. (Coleoptera: Curculionidae) and the pine processionary caterpillar, *Thaumetopoea pityocampa* Dennis and Schiff (Lepidoptera: Thaumetopoeidae) (*Hyllobius* and *Thaumetopoea*, respectively, hereafter). Both herbivores were chosen because they attack a variety of coniferous species and thus are considered generalist conifer herbivores. Each herbivore feeds on conifer tissues with contrasting fitness value: *Hyllobius* attacks stem phloem while *Thaumetopoea* feeds on needles. Their native distributional range is within the geographical limits of Europe and concurs with native European *Pinus* species having high probability to attack them because of their extensive distribution area.

*Hyllobius* occurs naturally in Europe and northern Asia (Scott & King 1974) where it is one of the most harmful native pests affecting the regeneration of managed coniferous forests in many parts of Europe (Långström & Day 2004). This pine weevil is a polyphagous herbivore that feeds on the bark and phloem of young conifer seedlings (Wallertz 2005), mainly affects *Pinus* and *Picea* species, leading to high seedling mortality (Nordlander *et al.* 2003). *Hyllobius* can cause extensive tree mortality at young ages and major growth losses because of leader loss (Lieutier *et al.* 2004). As an example, the pine weevil has been estimated to cause the death of up to 80% of coniferous seedlings planted following clear-cutting (von Sydow & Birgersson 1997; Orlander & Nilsson 1999; Nordlander, Nordenhem & Hellqvist 2008).

*Thaumetopoea*, the pine processionary, is a defoliator naturally distributed in the Mediterranean Basin and southern Europe and may eventually feed on several coniferous genera. It attacks mainly plants of the genus *Pinus* (Devkota & Schimidt 1990). Its outbreaks inflict serious economic losses (Hódar, Zamora & Castro 2002). Larvae feed in conifer needles of juvenile and adult pines causing them to dry and

fall. Damage by the pine processionary weakens and reduces the growth of juvenile trees (Lieutier *et al.* 2004), lowers the reproductive capacity of adult ones (Hódar, Castro & Zamora 2003) and even causes death when trees are heavily defoliated. The ability of *Thaumetopoea* to develop varies widely depending on the pine species and its living conditions (Devkota & Schimidt 1990; Masutti & Battisti 1990; Hódar, Zamora & Castro 2002).

Adult weevils were trapped in the field following the method described by Moreira *et al.* (2008), stored in culture chambers at 15 °C and fed fresh twigs for maximum 2 weeks before the experiment began. Entire *Thaumetopoea* caterpillar nests were collected directly from infested trees, labelled and maintained as above.

### GREENHOUSE EXPERIMENTAL DESIGN

We conducted a two-factorial greenhouse experiment with pine species and induction of plant defensive responses by exposing living seedlings to two insect herbivores as the main factors (herbivory-induction treatments, hereafter). The experiment followed a randomized split-plot design replicated in 10 blocks, with herbivory-induction (three levels: control and herbivory by *Hyllobius* and by *Thaumetopoea*) as the whole factor and pine species (nine levels) as the split factor. However, because of lack of enough pine seedlings, only six pine species (CAN, PTR, SYL, COU, RAD and ROX) were induced with *Thaumetopoea*. In total, we grew 240 pine seedlings, corresponding to 10 blocks × two treatments (control and herbivory by *Hyllobius*) × nine species, plus 10 blocks × one treatment (herbivory by *Thaumetopoea*) × six species.

In October 2008, at the Forestry Research Centre of Lourizán (Pontevedra, Spain), 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, Haifa Chemicals Ltd., Israel) and covered with a 1- to 2-cm layer of sterilized sand. Pots were placed in a greenhouse with controlled light (12 h light) and temperature (10–25 °C at night and day, respectively) and daily irrigation. Plants were supplemented monthly with a solution of micronutrients to avoid nutritional deficiencies according to previous experience (see complete fertilization treatment in Table 2a of Appendix S2, Supporting information from Sampedro, Moreira & Zas 2011b). Foliar nutrition was applied monthly using chelated iron.

Before the herbivory-induction treatment began, weevils were kept individually without food for 48 h in labelled Petri dishes with a moist filter paper (15 °C, dark) and then weighed. Similarly, *Thaumetopoea* nests were carefully opened, 2nd-instar larvae randomly separated into groups of 10 caterpillars and starved and weighed as above. In September 2009, we applied the herbivory-induction treatments (herbivory by *Hyllobius* and by *Thaumetopoea*). In the herbivory by *Hyllobius*, one pre-weighed weevil was placed on each pine seedling, allowed to feed for 5 days and then removed and weighed again. Damage inflicted by the weevil 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 five sections per seedling (i.e. 0–15 score) was considered to be the debarked area. For the herbivory by *Thaumetopoea*, as caterpillars are gregarious and move little from the place where they are deposited, we placed one pre-weighed group of 10 caterpillars in the top section and other in the bottom section of each living pine seedling. Because of the smaller size of *P. sylvestris* seedlings, only one group of 10 larvae was placed on this species. Caterpillars were allowed to feed on the needles for 6 days and then removed, counted and weighed. Foliar damage was

evaluated for the whole plant in a three-level scale: 0 = undamaged leaves, 1 = damaged leaves < 5, 2 = damaged leaves > 5 (i.e. 0–2 score). Each plant of the three herbivory-induction treatments was carefully covered with a nylon mesh to avoid either herbivore escape or interference among treatments.

One week after the application of herbivory-induction treatments, all pine seedlings were harvested and directly weighed to determine the total above-ground plant biomass. Just after harvesting, two basal 2.5-cm-long stem sections and two subsamples (1.5 g each) of needles randomly from the whole pool of needles of each plant were collected, labelled and immediately stored in ice-coolers to be used in the cafeteria bioassays.

#### CHEMICAL ANALYSES

In the current paper, we analysed the concentration of non-volatile resin and total phenolics in the stem and needles. Additionally, we analysed the concentration of condensed tannins in needles after herbivory by *Thaumatopoea*. Long-lived plants such as pine trees, which must cope with multiple enemies simultaneously, are likely to employ quantitative defences (*sensu* Feeny 1976) that offer increased resistance in higher concentrations. Although the relevance of different types of chemical defences against diverse herbivores in conifers is not fully known, it is clear that these are involved in direct resistance against pine defoliators and stem borers (see reviews by Franceschi *et al.* 2005; Mumm & Hilker 2006). Indeed, in previous research with Mediterranean pine species, we found that non-volatile resin and phenolics are inducible, responsive to herbivore damage and confer resistance to weevil feeding (Moreira, Sampedro & Zas 2009; Sampedro, Moreira & Zas 2011a,b; Zas, Moreira & Sampedro 2011; Moreira, Zas & Sampedro 2011).

The concentration of non-volatile resin in stems and needles was estimated gravimetrically following the procedure proposed by Sampedro, Moreira & Zas (2011b) and expressed as mg of non-volatile resin per gram plant tissue dry weight (d.w.). A 5-cm-long section of the low part of the stem or 2 g of fresh needles of each plant was collected, weighed, immediately frozen and stored at  $-30^{\circ}\text{C}$ . Plant material was cut in small sections, and resin compounds were quantitatively extracted twice with *n*-hexane in an ultrasonic bath, after which the plant material was recovered by filtration (Whatman GFF, Whatman, Kent, UK), the solvent in the tubes was evaporated to dryness, and the mass of the non-volatile resin residue was determined gravimetrically with a precision scale. This gravimetric determination of non-volatile resin was well correlated with the concentration of the diterpenoids fraction as quantified by gas chromatography in previous trials ( $r = 0.921$ ,  $P < 0.0001$ ; Sampedro, Moreira & Zas 2011b).

The total phenolics in the needles and stem (and condensed tannins in the needles) were extracted and analysed as described by Sampedro, Moreira & Zas (2011b). A subsample of needles (*c.* 2 g dry weight) or of stem (*c.* 300 mg dry weight) was also weighed, oven-dried ( $45^{\circ}\text{C}$  to constant weight) and then manually ground in a mortar with liquid nitrogen. Briefly, needles were extracted with aqueous methanol (1 : 1 vol : vol) in an ultrasonic bath, and total phenolics in the methanolic extract were analysed by the Folin-Ciocalteu method, with colorimetric determination in a microplate reader at 740 nm using tannic acid as standard. The concentration of total phenolics is thus expressed as mg of tannic acid equivalents per gram plant tissue dry weight. Needle-condensed tannins in the aqueous methanol extracts were determined by the procyanidine method as in Baraza *et al.* (2004). The methanolic extract was mixed with acidified butanol and a ferric ammonium sulphate solution, allowed to react in a boiling water bath for 50 min and then cooled rapidly on ice. The concen-

tration of condensed tannins in this solution was determined colorimetrically in a Biorad 650 microplate reader at 550 nm, using as standard purified condensed tannins of quebracho (*Schinopsis balansae* Engl.; Droguería Moderna, Vigo, Spain). Needle concentration of condensed tannins was thus expressed as mg of quebracho equivalents per gram needle dry weight. Because of lack of plant material, *P. sylvestris* was not included in the chemical analyses of non-volatile resin in needles and total phenolics in stem.

#### CAFETERIA BIOASSAYS

Independent cafeteria bioassays were conducted with *Hylobius* and with *Thaumatopoea* in six pine species: CAN, PTR, SYL, COU, RAD and ROX. In the *Hylobius* bioassay, one weevil (starved and weighed as explained above) was allowed to feed on a 2.5-cm-long section of basal stem for 48 h in a Petri dish with a moist filter paper (dark,  $18^{\circ}\text{C}$ ). The volume of tissue ingested by the weevil ( $\text{mm}^3$ ) was estimated after measuring the debarked area by the weevil with a millimeter grid, and the depth of the gnawing with an electronic gauge. We conducted two replicates per plant, except for *P. sylvestris*, for which only one replicate was made because of insufficient plant material.

In the *Thaumatopoea* bioassay, groups of ten 2nd- or 3rd-instar caterpillars (starved and weighed as above) were allowed to feed on fresh needles for 7 days in Petri dishes as described above. A parallel series of needles was immediately dried at  $65^{\circ}\text{C}$  to constant weight to determine percentage of dry matter. The specific ingestion of needles (g needles d.w.) by the caterpillars was calculated as the dry food ingested. The percentage of caterpillar survival at the end of bioassays was also registered as an indicator of plant defences. As in the *Hylobius* bioassay, two replicates were performed per seedling except for *P. sylvestris*, for which we included only one replicate. In total, 330 Petri dishes were used in the bioassay (five species  $\times$  three treatments  $\times$  10 plants  $\times$  two replicates, and one species (SYL) with three treatments  $\times$  10 plants  $\times$  one replicate).

#### STATISTICAL ANALYSES

Differences among pine species in whole-plant damage to seedlings, measured as debarked area by *Hylobius* and foliar damage by *Thaumatopoea*, were analysed in treated plants using a generalized linear model:  $Y_{jk} = \mu + \text{SP}_j + B_k + \varepsilon_{jk}$ , where  $\mu$  is the general mean,  $\text{SP}_j$  and  $B_k$  are the main effects of the species  $j$  ( $\text{SP}_j = 1-9$  and  $\text{SP}_j = 1-6$  in herbivory for *Hylobius* and by *Thaumatopoea* treatments, respectively) and block  $k$  ( $B_k = 1-10$ , respectively). This analysis was performed with the PROC GLIMMIX procedure of the SAS System (Littell *et al.* 2006) with normal error and an *identity* link function, while differences among pine species in constitutive chemical defences were analysed in control untreated plants using the same equation model as before but using PROC MIXED procedure of the SAS System.

The effects of herbivory-induction treatments and pine species on chemical defences were analysed with the following mixed model:  $C_{ijk} = \mu + T_i + \text{SP}_j + B_k + T_i * \text{SP}_j + T_i * B_k + \varepsilon_{ijk}$ , where  $\mu$  is the overall mean,  $T_i$  and  $\text{SP}_j$  are the main effects of induction treatments  $i$  ( $T_i = 0, 1, 2$ ), species  $j$  ( $\text{SP}_j = 1-9$  and  $\text{SP}_j = 1-6$  in herbivory for *Hylobius* and by *Thaumatopoea* treatments, respectively),  $T_i \times \text{SP}_j$  and  $B_k \times T_i$  are the corresponding interactions and  $\varepsilon_{ijk}$  is the experimental error, while block  $k$  ( $B_k = 1-10$ ) and the  $B_k \times T_i$  interaction were considered random factors to analyse the main factor  $T$  with the appropriate error terms (Littell *et al.* 2006). Because of the different number of pine species included in the *Hylobius* and

*Thaumetopoea* herbivory-induction treatments, the effects of induction were analysed independently for each treatment together with the control. Plant biomass was previously checked not to covariate with the response variables and thus was not included in the model.

The cafeteria bioassays were analysed with an hierarchical mixed model in which we included the  $B_k \times T_i \times SP_j$  interaction as a random effect (split-split design), to account for the autocorrelation of the two Petri dishes used for each pine seedling (Littell *et al.* 2006), and to analyse the species factor with the appropriate degrees of freedom. Insect weight was included as a covariate in the analyses. Relationships between damage in cafeteria bioassays and chemical defences were carried out across pine species ( $n = 6$ ).

When there were significant differences between species, we analysed whether native pine species differed from alien species by including the factor Range (alien or native) and nesting the factor Species within the factor Range in the mixed models.

All analyses were performed with the PROC-MIXED procedure of the SAS System. When needed, normality was achieved by log-transforming the original variables. Equality of residual variance across treatments was tested in all cases, and residual heterogeneity variance models were used when significant deviations were found (Littell *et al.* 2006). Data are shown as least square means  $\pm$  standard error of the mean (SEM). When main effects or differences between species were significant, pairwise differences were tested for significance using the LSMEANS statement.

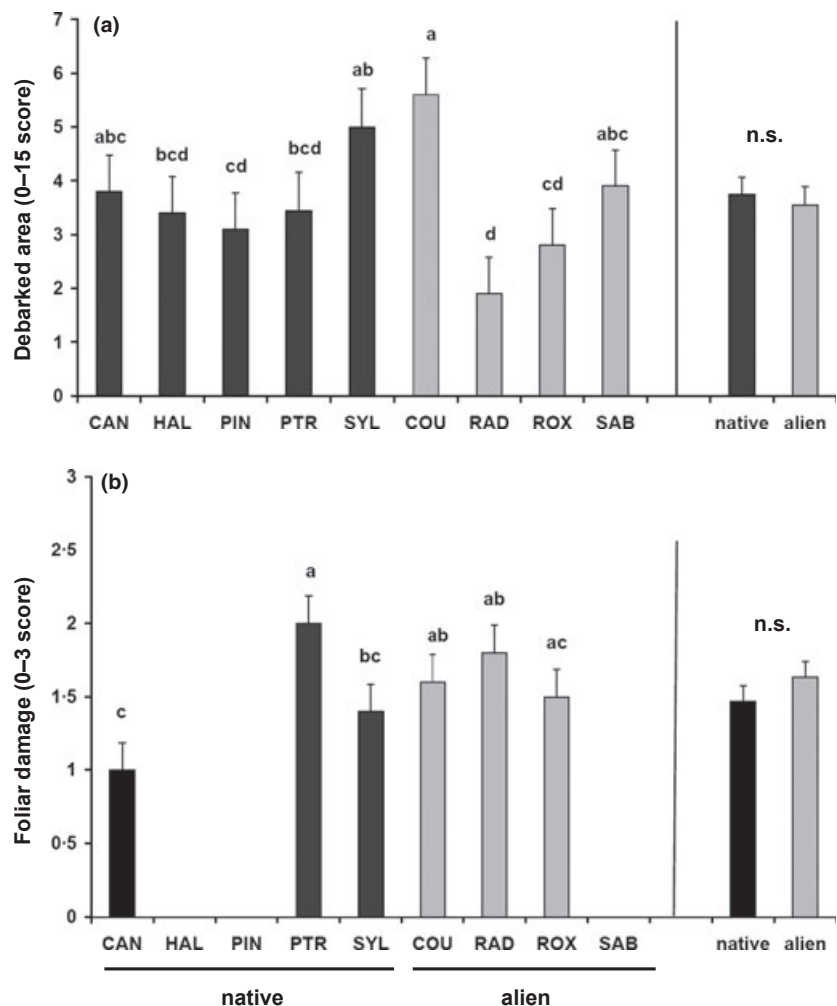
## Results

### SEEDLING DAMAGE

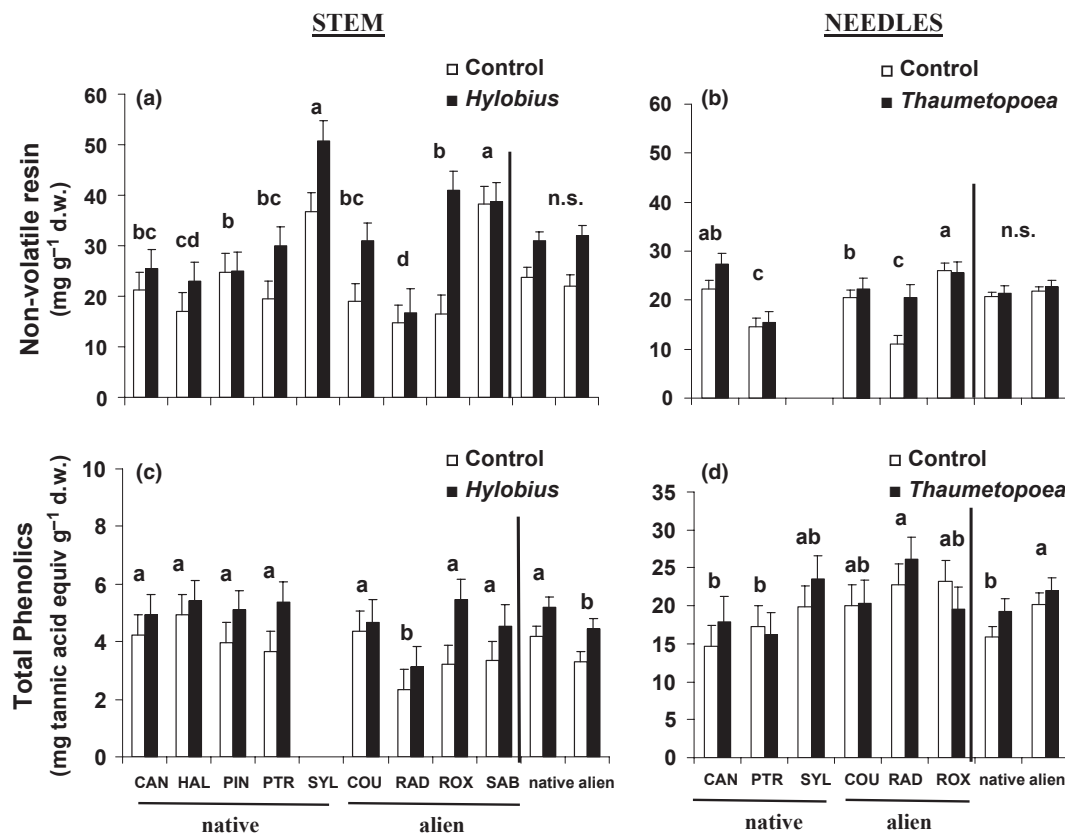
Seedling damage by *Hylobius* and *Thaumetopoea* after herbivory-induction treatments significantly differed among pine species (*Hylobius*:  $F_{8,70} = 2.66$ ,  $P < 0.05$ ; *Thaumetopoea*:  $F_{5,45} = 3.4$ ,  $P < 0.05$ ) (Fig. 1). The relative resistance of the pine species to both herbivore species was not consistent. For example, the least damaged pine by *Thaumetopoea* was *P. canariensis*, while stems of this pine species showed intermediate resistance to *Hylobius* (Fig. 1). Additionally, herbivore damage did not significantly differ between alien and native pine species (*Hylobius*:  $F_{1,70} = 0.19$ ,  $P = 0.66$ ; *Thaumetopoea*:  $F_{1,45} = 1.19$ ,  $P = 0.28$ ).

### CONSTITUTIVE AND INDUCED CHEMICAL DEFENCES

Constitutive concentration of most of chemical defences (undamaged seedlings) varied significantly among pine species (non-volatile resin in stem:  $F_{8,71} = 18.88$ ,  $P < 0.001$ , Fig. 2a; non-volatile resin in needles:  $F_{7,61} = 13.71$ ,  $P < 0.001$ , Fig. 2b; total phenolics in needles:  $F_{8,72} = 2.17$ ,



**Fig. 1.** Damage in European native (black bars) and alien (grey bars) pine species after exposure of living seedlings to real herbivory by (a) the pine weevil *Hylobius abietis* and (b) the pine processionary *Thaumetopoea pityocampa*. Bars are LS means  $\pm$  SEM. Different letters above columns indicate significant differences ( $P < 0.05$ ) among pine species or between range (native vs. alien). Species abbreviations: *Pinus canariensis* (CAN), *Pinus halepensis* (HAL), *Pinus pinea* (PIN), *Pinus pinaster* (PTR) and *Pinus sylvestris* (SYL) as native species, and *Pinus coulteri* (COU), *Pinus radiata* (RAD), *Pinus roxburghii* (ROX) and *Pinus sabiniana* (SAB) as alien species.



**Fig. 2.** Effects of the herbivory-induction treatments on the concentration of (a) non-volatile resin and (c) total phenolics in the stem in plants exposed to herbivory by the phloem feeder *Hylobius abietis*; and (b) non-volatile resin and (d) total phenolics in the needles in plants exposed to herbivory by the defoliating caterpillar *Thaumetopoea pityocampa*. The species are grouped into native and alien species in Europe. Bars are LS means  $\pm$  SEM. Different letters above columns indicate significant differences ( $P < 0.05$ ) among pine species or between range (native vs. alien). n.s. = non-significant differences. See Fig. 1 legend for species abbreviations.

$P = 0.04$ , Fig. 2d; condensed tannins in needles:  $F_{8,70} = 7.46$ ,  $P < 0.001$ ). *Pinus sabiniana* and *P. sylvestris* were the species with the greatest concentration of non-volatile resin in the stem (Fig. 2a). *Pinus roxburghii* showed the highest concentration non-volatile resin in the needles (Fig. 2b) while *P. radiata* and *P. roxburghii* showed the highest concentration both of total phenolics (Fig. 2d) and of condensed tannins in the needles. Only constitutive concentration of total phenolics in the stem did not significantly vary among pine species ( $F_{7,63} = 1.92$ ,  $P = 0.08$ ; Fig. 2c). Alien pine species registered significantly greater constitutive concentration of total phenolics and condensed tannins in the needles than did native pine species (total phenolics:  $F_{1,72} = 6.22$ ,  $P < 0.05$ , Fig. 2d; condensed tannins:  $F_{1,70} = 4.80$ ,  $P < 0.05$ ). Contrarily, native pine species showed significantly greater constitutive concentration of stem total phenolics than alien pine species ( $F_{1,63} = 4.76$ ,  $P < 0.05$ ; Fig. 2c). However, alien and native pines did not differ in their constitutive concentration of stem and needle non-volatile resin (stem non-volatile resin:  $F_{1,71} = 1.79$ ,  $P = 0.18$ , Fig. 2a; needle non-volatile resin:  $F_{1,61} = 0.78$ ,  $P = 0.38$ ; Fig. 2b).

The exposure to real herbivore by *Hylobius* affected the concentration of non-volatile resin and total phenolics in the stem (Table 1a). Overall, the concentration of non-volatile resin in seedlings exposed to *Hylobius* was 25% greater than those in

undamaged control seedlings (Fig. 2a). Moreover, the induction of stem non-volatile resin after herbivory by *Hylobius* differed among the pine species, as indicated by the significant species  $\times$  treatment interaction (Table 1a, Fig. 2a). Non-volatile resin concentrations in the stem of *P. pinaster*, *P. sylvestris*, *P. coulteri* and *P. roxburghii* significantly increased after exposure to the weevil, while no significant changes were detected in the other pine species (Fig. 2a). We found no differences between alien and native pine species ( $F_{1,137} = 1.21$ ,  $P = 0.27$ ). The concentration of total phenolics in the stem differed between treatments and among species (Table 1a). Overall, the concentration of total stem phenolics in seedlings exposed to *Hylobius* was 23% greater than those in undamaged control seedlings (Fig. 2c). *Pinus halepensis* and *P. radiata* had the highest and the lowest concentration of total phenolics in stem, respectively. Moreover, native pines showed significantly greater total phenolics concentration than alien pines ( $F_{1,120} = 5.53$ ,  $P < 0.05$ , Fig. 1b).

After real herbivory by *Thaumetopoea*, only non-volatile resin in needles significantly differed between treatments and among species (Table 1b, Fig. 2b,d). Pine species exposure to herbivory by *Thaumetopoea* had greater non-volatile resin concentration in needles than control seedlings, and *P. roxburghii* and *P. pinaster* showed the greatest and lowest induction of non-volatile resin concentration in needles,

**Table 1.** Results of the mixed models with species and herbivory-induction treatment as fixed factors, and concentration of non-volatile resin and of total phenolics as dependent variables. Analyses were performed independently to compare control plants and (a) pine seedlings with induced herbivory from the large pine weevil *Hylobius abietis* and (b) pine seedlings with induced herbivory from caterpillars of the pine processionary moth *Thaumetopoea pityocampa*. Significant P-values ( $P < 0.05$ ) are given in bold

(a) Nine pine species in control and after herbivory-induction by *Hylobius*

Source	Non-volatile resin in the stem			Total phenolics in the stem		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
Block	9, 9	0.43	0.885	9, 9	1.23	0.383
Treatment ( <i>T</i> )	1, 9	12.55	<b>0.006</b>	1, 9	9.23	<b>0.014</b>
Species (SP)	8, 137	13.33	<b>&lt; 0.0001</b>	7, 120	2.12	<b>0.046</b>
<i>T</i> *SP	8, 137	2.46	<b>0.015</b>	7, 120	0.42	0.891

(b) Six pine species in control and after herbivory-induction by *Thaumetopoea*

Source	Non-volatile resin in the needles			Total phenolics in the needles		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
Block	9, 9	1.93	0.17	9, 9	2.66	0.080
Treatment ( <i>T</i> )	1, 9	4.72	<b>0.058</b>	1, 9	0.31	0.590
Species (SP)	4, 68	8.52	<b>&lt; 0.0001</b>	5, 85	2.28	<b>0.053</b>
<i>T</i> *SP	4, 68	1.5	0.21	5, 85	0.52	0.759

respectively (Fig. 2b). However, we found no differences between alien and native pine species (non-volatile resin in needles:  $F_{1,68} = 0.42$ ,  $P = 0.51$ ). The concentration of condensed tannins in the needles was not affected by *Thaumetopoea* treatment ( $F_{1,9} = 0.10$ ,  $P = 0.76$ ).

In addition, chemical defences in needles against *Hylobius* herbivory and in stem against *Thaumetopoea* herbivory showed no significant changes after exposure to both generalist herbivores (see Table 2 and Fig. 1 of Appendix S2, Supporting information).

#### CONSUMPTION PREFERENCES IN THE CAFETERIA BIOASSAYS

Pine species differed significantly in the consumed volume of stem by *Hylobius* in the cafeteria bioassays (Table 2a). *Pinus sylvestris* was the least damaged while *P. coulteri* and *P. canariensis* were the most (Fig. 3a). We found a marginally significant negative relationship (at the species level) between the amount of tissue consumed by *Hylobius* in the cafeteria bioassays and the concentration of non-volatile resin in the stem ( $r^2 = 0.46$ ,  $P = 0.06$ ,  $n = 6$ ). Consumption by *Hylobius* was not significantly affected by the herbivory-induction treatment (Table 2a). Specifically, stems from seedlings previously exposed to herbivory by *Hylobius* were consumed as much as were those from control seedlings (Fig. 3a). How-

**Table 2.** Results of the mixed model for the cafeteria bioassays with species and herbivory-induction treatment as fixed factors. Analyses were performed independently to compare control plants and (a) the consumed volume of stem by the large pine weevil *Hylobius abietis* and (b) the specific ingestion of needles and caterpillar survival of the pine processionary *Thaumetopoea pityocampa* as dependent variables. Nine pine species were tested with the weevil, and six were tested with the caterpillar, so that independent analyses were performed for each insect bioassay. Significant P-values ( $P < 0.05$ ) are given in bold

(a) *Hylobius* cafeteria bioassay

Source	Consumed volume of stem		
	d.f.	<i>F</i>	<i>P</i>
Block	9, 9	5.34	<b>0.01</b>
Treatment ( <i>T</i> )	1, 9	0.23	0.645
Species (SP)	5, 89	8.71	<b>&lt; 0.0001</b>
<i>T</i> *SP	5, 89	0.51	0.766
Insect weight	1, 94	6.36	<b>0.013</b>

(b) *Thaumetopoea* cafeteria bioassay

Source	Specific ingestion of needles			Caterpillar survival		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
Block	9, 9	4.51	<b>0.017</b>	9, 9	0.71	0.690
Treatment ( <i>T</i> )	1, 9	0.01	0.938	1, 9	0.31	0.593
Species (SP)	5, 89	12.09	<b>&lt; 0.0001</b>	5, 90	7.65	<b>&lt; 0.0001</b>
<i>T</i> *SP	5, 90	1.42	0.225	5, 91	0.67	0.643
Insect weight	1, 64	1	0.320	1, 65	27.35	<b>&lt; 0.0001</b>

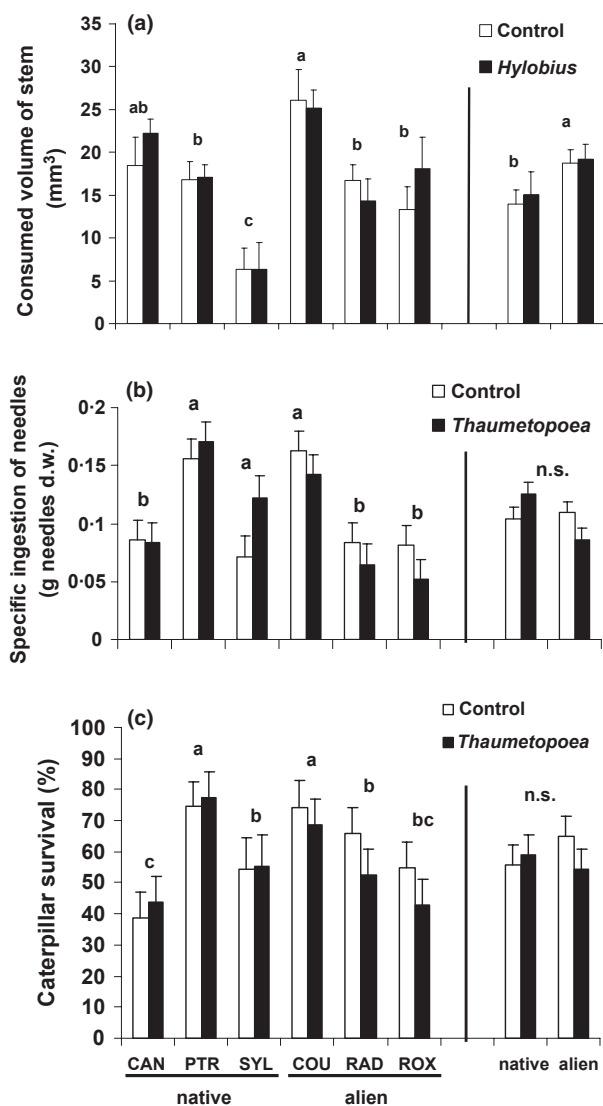
ever, alien and native pine species significantly differed in consumption ( $F_{1,89} = 7.69$ ,  $P < 0.005$ ), with alien pines on average were consumed more than native pine species.

Pine species also differed significantly in the ingestion of needles by *Thaumetopoea* caterpillars (Table 2b). Less-consumed needles had higher concentrations of condensed tannins ( $r^2 = 0.5$ ,  $P < 0.05$ ,  $n = 6$ ). Specifically, *P. pinaster* and *P. coulteri* were the most consumed pine species (Fig. 3b). These two pine species also showed the greatest caterpillar survival (Fig. 3c). Specific ingestion of needles and caterpillar survival were not significantly affected by herbivory-induction treatment (Table 2b; Fig. 3b,c). Also, specific ingestion of needles did not differ between native and alien pine species ( $F_{1,89} = 2.96$ ,  $P = 0.08$ ).

## Discussion

Given that alien pine seedlings were not less damaged than native pines when exposed to native generalist herbivores, our results do not support the predictions of the NEH. Our observations agree with a recent meta-analysis that found no differences in the damage caused by native herbivores with respect to coexisting alien and native species (Chun, van Kleunen & Dawson 2010).

*Hylobius* has been documented to feed on several conifer species, but there are no available studies that simultaneously



**Fig. 3.** Results of the cafeteria bioassays showing the feeding activity of *Hylobius abietis* (a), and feeding activity (b) and performance (c) of *Thaumetopoea pityocampa* when feeding on pine tissues from control seedlings (white bars) and seedlings previously induced by these insects (black and grey bars). Pine species are grouped from native to alien species in Europe. Bars are LS means  $\pm$  SEM. Different letters above columns indicate significant differences ( $P < 0.05$ ) among pine species or between range (native vs. alien). See Fig. 1 legend for species abbreviations. Insect weight was included as covariate in the models.

compare several alien and native pine species with regard to the preference of this insect. Under field conditions, *Hylobius* significantly feeds more on alien *P. radiata* than on native *P. pinaster* seedlings (Zas et al. 2006, 2008). However, studies evaluating the damage that other insect species cause to alien and native pines showed opposite results. For example, observational field studies have shown that the bark beetle *Tomicus piniperda* L. (Coleoptera: Curculionidae) attacks *P. pinaster* more than *P. radiata* (Lombardero, Vázquez-Mejuto & Ayres 2008).

With respect to herbivory by *Thaumetopoea*, it reportedly feeds on several pine species across Europe, but the results on

the preference of this insect for different species are also contradictory. For example, in Greece, Avtzis (1986) found different resistance against this caterpillar among five pine species, *P. radiata* and *P. pinea* being the most and the least consumed, respectively. On the contrary, in Italy, *Thaumetopoea* caterpillars showed no preferences between different alien and native pine species (Tiberi et al. 1999).

Our results show that constitutive concentration of chemical defences differed among pine species, with greater concentrations of constitutive total phenolics in the stems of native pine species. The opposite trend was observed for constitutive phenolic compounds in the needles. Herbivory by *Hylobius* largely increased the concentration of non-volatile resin and total phenolics in the stem of some species. Moreover, as we hypothesized, native pines showed significantly greater concentration of total phenolics in stems than alien pines. However, some alien pine species surprisingly showed a quantitative induction of chemical defences in the stem after herbivory by *Hylobius* (see Fig. 2a,c), especially for non-volatile resin concentration. Increasing induced responses in those alien pines might be for several reasons. First, the coexistence of other insect herbivores belonging to the same feeding guild as *Hylobius*, such as *Pissodes* spp. in North America, might explain the induction of stem non-volatile resin by the weevil in the American origin *P. coulteri*. Secondly, the resource availability hypothesis (Coley, Bryant & Chapin 1985) assumes that fast-growing species will be less defended because they invest more in growth than do slow-growing species, which invest more in defences under the same environmental conditions (Blumenthal 2006). According to the pine classification of Rejmánek & Richardson (1996) and Grotkopp, Rejmánek & Rost (2002), only *P. radiata* is considered an invasive and fast-growing species in many parts of the world, while the alien pines studied here are considered mainly non-invasive species and slow-growing species. Thus, non-invasive species might be better defended than *P. radiata*, which might invest more in growth than in defences. On the other hand, the *Thaumetopoea* treatment only significantly increased the concentration of non-volatile resin in needles, without significant differences in inducibility between alien and native pines. However, the increase of needle non-volatile resin after the *Thaumetopoea* treatment was apparent in only a few species (see Fig. 2b). The weak quantitative induction of chemical defences by *Thaumetopoea* treatment might be due to several non-exclusive factors. First, our pine species might have not been damaged long enough by herbivores to provoke a defensive response (Gardner & Agrawal 2002; Underwood, Anderson & Inouye 2005). Second, although it is known that measurable responses to *Hylobius* by native pines can be found within 48 h (Sampedro, Moreira & Zas 2011a), the response of pines to *Thaumetopoea* caterpillars might be slower, requiring a greater lag time between the damage and the induced response (Underwood, Anderson & Inouye 2005). Furthermore, there is also a lag time from the translocation of the signal from damaged to undamaged leaves (Howe & Schaller 2008); the activation of a defensive response in undamaged tissues might require more time than



the length of our experiment (6 days) for quantitative changes in secondary chemical metabolites to be detected. Besides, the induction of defences might involve qualitative changes in secondary compounds rather than quantitative changes (Petrakis *et al.* 2005; Sampedro *et al.* 2010).

We expected that prior exposure of the seedlings to herbivores would influence the consumption of both generalist herbivores in the bioassays. When comparing alien vs. native pines, only in the *Hylobius* cafeteria bioassays were alien pine species consumed more than native species were, while no differences among range origin were found in the *Thaumetopoea* cafeteria bioassays. This difference in the *Hylobius* bioassays might be explained by the greater induction of total stem phenolics in native pine species after bark weevil attack. Perhaps inducible defences in native pines are more responsive to damage by native generalist enemies than those in alien pines, because of a shared evolutionary history (Joshi & Vrieling 2005; Zas, Moreira & Sampedro 2011). Cafeteria bioassays with insects provide useful information, but it should be noted that some other factors could affect the accuracy of the results. In other studies, cafeteria bioassays using *Hylobius* in *P. radiata* and *P. pinaster* have registered significant differences in consumption between species and induction treatments (Moreira, Sampedro & Zas 2009; Zas, Moreira & Sampedro 2011). However, our results from bioassays performed *Thaumetopoea* were not conclusive, because, although differences between some species were found, the herbivores showed little sensitivity to changes in the nutritional quality or other physical or chemical properties of the tissues offered, as reported in previous studies (Hódar, Zamora & Castro 2002). However, very little is known about the ability of pine trees to produce induced responses against those insects, as reflected by the present work.

Our study reports early plant resistance and response to generalist herbivores in several alien and native woody species. Overall, our results did not support the assumption based on the NEH that native herbivores would avoid alien plants because of a lack of recognition. Results from other studies are somewhat contradictory. Some show either no evidence that generalist herbivores avoided alien plants (Morrison & Hay 2011) or, by contrast, they show that generalist herbivores have greater impact on native than on alien species (Schaffner *et al.* 2011). Our findings agree with a recent meta-analysis that found no differences in the damage caused by native herbivores with respect to coexisting alien and native species (Chun, van Kleunen & Dawson 2010).

Our results suggest that inducible defences do not protect alien pines, in regions where they coexist with native congeners. This agrees with Lind & Parker (2010), who compared chemical deterrents in several invasive and co-occurring native plant species and found that alien species did not show higher deterrence to native generalist herbivores than did native species. Native generalist herbivores might be one reason why there is a low incidence of invasion by alien pines in Europe (Carrillo-Gavilán & Vilà 2010).

## Acknowledgements

We thank B. Santos, D. Blanco, A. García, L. Martínez, A. Pazos and E. Dios for greenhouse and laboratory assistances; S. Piñeiro and M. J. Villanueva for field assistance and maintenance of insects; and P. González, E. Manzano, A. Montero and A. Montesinos for their comments on a previous draft of the manuscript. Research was partially funded by the Spanish Ministerio de Ciencia e Innovación, projects Consolider-Ingenio MONTES (CSD2008-00040), COMPROPIN (AGL2010-18724) and CSIC (project 2008401153).

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Received 25 February 2011; accepted 27 September 2011

Handling Editor: Alison Brody

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Appendix S1.** Information on native distribution and geographical seed provenances of the *Pinus* species assayed.

**Appendix S2.** Results of the chemical analyses in needles for herbivory by *Hylobius abietis* and in stem for herbivory by *Thaumetopoea pityocampa*.

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