

1 **Tolerance and induced resistance in a native and an exotic pine species:**
2 **relevant traits for invasion ecology**

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4 Running title: Tolerance and induced defences in invasion ecology

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19 **Summary**

20 **1.** Current hypotheses predict contrasting roles for natural enemies in determining the success or
21 failure of plant invasions. Differences in plant induced resistance and tolerance to native
22 herbivores between native and exotic species might contribute to resolve this controversy.

23 **2.** We examined the differences between the native *Pinus pinaster* and the exotic *P. radiata* in
24 constitutive resistance, inducibility of chemical defences, realized resistance and tolerance to
25 the large pine weevil *Hylobius abietis* in NW Spain. In this region, both pine species closely
26 coexist and are threatened by the weevil, a harmful phloem feeder that causes extensive
27 mortality and growth reduction in young pine stands.

28 **3.** We performed two *in vitro* cafeteria bioassays, two induction experiments with direct
29 exposure to the weevil and spraying methyl-jasmonate and an exhaustive field study of the
30 genetic variation in tolerance and resistance in forestry genetic trials.

31 **4.** The weevil significantly preferred the native to the exotic pine when twigs were offered as
32 cut material in Petri dishes. However, the pattern in the field was the opposite, with greater
33 damage on the exotic. Inducibility of stem oleoresin did not differ between species when
34 elicited by the application of methyl-jasmonate. However, after a 72-h experimental exposure to
35 the weevil, stem resin content in the native pine was double that in the exotic pine, suggesting a
36 lower capability of the exotic pine to respond to the insect damage. In the field, family
37 relationships between early damage and several pine fitness correlates revealed a significantly
38 greater tolerance of the native pine to the insect damage. Furthermore, only the native pine
39 showed genetic variation in tolerance to the damage.

40 **5. Synthesis.** The preference of the herbivore for the native species was counterbalanced by a
41 lower capability for expressing induced resistance to the weevil and reduced tolerance in the
42 exotic species, resulting in no apparent fitness advantage of the exotic *P. radiata* over the native
43 *P. pinaster*. Differences in inducibility by and tolerance to native enemies between exotic and

44 native host congeners emerge as key traits for understanding how native enemies might
45 contribute to preventing an introduced species from becoming invasive.

46

47 **Key-words:** biological invasions, biotic resistance hypothesis, enemy release hypothesis,
48 exotic plants, herbivory, *Hylobius abietis*, invasion ecology, *Pinus pinaster*, *Pinus radiata*,
49 plant–herbivore interactions

50

51 **Introduction**

52 There are currently several much-debated hypotheses about the differences that should be
53 expected in the impact of specialist and generalist herbivores on native and exotic plant
54 species and how these differences may explain the invasive behaviour of some exotics when
55 planted out of their natural ranges (Catford, Jansson & Nilsson 2009 and references therein).

56 In particular, the enemy release hypothesis (ERH) predicts a lower impact of herbivores on
57 introduced plants because specialist enemies of the introduced plants are absent in the new
58 range, and generalist enemies would be better adapted to consume native than novel exotic
59 species (Keane & Crawley 2002, Maron & Vilá 2001). Alternatively, the biotic resistance
60 hypothesis (BRH) predicts that native herbivores can limit invasions as introduced species
61 may be poorly adapted for defending against them (Elton 1958, Maron & Vilá 2001).

62 Lacking a common evolutionary history with the resident herbivores, exotic plant species
63 may be particularly vulnerable to the new enemies in the introduced range, as they have
64 been not selected to evolve defences against them (Parker, Burkepile & Hay 2006).

65 These hypotheses have been widely tested in different plant–animal systems, and
66 results are controversial (Colautti *et al.* 2004, Chun, van Kleunen & Dawson 2010, Keane &
67 Crawley 2002, Levine, Adler & Yelenik 2004, Liu & Stiling 2006, Maron & Vilá 2001,
68 Parker, Burkepile & Hay 2006, Parker & Hay 2005). There is increasing evidence that

69 richness of herbivorous insects, particularly of specialists, is greater in the native than in the
70 introduced ranges. This apparent release from enemies, however, is only partially translated
71 into a lower incidence of herbivory on the exotics than on the native congeners (Colautti *et*
72 *al.* 2004, Liu & Stiling 2006), whereas differences in damage may not necessarily result in
73 greater performance of the introduced species (Chun, van Kleunen & Dawson 2010).
74 Results are especially variable and inconclusive when the incidence of herbivory in exotic
75 and native congeners occurring in the same community is compared (community studies
76 *sensu* Colautti *et al.* 2004). Some studies found exotics to be more damaged or more
77 impacted in terms of fitness by herbivores than were native species (e.g. Morrison & Hay
78 2011, Parker & Hay 2005), whereas others found the opposite (e.g. Hill & Kotanen 2009,
79 Siemann & Rogers 2003). These apparently contradictory results have prompted
80 considerable research efforts during recent decades. Surprisingly little attention, however,
81 has been paid to exploring the role played by different plant defensive strategies in
82 explaining the observed patterns.

83 Plant defences against herbivores include two different and complementary
84 mechanisms: those aiming for reducing the damage (resistance) and those aiming for
85 reducing the impact of damage on plant fitness (tolerance). Resistance can be further
86 divided into constitutive resistance, which is permanently expressed irrespective of the
87 incidence of herbivores, and induced resistance, which includes traits that are activated in
88 response to herbivore attack. All these mechanisms are considered adaptive and have
89 evolved in response to the selection pressure imposed by the herbivores with which they
90 have shared an evolutionary history (Núñez-Farfán, Fornoni & Valverde 2007). Although
91 the simultaneous evolution and expression of different mechanisms can be constrained
92 (Koricheva, Nykanen & Gianoli 2004, Leimu & Koricheva 2006), the combination of
93 different defensive strategies appears to be quite common and advantageous, especially in

94 trees (Leimu & Koricheva 2006). Their large size and long lifespan make trees very
95 apparent to a wide range of herbivores, and commonly their resistance mechanisms do not
96 completely exclude damage (Haukioja & Koricheva 2000).

97 Interpretation of the results from studies on invasion ecology may substantially
98 differ if different plant defensive strategies are considered (Ashton & Lerdau 2008, Chun,
99 van Kleunen & Dawson 2010). For instance, Lombardero *et al.* (2008) found that the attack
100 of a bark beetle was significantly greater in a native pine than in a co-occurring exotic pine,
101 but the impact of the insect on pine growth was notably greater in the exotic. Thus, their
102 results support the predictions of the ERH when damage (i.e. resistance) is considered, but
103 they agree with the predictions of the BRH when the impact of herbivores on plant fitness
104 (i.e. tolerance) is taken into account. Native and introduced species can therefore differ in
105 how they tolerate the damage inflicted by native enemies. However, to date, little attention
106 has been paid to exploring how these differences may influence the likelihood of a given
107 introduced species of becoming invasive (Ashton & Lerdau 2008, Chun, van Kleunen &
108 Dawson 2010).

109 On the other hand, induced responses to herbivory caused by native insects may be
110 elicited more strongly in native than in alien plants (Stout & Bostock 1999). There is
111 evidence that plants have evolved sophisticated mechanisms to detect herbivore-derived
112 molecules that act as signals of herbivore damage and mediate the specificity in the
113 response in plant defence responses (Heil 2009, Hilker & Meiners 2010). Induced responses
114 to the damage of native herbivores may therefore be more efficiently elicited in native plant
115 species, which have coevolved with the herbivore, than in exotics, which may lack
116 mechanisms to recognize the native insect as a harmful enemy. This would imply that native
117 herbivores could limit invasions, as suggested by the BRH, but as far as we know, no
118 previous studies have specifically tested this appealing idea.

119 The aim of this paper was to explore the role of different defensive strategies in
120 explaining the diverse impact of a harmful native insect herbivore on the native *P. pinaster*
121 and the exotic *P. radiata*. Specifically, we analysed the differences between the two pine
122 species in i) constitutive resistance to this insect, by means of *in vitro* bioassays, ii)
123 inducibility of chemical defences elicited by chemical elicitors and insect feeding, iii)
124 realized resistance (constitutive plus induced) and iv) short- and medium-term tolerance to
125 the damage, and their genetic variation, under field conditions. Although invasive in many
126 countries of the southern hemisphere, *P. radiata* is not invasive in Europe (Carrillo-Gavilán
127 & Vilá 2010). It has been suggested that the populations of phytophages and pathogens from
128 closely related host species in the introduced area might contribute to the inability of this
129 species to invade in Europe, but this hypothesis remains untested. We aimed to gain insight
130 into the implications of constitutive and induced resistance and of tolerance and resistance
131 strategies in explaining how native herbivores might contribute to prevent an introduced
132 species from becoming invasive.

133

134 **Material and Methods**

135 *Study system*

136 Our study was carried out in the coastal area of Galicia (NW Spain), where two main pine
137 species currently dominate the forested area: the native *Pinus pinaster* and the introduced *P.*
138 *radiata*. The former, Maritime pine, occurs naturally in the western part of the
139 Mediterranean basin, where it is an important forest species with high ecological and
140 economic value. The natural distribution of radiata pine is restricted to a very small area in
141 California (US). Nowadays, however, it is among the most widely planted tree species in the
142 world, especially in the southern hemisphere (Lavery & Mead 1998), where no pine species
143 occurs naturally, and where this pine species is considered a threatening invasive plant

144 causing important economic and ecological problems (Richardson & Higgins 1998). In
145 Spain, *P. radiata* was introduced in the nineteenth century, although it was not widely
146 planted until the mid-twentieth century. Here, however, it rarely spreads from planting sites
147 (Carrillo-Gavilán & Vilá 2010).

148 In Spain, both pine species coexist closely, are planted in similar sites and exhibit
149 similar ecological habits. They are light pioneer species with high growth rates and similar
150 dispersal strategies (Barberó *et al.* 1998, Lavery & Mead 1998). As in other sun-loving
151 colonizer species, early growth is related to plant fitness, because only the fast growers can
152 take their crowns to the vital sunlight and thus survive and reproduce. Because they are
153 obligate seeders living in fire-prone regions, early reproduction is also crucial for fitness of
154 the species (Barberó *et al.* 1998, Richardson & Higgins 1998).

155 In Spain, both pines are attacked by diverse insect herbivores that cause important
156 growth reduction and mortality, especially at the seedling stage. The native large pine
157 weevil, *Hylobius abietis*, in particular, stands out as an herbivore that directly affects pine
158 fitness by causing extensive tree mortality at young ages (Lieuter *et al.* 2004). This pine
159 weevil is a polyphagous herbivore that feeds on the bark and phloem of conifer seedlings
160 and small branches of adult trees, mainly *Pinus* sp. and *Picea* sp. If the damage is intense,
161 the wounds on the stems of young seedlings may cause stem girdling and, consequently, the
162 loss of the leader and even the death of the seedling (Lieuter *et al.* 2004). This weevil occurs
163 naturally in Europe and northern Asia where it is one of the most harmful pests affecting the
164 regeneration of managed coniferous forests. Clear-felling of conifers provides ideal
165 conditions for weevil breeding, leading to outbreaks that can severely damage the natural or
166 artificial regeneration in the cleared area (Nordlander 1991). After one or two years, adults
167 fly away to find new breeding sites. Although weevils can also feed on small branches of
168 adult stands and in young stands established on areas not previously occupied by conifers,

169 the damage inflicted on those stands, and its impact on plant fitness, is minimal (Orlander *et*
170 *al.* 2000). Indeed, this insect herbivore is considered a ‘silvicultural pest’ that causes serious
171 damage only after the clear-felling of large areas of conifers (Lieuter *et al.* 2004). Genetic
172 variation in resistance to this insect has been found in both pine species (Zas *et al.* 2008, Zas
173 *et al.* 2006), although the weevil has only coexisted with the native *P. pinaster*, not with the
174 exotic *P. radiata* (Lieuter *et al.* 2004).

175 To address our research questions we used three different and independent
176 experimental approaches. First, we evaluated the realized resistance and tolerance to *H.*
177 *abietis* damage in an extensive field genetic trial with both pine species naturally infected by
178 the weevil, where damage, subsequent growth and other fitness correlates were assessed
179 over five years. Additionally, we studied pine growth in a neighbouring identical twin trial
180 not attacked by the weevil. Second, the preference of adult weevils for cut twigs of the two
181 pine species was evaluated in *in vitro* feeding bioassays. Third, differences in inducibility
182 between the two pines were evaluated by assessing the increase in concentration of chemical
183 defences (stem resin) after exposing the pine seedlings to either chemical induction with
184 methyl jasmonate (MeJa) or biotic induction by direct *H. abietis* feeding.

185

186 *Field trials*

187 A family × fertilization trial of *P. pinaster* and *P. radiata*, located in Rianxo (Galicia, NW
188 Spain; 42.36° N; 8.46° W; altitude 90 m a.s.l.) and planted in March 2003, was naturally
189 infected by the large pine weevil *H. abietis* soon after planting. The site had previously been
190 occupied by a mature stand of *P. pinaster*, clearcut in 2002. Weevils were attracted to the
191 cut area by the volatiles emanating from the fresh conifer stumps, where females lay their
192 eggs (Nordlander 1991). Both the newly arrived adults and the new generation emerging
193 from the stumps caused an intensive *H. abietis* attack on the established seedlings that lasted

194 until the end of the second season. All the seedlings of the two trials had the appropriate size
195 for being fed on by the weevil, with diameter distributions at the time of damage (99%
196 confidence intervals of 4.8-16.6 mm and 4.4-15 mm for *P. radiata* and *P. pinaster*,
197 respectively) within the range of twig diameters that the weevil prefers (Orlander *et al.*
198 2000). The spatial analysis of the damage severity across all the seedlings in the trial
199 revealed that the infestation was spatially uniform in the experimental area (see variograms
200 in Zas *et al.*, 2006, 2008).

201 Two adjacent independent trials were established, one where the main species was *P.*
202 *radiata* and *P. pinaster* was used as a control, and the other with *P. pinaster* as the main
203 species and *P. radiata* as the control. The experimental layout, exactly the same for both
204 species, consisted of a split-plot design superimposed across a randomized block design
205 with 10 blocks, with 9 fertilization treatments randomly assigned to the 9 whole plots in
206 which each block was divided and 32 genetic entries randomly distributed within each
207 whole plot. Fertilization (see details in Zas *et al.* 2006, 2008) was not a central focus of the
208 present study but was considered in the statistical analyses to account for the corresponding
209 variation.

210 The plant material for each species consisted of open-pollinated families of mother
211 trees from natural stands or mature plantations in the coastal area of Galicia. In the *P.*
212 *pinaster* section, the 32 genetic entries included 28 half-sib families and 3 commercial
213 seedlots of *P. pinaster* without family structure. One mixed seedlot of *P. radiata* was
214 interspersed within the *P. pinaster* seedlings to complete the 90 main rectangular
215 experimental units (whole plots) of 31+1 plants (8 columns \times 4 rows). Conversely, in the *P.*
216 *radiata* section there were 30 half-sibs and 1 commercial seedlot of *P. radiata*, with a mixed
217 seedlot of the material included in the attached *P. pinaster* experiment. In total, within each
218 trial, 2790 seedlings of the main pine species were interspersed with 90 seedlings of the

219 opposite species, providing a unique experimental design for studying the feeding behaviour
220 of *H. abietis* on these species.

221 Plant growth (total height and basal stem diameter) was measured yearly from
222 December 2003 to December 2007 (1 to 5 growing seasons after planting) in all plants of
223 the two trials. Reproductive effort of each tree was also evaluated at age five and considered
224 as another early fitness correlate. Reproductive effort was assessed according to Climent *et*
225 *al.* (2008) as the total count of cones per tree, including the two female strobili cohorts that
226 grow together in the crown.

227 *Hylobius abietis* damage was measured after the first growing season (February
228 2004) by evaluating the wounds in the stem caused by the insect. The stem height of each
229 tree was divided in 10 sections; the damage was measured in each section according to a
230 four-level wounding scale (0 = undamaged, 1 = some wounds, 2 = many wounds, and 3 =
231 full of wounds or death due to stem girdling below the given section). The weevil damage
232 on each plant was the sum of the 10 stem sections (0-30 score, Zas *et al.* 2006). We assumed
233 that the lower the site of the stem girdling, the smaller the amount of living tissue remaining
234 alive, and thus the greater the damage caused to the plant. We accounted for this by
235 assigning the maximum score to all stem sections recorded above the site of girdling.

236

237 *Cafeteria* experiments

238 Two independent *in vitro* feeding bioassays, a two-choice and a non-choice test, were
239 performed to test for differences in the preference of the weevil for the two species.

240 Ingestion rate (debarked area) was considered a proxy of constitutive resistance against the
241 pine weevil between the two pine species.

242 Plant material for both experiments was randomly selected from the open-pollinated
243 families included in the field trials. Seeds were sown in May 2006 and seedlings were

244 cultivated in 2-L pots filled with 1:1 peat:perlite (v:v) in a greenhouse, following
245 conventional nursery watering and fertilizing protocols. One year after they were sown,
246 seedlings of each pine species were grouped in pairs of similar basal diameter and
247 harvested; needles were gently removed and stems placed on ice in coolers.

248 For the two-choice experiment, three 2.5-cm long twigs (upper, middle and lower
249 thirds of the stem) from each seedling were sampled and paired with twigs belonging to the
250 other pine species according to their relative position within the plant. Each pair of fresh
251 cuttings was placed in an 8-cm Petri dish with a moistened filter paper and offered to one
252 preweighed pine weevil for 48 h at room temperature (c. 22 °C). The total number of plates
253 was 60, corresponding to 20 pairs of seedlings of similar diameter, with 3 stem twigs per
254 seedling. Sampling several twigs from each seedling allowed us to account for the possible
255 effects of heterogeneous levels of defences along the stems of pine seedlings.

256 For the non-choice feeding test we used 20 seedlings of each species which were
257 grouped according to their diameter into 5 groups, with four plants in each group. We
258 sampled 5 twigs of 2.5 cm from each seedling. Twigs of the four seedlings of each group
259 were grouped according to their position along the stem and offered together in Petri dishes
260 to one preweighed weevil as above. This allowed us to analyse the sum of the debarked
261 areas in the four twigs of each plate, without losing information regarding the possible
262 effects of the seedling size and the part of the stem from which the twigs were sampled. The
263 total number of plates was 50, corresponding to 2 species, 5 diameter groups and 5 twigs per
264 seedling.

265 Pine weevils were collected at a neighbouring clear-felled *P. pinaster* forest (see
266 Moreira *et al.* 2008a) two weeks before the experiments and maintained at room
267 temperature in the laboratory in plastic buckets with moistened sawdust and bark and twigs
268 of both pine species. Sex was not considered in the bioassays because previous studies have

269 revealed little difference in feeding behaviour between sexes (e.g. Borg-Karlson *et al.*
270 2006). The weevils were starved for 24 h before the test period.

271 After 48 h, weevils were removed and the debarked area in each twig was estimated
272 with the aid of a transparent millimetric grid.

273

274 *Induction experiments*

275 In order to assess weevil-driven induced responses, we covered 40 one-year-old seedlings of
276 each pine species with a plastic net cage (plant material and culture as above). We confined
277 1 preweighed adult weevil within the net in half of the seedlings following a completely
278 factorial design with 20 replicates. After a 72-h feeding period, weevils were removed,
279 debarked area was evaluated according to the same scale as in the field trials, and seedlings
280 were harvested to analyse the concentration of resin acids in the stem. The whole stem was
281 cut in 5-mm sections, extracted twice with hexane in an ultrasonic bath (15 min at 20 °C)
282 and then for 24 h at room temperature, and the non-volatile resin residue was determined
283 gravimetrically and referred to the dry mass of each stem (Sampedro, Moreira & Zas 2011).
284 Resin concentration in pine stems has been shown to be inversely related to wounding by
285 the weevils (Moreira, Sampedro & Zas 2009), and thus represents an effective resistance
286 trait against this insect.

287 In order to check for differences between species in their inducibility potential, we
288 evaluated the pine response in chemical defences to the exogenous application of MeJa.
289 Methyl jasmonate is a well-known plant phytohormone involved in the signalling and
290 triggering of induced responses after insect damage. Experimental MeJa application to
291 conifer seedlings has been reported to induce chemical defensive responses similar to those
292 caused by insects (Moreira, Sampedro & Zas 2009, Sampedro, Moreira & Zas 2011), and it

293 has been shown to be very useful as a reference induction value for comparing induction
294 capabilities to different agents (Hudgins, Christiansen & Franceschi 2004).

295 Twelve seedlings from each pine species were cultivated as described before, and
296 during the second growth period (August 2008) half of the seedlings were sprayed with a
297 suspension of 80 mM MeJa (cat. #39270-7; Sigma-Aldrich, St. Louis, MO, USA) in
298 deionized water with 2.5 % (v/v) ethanol; the remaining half was treated only with the
299 carrier solution. Both treatments were applied with a hand sprayer to runoff at an average
300 dose of about 3 mL of solution per plant. The MeJa was applied in a different space of the
301 greenhouse, and the seedlings remained in that isolated room for 24 h to allow the
302 volatilization of excess MeJa. Then, all seedlings were distributed in the greenhouse
303 according to a factorial randomized block design with 6 blocks. Fifteen days after the
304 application of the induction treatments, i.e. when MeJa effects are assumed to be maximized
305 (Martin *et al.* 2002), the plants were harvested and the non-volatile resin content in the stem
306 was determined as described before.

307

308 *Statistical analyses*

309 All analyses were carried out in SAS System, using the GLIMMIX and MIXED procedures
310 for analyses of generalized and general linear mixed models, respectively (Littell *et al.*
311 2006).

312 Damage data from the field experiments was analysed with a generalized mixed
313 model appropriate for split-plot designs, in which the two main factors (fertilization and
314 genetic entry) are applied to nested experimental units of different size. The dependent
315 variable, which ranged from 0 to 30, was assumed to be similar to a count-data variable and
316 was thus modelled with a Poisson distribution. Fertilization, genetic entry and fertilization
317 by genetic-entry interaction were considered fixed effects. Blocks and the block \times

318 fertilization interaction (i.e. the whole plots) were considered random factors in order to
319 analyse each factor with the appropriate error term (Littell *et al.* 2006). To avoid any
320 possible confounding effects associated with size differences between the two pine species,
321 we also included the diameter at the time of the attack as a covariate in the model. Within
322 each site and species, differences in consumption between the pine species by the pine
323 weevil were analysed with specific contrasts on linear combinations of the family means,
324 using the CONTRAST statement of the GLIMMIX procedure in SAS. Family least-squares
325 means were derived from the generalized mixed models and used to estimate the
326 relationship between the damage by the insect and pine survival at the family level. Survival
327 being a clear component of pine fitness, the slope of this relationship reflects the tolerance
328 to the insect attack (Simms 2000). How pines were able to recover growth after insect
329 damage was also considered as a measure of tolerance to the insect. Thus, for each pine
330 species, we estimated the relationship at the family level between damage by the insect and
331 consecutive pine growth one and five years after the attack. Consecutive pine growth was
332 measured as the relative growth rate in height or diameter after the attack (e.g. $(H_5 - H_0) / H_0$,
333 where H_5 and H_0 are the pine heights in year five and in the year of the attack). This
334 measure was considered to represent the relative investment of plant resources in recovering
335 after attack. Differences in tolerance between the two pine species were estimated by testing
336 for differences in the slope of these regressions with an analysis of covariance (ANCOVA).
337 To double check that the differences in the slopes between the two species are not due to
338 different ranges of weevil damage, we further confirmed the results of this analysis using
339 only the common range of weevil damage (i.e. restricting the analyses to scores from 12 to
340 20).

341 Intraspecific genetic variation for tolerance to *H. abietis* damage was evaluated by
342 analysing different pine fitness correlates with a general mixed model equivalent to that

343 described before for the analysis of weevil damage, but including the damage and damage ×
344 family interaction as covariates (Pilson 2000). A significant family × damage interaction
345 indicates that the effect of damage on pine fitness, that is tolerance, varies among families.

346 The two-choice test bioassay was analysed with a doubly repeated-measures analysis
347 where the two dimensions of repeated measures were represented by the pair of pine
348 seedlings (with three parts of the same subjects) and the Petri dish in which they shared the
349 same pine weevil. Analysis was performed with a repeated-measures mixed model
350 including the species as a between-subject factor, and the stem part and their interaction as
351 within-subject factors. The Petri dish and the pine weevil weight were also included in the
352 model as a random effect and a fixed covariate, respectively. The covariance structure of the
353 repeated measures was modelled with the Huynh–Feldt structure, as it gave the lowest
354 Akaike information criteria and was consistent with the sample schedule (Littell *et al.*
355 2006). The Kenward–Roger correction was used for the estimation of the degrees of
356 freedom, as recommended by Littell *et al.* (2006, p. 188).

357 The non-choice experiment was analysed with a repeated-measure mixed model
358 where the five stem parts are repeated measures of the same subjects. The mixed model was
359 fitted for the sum values of the four wounding-area values per plate, and the pine weevil
360 weight was included as a covariate. A first-order autoregressive covariance structure was
361 used to model the covariance of the repeated measures. Degrees of freedom were again
362 estimated by the Kenward–Roger method.

363 The induction of chemical defences by either weevil feeding or MeJa application
364 was analysed with a factorial ANCOVA using the debarked area as covariate and assuming
365 all effects (species, induction treatment and their interaction) as fixed factors.

366

367

368 **Results**

369 *Weevil damage on the two pine species*

370 Results from the field trials indicate that *H. abietis* fed significantly more on *P. radiata* than
371 on *P. pinaster* seedlings. The *P. radiata* seedlings growing interspersed within the *P.*
372 *pinaster* trial were attacked the most (Fig. 1a; see also Table S1 in Supporting Information),
373 and they were significantly more consumed than 24 of the 31 *P. pinaster* genetic entries in
374 the trial (data not shown). Consistently, the *P. pinaster* seedlings growing interspersed
375 within the *P. radiata* trial were significantly less attacked (Fig. 1b, Table S1), and 26 out of
376 the 31 *P. radiata* genetic entries were significantly more damaged than the control *P.*
377 *pinaster* seedlings (data not shown).

378 However, pine weevils preferred *P. pinaster* (the native) more than *P. radiata* (the
379 exotic) twigs when both species were offered as cut material in Petri dishes (Fig. 2,
380 Appendix S2). Weevil feeding was significantly more intense in *P. pinaster* than in *P.*
381 *radiata*, both in the two-choice (Fig. 2a, Table S2A) and in the non-choice (Fig. 2b, Table
382 S2B) experiments.

383

384 *Short-term tolerance to pine weevil damage*

385 The relationship at the family level between actual damage and subsequent survival in the
386 field was negative and very strong in both species (Fig. 3). Damage by the pine weevil
387 explained more than 50% of the family variation in survival. The slopes significantly
388 differed between the two pine species (ANCOVA $F_{1,58} = 9.34$, $p = 0.003$), with a steeper
389 relation in the case of *P. radiata*, indicating that the native *P. pinaster* was more tolerant of
390 the damage than the exotic *P. radiata* (Fig. 3). Results remained the same when the analysis
391 was restricted to the common range of damage scores for the two species (scores 12-20)
392 (ANCOVA $F_{1,51} = 4.53$, $p = 0.038$). The relationships with survival at age 5 followed the

393 same trends, confirming the relevance of the early attack as a determinant of medium-term
394 mortality.

395

396 *Medium-term tolerance to the pine weevil damage*

397 The family relationship between insect damage and the immediate consecutive pine growth
398 in *P. pinaster* was significant and negative (Fig. 4a). Pine growth after insect damage was
399 less in those families that were more damaged and greater in those where the damage was
400 less, indicating that the insect attack disrupted regular growth. This relationship, which
401 could be interpreted as early growth costs of tolerance or induced resistance, was not
402 significant in the exotic *P. radiata* (Fig. 4a), where differences in consecutive family growth
403 appeared to be unaffected by the damage level. Figure 4b summarizes the medium-term
404 vegetative tolerance, that is, the potential of each family for height recovery 5 years after the
405 insect attack, measured as 5- to 1-year relative growth regressed against the early damage.
406 Relative compensatory effort in the 5 years following attack was greater in the more
407 damaged families of *P. pinaster*, whereas we found no significant response in *P. radiata*.
408 Comparing the growth patterns in the studied stands with those in two neighbouring twin
409 trials of the two pine species that remained free of weevil damage also suggested that the
410 native pine was better able to recover from weevil damage (see Appendix S3). The negative
411 effect of the weevil damage on pine growth disappeared at age five in *P. pinaster*, but still
412 remained significant in *P. radiata*, suggesting a compensatory growth in the native but not
413 in the exotic.

414

415 *Genetic variation in tolerance*

416 Growth and survival during the period studied were strongly determined by the damage
417 produced by the pine weevil at age 1, as evidenced by the significant covariation of the

418 initial damage on the studied variables in the ANCOVA analyses (Table 1). We found
419 evidence of genetic variation for tolerance to *H. abietis* in *P. pinaster*, but not in *P. radiata*.
420 In the case of *P. pinaster*, the damage \times family interaction was marginally significant for
421 pine survival after the insect damage, representing the proximal effects of the attack, and
422 significant for pine height at age 5, suggestive of vegetative compensatory effort and
423 opportunity fitness. However, the covariation of weevil damage on the early reproductive
424 effort did not vary among the *P. pinaster* families (Table 1). We did not detect genetic
425 variation in tolerance in *P. radiata* in any case, nor was there covariation of weevil damage
426 with early reproductive effort in this species. Radiata pine families survived and grew
427 similarly irrespective of the level of damage suffered, whereas early cone production was
428 not affected by insect damage.

429

430 *Induced response of pine to weevil damage*

431 We did not detect significant differences between species in the constitutive resin content in
432 control plants (Fig. 5a). After exposure to insect feeding for 72 h, both species reacted to the
433 weevil damage by increasing the resin concentration in the whole stem. However, the
434 quantitative response in resin differed between the two species (significant species \times
435 induction interaction). The response of the native *P. pinaster* was 2-fold that of the exotic
436 pine, even though no significant differences were observed in the wounding intensity
437 between the species ($F_{1,27} = 1.2$, $p = 0.274$).

438 The application of MeJa also significantly increased the resin content in the two pine
439 species. The response to MeJa application was, however, similar in the two pines (no
440 significant species \times MeJa interaction, Fig. 5b). Thus, the resin content in the stem of both
441 constitutive and MeJa-induced plants did not differ between species, suggesting similar
442 physiological capabilities to express MeJa elicited induction.

443 **Discussion**

444 *Induced resistance and biological invasions*

445 In contrast to the expectations of the BRH, the large pine weevil consumed more on the
446 native *P. pinaster* than on the exotic *P. radiata* when both species were offered as cut
447 material in *in vitro* bioassays. As cut stem twigs are unable to respond to the damage caused
448 by the weevils, results of the *in vitro* bioassays likely reflect the variation in preference due
449 to constitutive resistance between the two species, which appeared to be higher in the exotic
450 than in the indigenous pine species. However, under field conditions, damage by the pine
451 weevil was clearly greater on the exotic than on the native species, suggesting lower
452 effective resistance of the former. Both species were planted simultaneously, and the pine
453 weevil attack started early after planting and lasted several months until weevil damage was
454 assessed. During this time, weevils were continuously feeding on the pine seedlings, which
455 should have had enough time to respond by activating induced resistance mechanisms. As
456 an evidence of this, the resin canal density in the xylem of the *P. pinaster* seedlings in the
457 attacked stand was nearly twice as high as that found in the nearby unattacked stand; the
458 differences were attributed to the defensive response induced by the weevil damage
459 (Moreira *et al.* 2008b).

460 The results of our induction experiment revealed large differences between the
461 species in the induction of resin in the stem after wounding. Although the damage inflicted
462 by the weevil during the induction experiment did not differ between pine species, the
463 increment in the resin content in the indigenous species just 72 hours after insect damage
464 was more than 2-fold that in the non-indigenous species. Moreover, differences between
465 species in this main resistant trait were only significant after exposure to the insect.
466 Conversely, the response to the application of MeJa did not differ between the species,
467 suggesting similar MeJa inducible physiological capabilities (i.e. inducibility potential)

468 between the two species. On the basis of the differences between the response to the pine
469 weevil and to the MeJa application, we infer, as predicted by the BRH, that the native pine
470 species is better adapted to this local insect, being able to elicit a stronger induced resistance
471 mechanism that likely will deter further damage. These results suggest that, because of the
472 greater induced resistance in the native species, the initial preference of the insect for the
473 native pine could switch with time towards greater damage on the exotic, as observed in the
474 field. The lack of preference between the two pine species shown by the weevil in the
475 induction experiment does not conflict with this idea, as the period of time that the weevils
476 were feeding on the seedlings (just 72 h) was probably insufficient for the induced defences
477 to become effective against the weevil.

478 Induced resistance is a key strategy for defending *Pinus* sp. against bark beetles and
479 phloem feeders (Franceschi *et al.* 2005, Lieuter *et al.* 2004) and varies greatly among
480 species of the Pinaceae (Hudgins, Christiansen & Franceschi 2004). Plants are known to be
481 able to recognize specific cues of their enemies and elicit the appropriate defensive
482 mechanisms (Heil 2009, Hilker & Meiners 2010). Thus, plant species can respond
483 specifically to different enemies (Lieutier 2002), with larger responses of the trees to the
484 enemies that normally attack them (Raffa 1991). Results presented here are in concordance
485 with this cue specificity of the induced responses, which were more intensely elicited in the
486 pine species with a common evolutionary history with the enemy.

487 Empirical evidences of the ability of plants to recognize different herbivorous
488 species and elicit differential responses against them are, however, equivocal (Heil 2009).
489 For example, the specificity of the tree response to the bark beetle–fungus association may
490 be more related to protein–protein interactions with the fungus species than to plant
491 recognition of the insect species (Karban & Kuc 1999). *Hylobius abietis* could also vector

492 different native pathogenic fungus species to which *P. radiata* may be not adapted, but
493 information on the transmission of diseases by weevils is very limited (Lieuter *et al.* 2004).

494 Although other factors (e.g. the environmental and ontogenetic differences between
495 the field trials and the bioassays with greenhouse potted plants) may have differentially
496 affected the results, our findings indicate that the intensity and/or rapidity of the induced
497 responses to a native insect can differ between native and non-native host species. If
498 induced defences are more specific mechanisms than constitutive resistance (Franceschi *et*
499 *al.* 2005), the differences in inducibility between native and exotic species may be relevant
500 for understanding the role of local enemies in invasion ecology. The lower inducibility of
501 the exotic pine species supports the predictions of the BRH (Elton 1958), as it could imply
502 greater damage in the field, which could contribute towards preventing the exotic pine from
503 becoming invasive in the area. As pointed out by Orians & Ward (2010), however, little
504 attention has been paid to the implications of induced resistance in invasion ecology. To
505 date, only two studies have examined induced defences in exotic plants, comparing patterns
506 of inducible defences in native and introduced ranges (Cipollini *et al.* 2005, Eigenbrode *et*
507 *al.* 2008). These studies explore how induced defences have evolved after the relaxation of
508 herbivore pressure in the exotic range, using either chemical elicitors or mechanical
509 wounding to assess inducibility. However, responses to specific herbivore species can
510 potentially differ from those to artificial induction treatments (as observed in the present
511 study), and these differences could be highly relevant in the context of invasion ecology. To
512 our knowledge, results presented here are the first reporting how failure to elicit effective
513 induced responses against local herbivores might be a factor in impeding an introduced
514 species from becoming invasive.

515

516

517 *Tolerance in the context of biological invasions*

518 Differences in tolerance to herbivore damage can also be important in interpreting the
519 invasion ecology hypotheses (Ashton & Ler dau 2008, Chun, van Kleunen & Dawson 2010).
520 Besides the release from specialist enemies, the ERH also assumes a lower impact of
521 generalist enemies on the introduced species, because native consumers will be better
522 adapted to consume native, rather than exotic hosts (Keane & Crawley 2002). According to
523 the BRH, however, the exotic hosts may be either more heavily damaged by the resident
524 herbivores than native plants or just poorly adapted to tolerate the damage caused by novel
525 enemies with which they lack an evolutionary history (Parker & Hay 2005). This idea is
526 well accepted among foresters, who commonly consider that trees can be more susceptible
527 to pests and pathogens when they are planted off-site, i.e. outside the soil, climate system
528 and biotic interactions to which they are adapted (Lombardero, Vázquez-Majuto & Ayres
529 2008).

530 In support of the BRH, we found that the native pine was more tolerant of the
531 coevolved herbivore than the exotic pine, suggesting no fitness advantage of the exotic pine
532 species in its introduced area. For the same level of insect damage, survival of *P. pinaster*
533 families was higher than that of *P. radiata*, indicating a lower impact of the herbivore on the
534 former. Fitness evaluation is especially complicated in forest trees with long lifespans and
535 reproduction. However, early survival is a clear component of pine fitness, and thus the
536 relationship between survival and damage can be utilized to assess tolerance to the attack.
537 Early growth in light-demanding species such as the pines studied is another component of
538 plant fitness, which evaluates the opportunity of a tree to arrive to the dominant stratus, and
539 thus reproduce and contribute to the next generation. Slow-growing pines are expected to be
540 suppressed by dominant trees and die. Considering early growth, the native *P. pinaster*
541 appeared to be, again, more tolerant than the exotic *P. radiata*. Although the pine weevil

542 reduced the expected regular size of *P. pinaster* during the immediate following years after
543 the attack, the positive relationship between initial weevil damage and relative medium term
544 growth reveals that this pine species was able to likely compensate after just 5 years. In
545 contrast, in *P. radiata*, neither immediate consecutive growth nor medium-term relative
546 growth was related to initial damage, suggesting no compensatory growth capability in the
547 exotic pine. The differences in the growth trends in the attacked and unattacked stands were
548 in concordance with this finding, although the results from this comparison should be
549 interpreted with care as the two sites may not have been equally suitable for the two pine
550 species (see Appendix S3).

551 The detection of intraspecific genetic variation in tolerance of *P. pinaster* to the
552 insect supports the possibility of evolution of tolerance traits as an adaptation to this (and
553 other) local insects. The cost–benefits balance, together with the temporal and spatial
554 heterogeneity in the selective pressure imposed by these (and other) insects, would explain
555 why these apparent adaptive traits are not fixed within the native population and genetic
556 variation for tolerance is maintained.

557 The lower tolerance of the exotic pine species suggest poor adaptation to its new
558 range, which is in consonance with the predictions of the BRH. In a recent study,
559 Lombardero *et al.* (2008), studying the impact of the local bark beetle *Tomicus piniperda* on
560 the same pine species in the same region, also found the native *P. pinaster* to be more
561 tolerant than the exotic *P. radiata*. Although the insect damage was more than twice as great
562 in the native *P. pinaster* as in the exotic *P. radiata*, the productivity of the exotic was more
563 adversely affected, suggesting higher tolerance of the native species. The results were
564 consistent across a wide range of tree ages and beetle incidence levels. Additionally,
565 productivity of the non-native *P. radiata* was further reduced by the pathogen *Sphaeropsis*

566 *sapinea*, a fungus that seems to be associated with *T. piniperda* and was only present in the
567 exotic pine.

568 Few studies have considered the importance of tolerance in invasion ecology (Chun,
569 van Kleunen & Dawson 2010). After the formulation of the Evolution of Increased
570 Competitive Ability (EICA) hypothesis (Blossey & Notzold 1995), however, it has been
571 postulated that the invasiveness of exotic species might be related to increased tolerance of
572 the introduced populations as a result of a relaxation in herbivore pressure in the new range
573 (Stastny, Schaffner & Elle 2005, Zou, Rogers & Siemann 2008). However, *P. radiata* was
574 introduced in Spain very few generations ago, so few opportunities to evolve tolerance are
575 expected in this species. Besides, the lack of genetic variation in tolerance found in the
576 present study would further constrain the possibility to evolve towards higher tolerance. As
577 stated by Lombardero *et al.* (2008), the most obvious implications of EICA in forestry
578 would be for tree breeding programs, since natural selection is constrained in trees by their
579 long generation times.

580 In summary, we found that *P. pinaster* and *P. radiata* coexisting in NW Spain
581 differed markedly in their responses to a native insect herbivore. Although the insect
582 intrinsically preferred the native pine species with which it shared an evolutionary history,
583 the native *P. pinaster* responded more strongly to the attack and was more tolerant of it than
584 the exotic *P. radiata*. Thus, in this area, the exotic *P. radiata* shows no advantages
585 compared with the native pine against this enemy. These findings are in agreement with the
586 fact that *P. radiata* is not invasive in this area (Carrillo-Gavilán & Vilá 2010). Other authors
587 have already suggested that the failure of invasions by alien conifers in Central Europe
588 might be, among other reasons, due to the presence of pathogens and phytophages in the
589 introduced range because of the presence of native conifer congeners (Adamowski 2004,
590 Carrillo-Gavilán & Vilá 2010). Indeed, exotic conifers in Europe are known to be colonized

591 by as much herbivore insect species as native conifers (Bertheau *et al.* 2009), and the
592 majority of native insects species that colonize alien trees are generalist herbivores
593 (Bertheau *et al.* 2010). Additionally, there is some evidence that herbivory by generalist
594 vertebrates might limit the invasiveness of *P. radiata* in some areas of the Southern
595 Hemisphere (Becerra & Bustamante 2008). However, that *P. radiata* might shows signs of
596 invasiveness in Europe in the absence of herbivory and that this pine has become invasive in
597 other parts of the world because of escape from herbivory, remains untested. Based on the
598 results presented here, the weak ability to elicit induced defences and the low tolerance of
599 the exotic species against native herbivores appeared as key concepts for understanding how
600 native enemies may contribute to limit the invasiveness potential of exotic plants, as stated
601 by the BRH. Further studies are needed to confirm this by testing whether *P. radiata* may
602 show signs of invasiveness in the absence of herbivory in Europe.

603

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614

615

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763

764

765 **SUPPORTING INFORMATION**

766 Additional supporting information may be found in the online version of this article:

767

768 **Appendix S1 – Table S1** Results of the generalized linear mixed model for the analysis of
769 the damage by *H. abietis* in the *P. pinaster* and *P. radiata* field trials.

770

771 **Appendix S2 – Table S2A** Results of the mixed model for the analysis of the debarked area
772 in the two-choice feeding experiment.

773

774 **Appendix S2 – Table S2B** Results of the mixed model for the analysis of the debarked area
775 in the non-choice feeding experiment.

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777 **Appendix S3** Medium-term growth trends of both pine species in the attacked and the
778 healthy stands.

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781 supplied by the authors. Such materials may be re-organized for online delivery, but are not
782 copy-edited or typeset. Technical support issues arising from supporting information (other
783 than missing files) should be addressed to the authors.

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785 Table 1. Tolerance and genetic variation in tolerance to *Hylobius abietis* damage in *Pinus*
 786 *pinaster* and *P. radiata*. The table shows the F-values and corresponding P values of the
 787 mixed model for the covariation of the initial weevil damage (wounds) and damage × family
 788 interaction with several components of pine fitness. A significant damage × family
 789 interaction indicates that the effect of damage on the subsequent fitness, that is, tolerance,
 790 varied between families
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Fitness component	<i>Pinus pinaster</i>				<i>Pinus radiata</i>			
	Wounds at age 1		Wounds x family		Wounds at age 1		Wounds × family	
	F _{1,1876*}	p>F	F _{30,1876*}	p>F	F _{1,1244*}	p>F	F _{30,1244*}	p>F
Survival after attack	340.5	<0.001	1.5	0.051	583.5	<0.001	0.8	0.737
Height at year 5	152.3	<0.001	1.5	0.031	17.5	<0.001	1.3	0.133
Cone production year 5	16.5	<0.001	0.8	0.801	0.1	0.819	0.9	0.587

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 793 * Denominator degrees of freedom in the case of survival were 2394 for *P. pinaster* and
 794 2262 for *P. radiata*.
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Figure legends

Figure 1. Realized resistance of the native *Pinus pinaster* (black bars) and the exotic *P. radiata* (white bars) to the pine weevil *Hylobius abietis* in the field conditions. The pine weevil consumed more on the exotic *P. radiata* than on the native *P. pinaster*, both when *P. pinaster* was planted surrounded by *P. radiata* (a), and when *P. radiata* was planted surrounded by *P. pinaster* (b). The F ratio and associated p-values are presented for the specific contrast between species within the generalized mixed model (see also Table S1); sample size is indicated within each bar. Diameter did not differ between species.

Figure 2. Preference of the native large pine weevil *Hylobius abietis* for the native *Pinus pinaster* (black bars) and the exotic *P. radiata* (white bars) evaluated in two independent *in vitro* cafeteria experiments: a two-choice (a) and a non-choice (b) feeding tests (see complete results of the corresponding mixed models in Table S2A and Table S2B). N = 60 and N = 25, respectively. Bars represent the debarked area consumed by adult individuals in 2.5 cm long twigs of both tree species. Twig diameter was used as covariate. Least-squares means \pm SE.

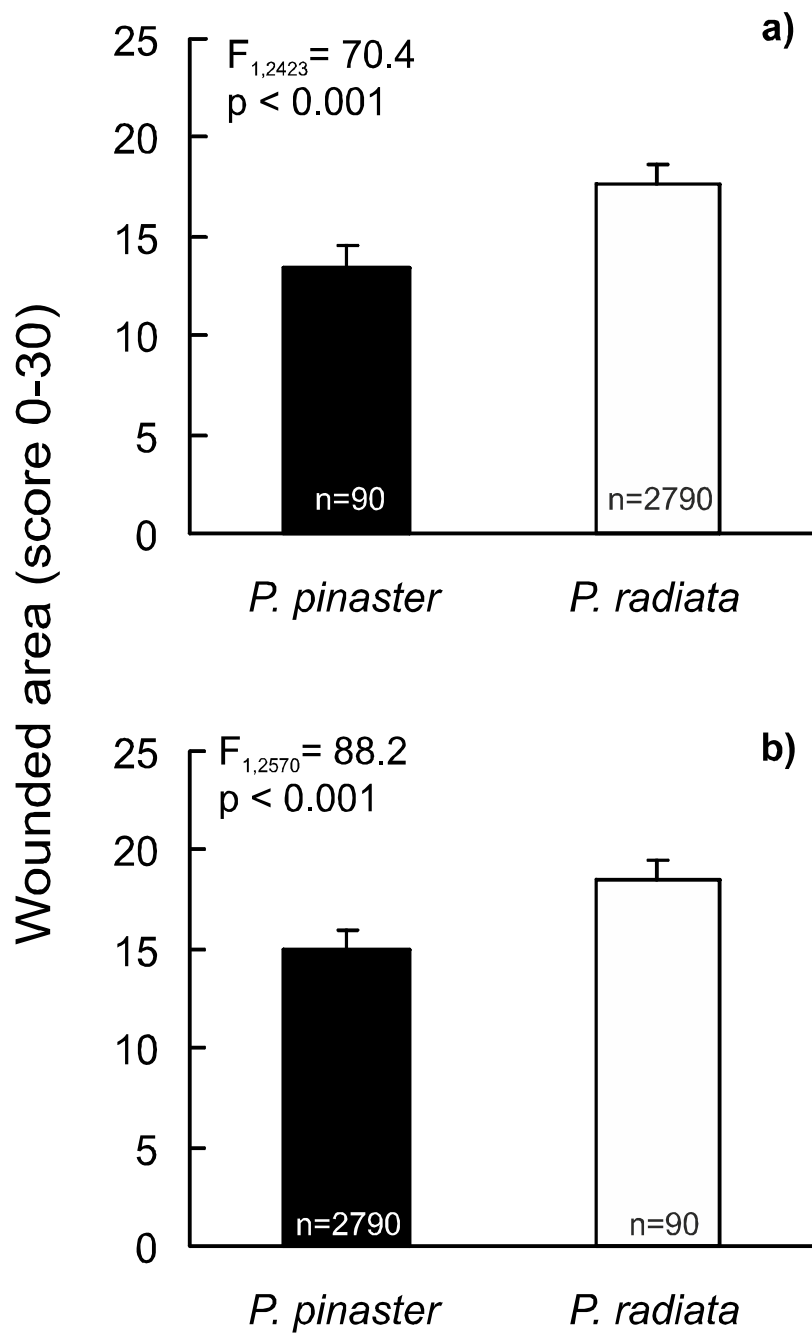
Figure 3. Short term tolerance to *Hylobius abietis* damage as revealed by the family relationship between *H. abietis* damage and pine survival one year after the insect attack for *Pinus pinaster* (filled dots) and *P. radiata* (open dots). Seedlings were planted in the same field trial, naturally and homogeneously infected by the pine weevil. Both relationships were significant at $p < 0.001$. Each dot is an open-pollinated family least-squares mean (N = 90).

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822
823 Figure 4. Family relationship between *Hylobius abietis* damage and subsequent pine growth
824 in *Pinus pinaster* (black dots) and *P. radiata* (open dots). (a) Early relative pine growth is
825 the relative diameter growth rate one year after the insect attack $((D_1 - D_0)/D_0)$, where D_0 and
826 D_1 are the stem pine diameter in the year of the attack and one year after weevil damage. (b)
827 Medium-term compensatory growth, measured as the relative growth rate 5 years after the
828 attack $((H_5 - H_0)/H_0)$, where H_5 and H_0 are pine height at age 5 and at the year of insect attack,
829 respectively. Seedlings were planted in the same field trial naturally and homogeneously
830 infected by the pine weevil. Each dot is an open-pollinated family least-squares mean ($N =$
831 90).

832
833 Figure 5. Inducibility of pine chemical resistance elicited by the pine weevil (a) and by the
834 exogenous application of methyl jasmonate (MeJa) (b) in *Pinus pinaster* (black bars) and *P.*
835 *radiata* (white bars) seedlings. P values in the associated tables resume the results from the
836 mixed models. Both species responded significantly to both chemical and biotic induction.
837 Although response to MeJa was similar, response to weevil feeding markedly differed
838 between the two species. Within each induction treatment (Control, Induced) differences
839 between species are indicated by the associated significance levels (n.s.= no significant; ***
840 = $p < 0.001$). Least-squares means \pm SE of two independent experiments are shown: $n = 20$ in
841 (a) and $n = 6$ in (b).

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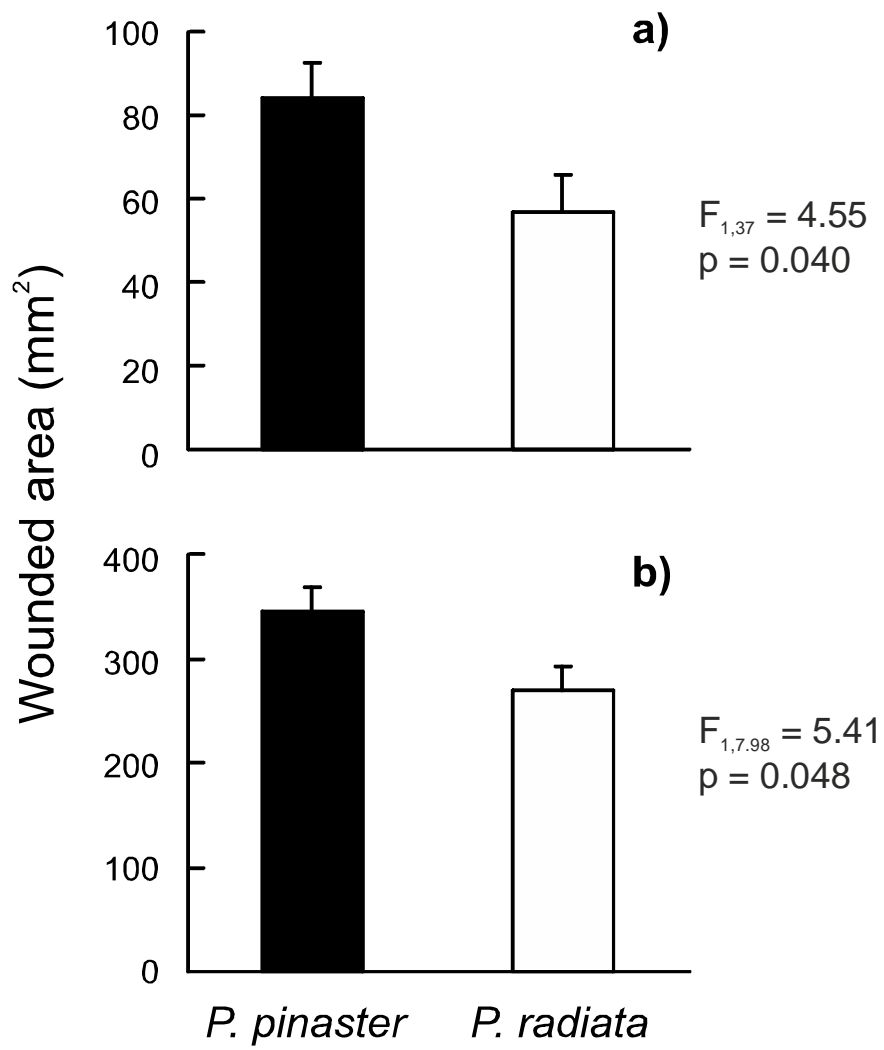
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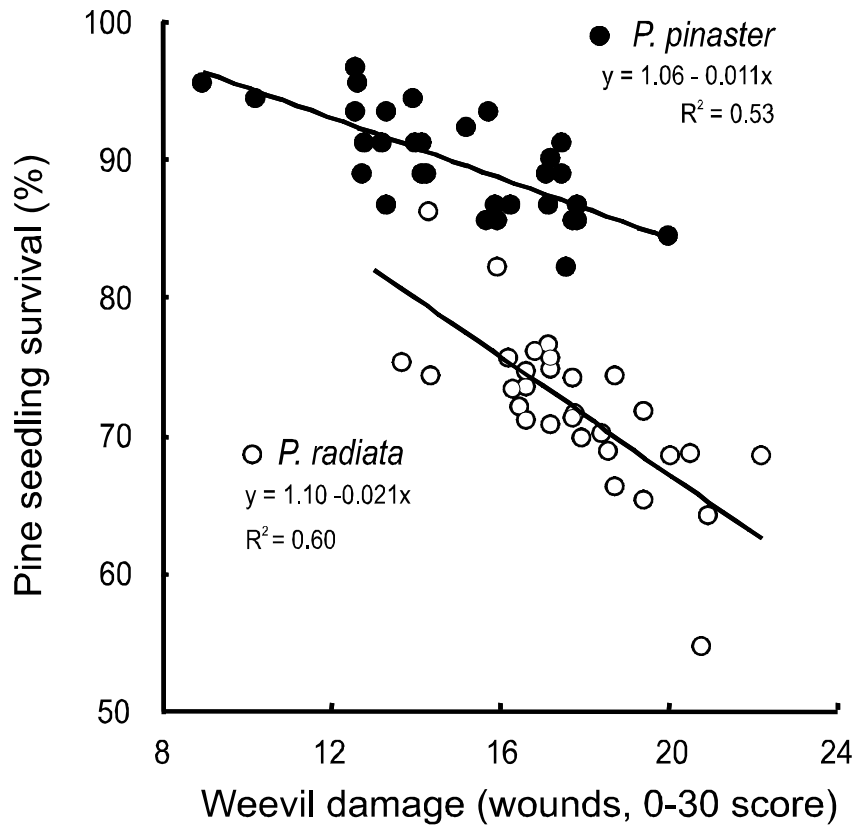
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9 Figure 2

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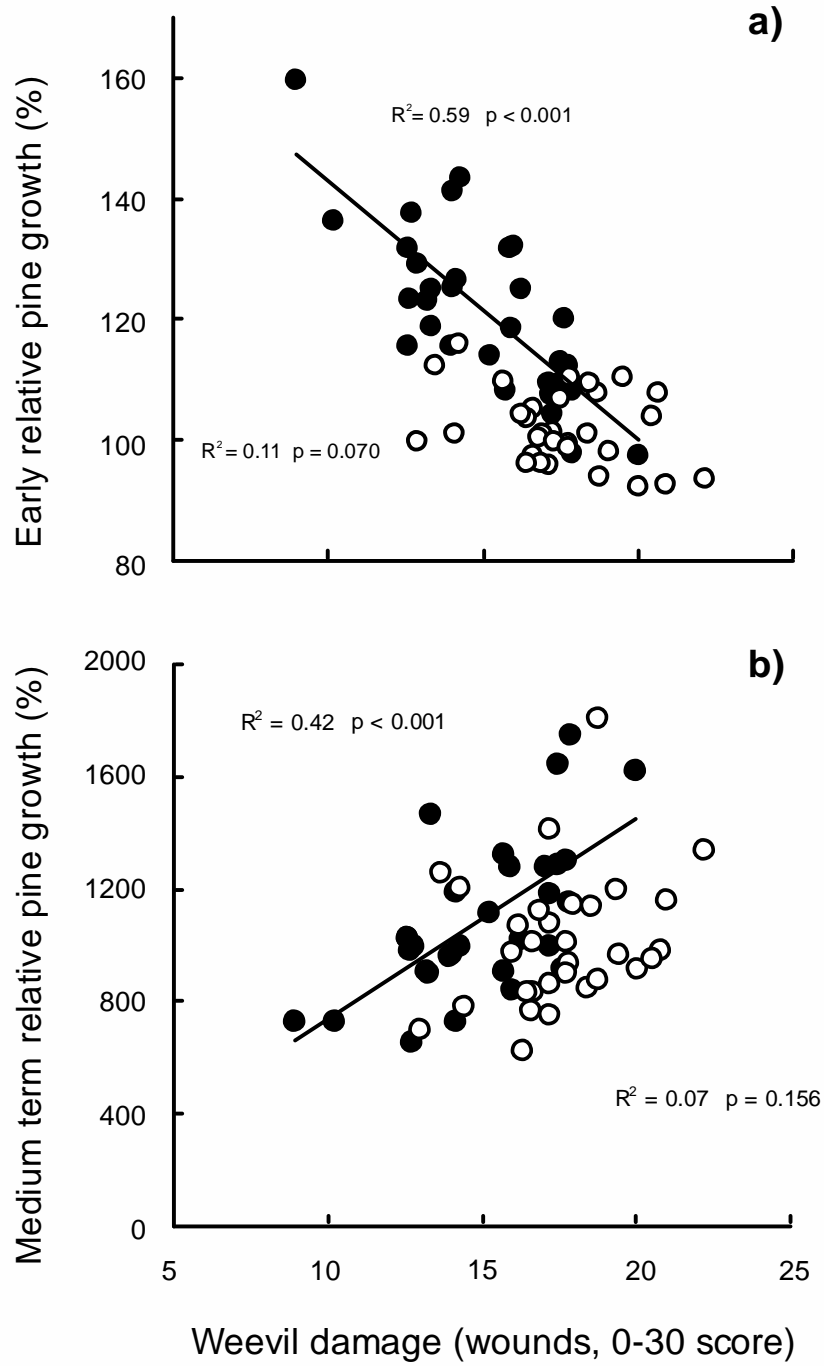
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Figure 3

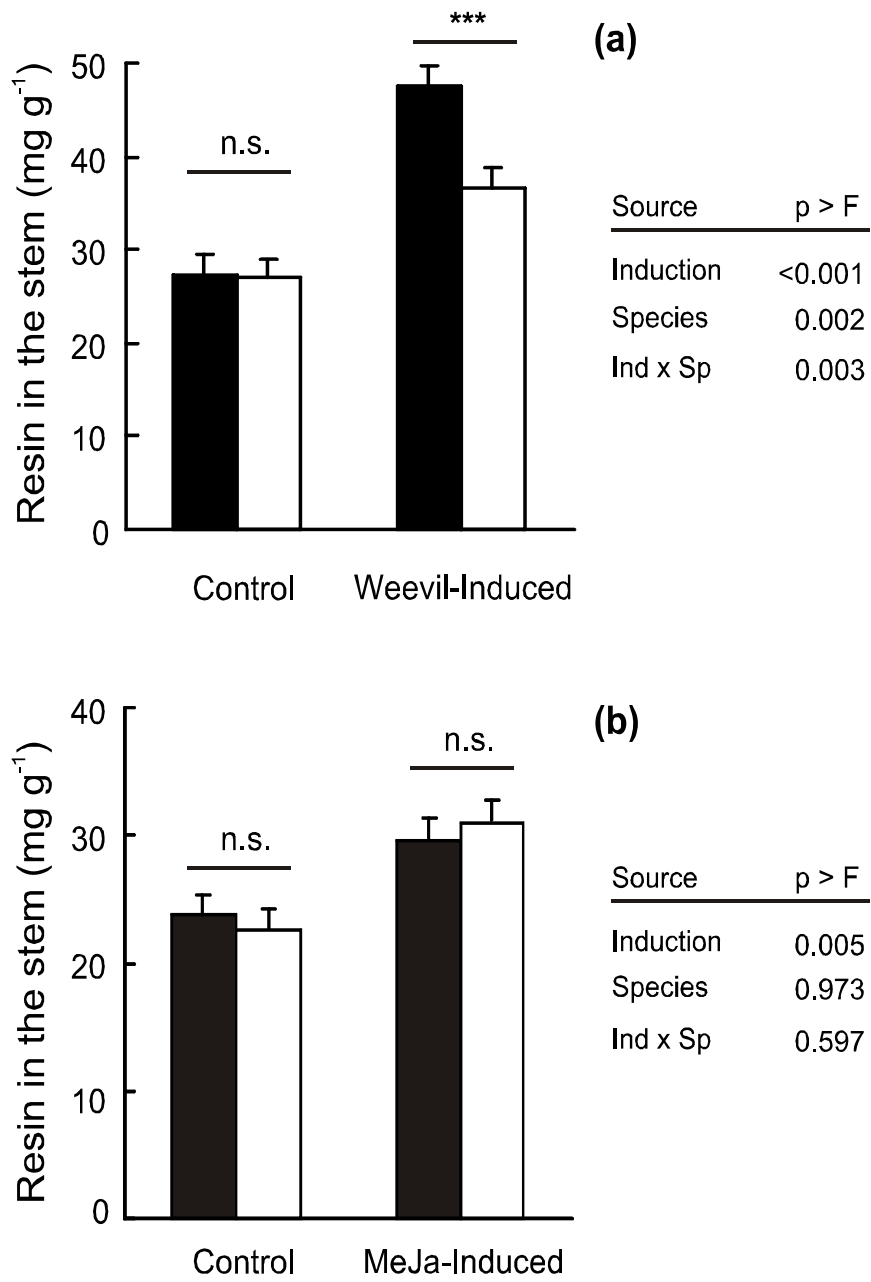
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