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Abstract: Chemical elicitors and mechanical treatments simulating real insect herbivory have been increasingly used to study induced defensive responses in woody plants. However, quantitative comparisons of plant chemical defences elicited by real and simulated herbivory have received little attention. In this paper we compared the effects of real herbivory, simulated herbivory using chemical elicitors, and mechanical damage treatments on the quantitative secondary chemistry of *Pinus pinaster* juveniles (non-volatile resin in the stem and total phenolics in the needles). The real herbivory involved *Hylobius abietis* (a phloem feeder) and *Brachyderes lusitanicus* (a defoliator); the chemical elicitors to simulate herbivory were 40 mM methyl jasmonate (MJ) and 20 μ M benzothiadiazole (BTH); and the mechanical treatments used involved phloem wounding and needle clipping. We also performed an additional experiment for assessing at what extent insect extracts could increase plant responses over mechanical damage.

Chemical induction with MJ, mechanical wounding and real phloem herbivory by *H. abietis* all produced quantitatively similar results, increasing the concentration of resin in the stem and total phenolics in the needles by equivalent magnitudes. Exogenous application of BTH increased the concentration of phenolic compounds in pine needles, but did not have the same effect on stem resin. Contrastingly, needle clipping and *B. lusitanicus* feeding did not induce pine chemical defences, neither in terms of resin in the stem nor phenolics in the needles. Mechanical damage followed by the application of extracts from the insects *B. lusitanicus* and *H. abietis* on the wounds did not increase the responses in comparison to mechanical damage alone. The fact that strong induced responses elicited by phloem wounding insects are equally elicited by phloem injuries suggests that defences in pine trees are raised with low specificity regarding biotic enemies. Our results have great relevance for the interpretation of methodological approaches using needle clipping and stem wounding, and for supporting future experiments using MJ and mechanical wounding as simulated herbivory treatments for the induction of conifer defences.

Suggested Reviewers:

1 **Quantitative comparison of chemical, biological and mechanical induction of**
2 **secondary compounds in *Pinus pinaster* seedlings**

3

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25 **Abstract**

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29 herbivory have received little attention. In this paper we compared the effects of real
30 herbivory, simulated herbivory using chemical elicitors, and mechanical damage treatments
31 on the quantitative secondary chemistry of *Pinus pinaster* juveniles (non-volatile resin in the
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35 mechanical treatments used involved phloem wounding and needle clipping. We also
36 performed an additional experiment for assessing at what extent insect extracts could
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43 induce pine chemical defences, neither in terms of resin in the stem nor phenolics in the
44 needles. Mechanical damage followed by the application of extracts from the insects *B.*
45 *lusitanicus* and *H. abietis* on the wounds did not increase the responses in comparison to
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49 the interpretation of methodological approaches using needle clipping and stem wounding,
50 and for supporting future experiments using MJ and mechanical wounding as simulated
51 herbivory treatments for the induction of conifer defences.

52

53 **Keywords:** induced defences, benzothiadiazole, methyl jasmonate, needle clipping, phloem
54 wounding, conifers, salicylic acid

55

56 **Introduction**

57 During recent decades several chemical elicitors and mechanical treatments mimicking
58 insect herbivory have been used in manipulative studies of plant immunity and induced
59 defensive responses, both in herbaceous plants (reviewed by Chen 2008) and, to a lesser
60 extent, in woody plants (reviewed by Eyles et al. 2009). Particularly in trees, experimental
61 mechanical damage such as phloem wounding (e.g. Lombardero et al. 2006; Knebel et al.
62 2008) or leaf clipping (e.g. Baraza et al. 2004) have been reported to induce plant chemical
63 responses similar to those caused by insect herbivory. Lombardero et al. (2006) observed
64 that a wounding treatment using a bark chipper to remove two strips of bark and phloem
65 significantly increased the resin flow of *Pinus resinosa* trees. Baraza et al. (2004) found that
66 a leaf clipping treatment using scissors to simulate ungulate attacks significantly increased
67 phenolic compounds in leaves of *Quercus pyrenaica*. Artificial activation of wound
68 signalling pathways through the exogenous application of methyl jasmonate (MJ) has also
69 been successfully used for studying the damage-associated changes in primary and
70 secondary metabolism in several species of tree (Holopainen et al. 2009 and references
71 therein). For example, Sampedro et al. (2011a) observed that exogenous application of 22
72 mM MJ significantly increased resin in the stems and phenolics in the needles of *P. pinaster*

73 seedlings, while reducing primary and secondary growth rates. Moreover, they found
74 relevant genetic variation in those induced responses. The same authors also found that only
75 48 h after exposure to the large pine weevil *H. abietis*, damaged pine seedlings increased
76 their resin content in the exposed stem section by 300% compared to control plants
77 (Sampedro et al. 2011b). The use of other chemical elicitors of plant defensive responses in
78 woody plants, such as ethylene (Hudgins and Franceschi 2004) or benzothiadiazole (BTH, a
79 synthetic chemical analogous of salicylic acid; Sklodowska et al. 2010) has been relatively
80 little studied, however.

81 The combination of mechanical, chemical and biotic induction of chemical defences
82 in the same experimental approach can be very useful for enhancing our understanding of
83 the ecology and physiology of plant induced defences, particularly for distinguishing the
84 effects of defensive elicitation from those derived from tissue damage or loss. Recent
85 advances have shown that both the signalling from injured tissues and the biotic recognition
86 of herbivore-associated molecules could play an essential role in plant defensive induction
87 (Mithöfer and Boland 2008; Heil 2009; Hilker and Meiners 2010). It is known that
88 simulated herbivory treatments such as MJ and mechanical wounding are able to induce
89 defensive responses that provide effective resistance in juvenile pine trees (Heijari et al.
90 2005; Sampedro et al. 2011b). To be representative of real herbivory, however, the effects
91 of chemical and mechanical elicitors should be within the same quantitative range as those
92 elicited by real herbivory. Quantitative comparisons between defensive responses induced
93 by real and simulated herbivory have received little attention, and in particular no
94 information is available for young conifer trees despite the fact that they are increasingly
95 used as model examples of long-lived woody plants.

96 In this study, we compared the effect of several real and simulated herbivory

97 treatments potentially used in experimental studies of induced defences on the quantitative
98 secondary chemistry of *P. pinaster* juveniles, specifically the concentration of resin in the
99 stem and total polyphenolics in the needles, two traits positively related with resistance
100 against pathogenic fungi and insect herbivores (e.g. Franceschi et al. 2005; Keeling and
101 Bohlmann 2006 and references therein). The specific objectives were: 1) to discover the
102 extent to which needle clipping and phloem wounding could elicit similar responses to those
103 caused by real herbivores, i.e., to check whether specific defence responses to insect
104 herbivores differ from stress responses due to mechanical damage; 2) to compare the effect
105 of known plant hormones involved in plant defensive responses with the responses observed
106 after real herbivory.

107

108 **Material and methods**

109 The experiment consisted in a greenhouse experiment following a completely randomized
110 block design with seven treatments of defensive induction, two sampling times and eight
111 replicated blocks.

112 *P. pinaster* seedlings from the coastal population of Galicia (NW Spain) were
113 individually grown in 1 L containers filled with peat and perlite (1:1 v:v), fertilized with 12
114 g of a slow release fertilizer (Multicote® N:P:K 15:15:15) and grown in a greenhouse with
115 controlled temperature (25° C at day and 18° C at night) and daily irrigation. On 20 July
116 2009, when seedlings were 12 months old, we manipulated the defensive chemistry of the
117 plants with the following treatments: i) MJ, plants were sprayed over the foliage to run off
118 with a solution of 40 mM methyl jasmonate (MJ; Sigma-Aldrich, #39270-7) in deionized
119 water with ethanol 2.5 % (v:v); ii) BTH, plants were sprayed with a solution of 20 µM BTH
120 (Syngenta Bion® 50 WG wettable powder, 50% active ingredient) in deionized water with

121 ethanol 2.5 % (v:v); iii) HYL, two adults of the large pine weevil *Hylobius abietis*
122 (Coleoptera: Curculionidae) were confined into a fine-mesh cage covering the whole
123 aboveground part of each living seedling; iv) BL, one adult of *Brachyderes lusitanicus*
124 (Coleoptera: Curculionidae) was confined as above; v) mechanical wounding (MW), three 3
125 mm diameter wounds was made through the phloem until the xylem along the stem
126 simulating the wounds made by the large pine weevil; vi) clipping (CLIP), about 25% of
127 needle length was removed in all the needles with laboratory scissors; and vii) control,
128 untreated plants.

129 Exogenous application of MJ and BTH were considered as chemical induction
130 treatments. The concentration of MJ was determined according to plant size and previous
131 studies with pine seedlings (Moreira et al. 2009; Sampedro et al. 2011a); concentration of
132 BTH was previously checked to be within the effective range but below those leading to
133 evident toxicity (Moreira et al. unpublished data).

134 Exposure to insect feeding by the pine weevils *H. abietis* (a phloem-feeder) and *B.*
135 *lusitanicus* (a defoliator) were considered as biological induction treatments (real
136 herbivory). *H. abietis* feeds extensively on the bark and phloem of conifer seedlings,
137 causing important mortalities in young conifer regeneration (e.g. Zas et al. 2006). Previous
138 studies have shown that *P. pinaster* seedlings exhibit a strong resin reaction in response to
139 feeding by this insect (Sampedro et al. 2011b). *B. lusitanicus* feeds occasionally on the
140 needles of pine species in the NW Iberian Peninsula (P. Mansilla, personal communication),
141 causing moderate damage to pine seedlings. Adults of both insect species were caught in the
142 field a few days before the experiment, reared on pine seedlings, and starved in Petri dishes
143 with a moist filter paper at 18 °C for 24 h prior to the experiment. Insects were allowed to
144 feed on the experimental plants for 72 h. All the plants were damaged by both herbivory

145 insects. Moreover, damage was similar in all the plants.

146 Phloem wounding and needle clipping were considered as mechanical inductors. The
147 intensity of both mechanical treatments was adjusted in order to cause similar levels of
148 damage to the phloem and needles as were expected in the treatments with real herbivory.
149 They were calculated based on previous experience with feeding bioassays with *H. abietis*
150 (Sampedro et al. 2011b) and after observing the eating habits of *B. lusitanicus* both in the
151 field and in laboratory rearing conditions. In the event, the debarked area by MW in each
152 plant was similar to those observed in HYL treatment, but needle damage by clipping was
153 slightly greater than that caused by living *B. lusitanicus*.

154 On 5 August 2009, two weeks after the application of treatments, all the pine
155 juveniles of four randomly chosen blocks were harvested, separated into stems and needles
156 and transported to the lab into ice coolers. Stem samples were frozen and preserved at -30
157 °C for analysis of resin and needle samples were oven dried (45 °C to constant weight) and
158 then manually grounded in a mortar with liquid N₂ for analysis of total phenolics. Plants of
159 the remaining four blocks were harvested on 20 August 2009, four weeks after the
160 application of treatments.

161 On 20 January 2011, we conducted a second additional experiment (using one-year-
162 old pine juveniles and the same greenhouse conditions as above) aimed to separate specific
163 defence responses to insect herbivores from stress responses due to mechanical damage. To
164 this end we compared the effect of mechanical damage treatments with and without the
165 application of insects extracts over the wounds, in order to indentify whether recognition of
166 herbivore associated molecular patterns was in part responsible for the elicitation (Mithöfer
167 and Boland 2008). We prepared extracts from *H. abietis* and *B. lusitanicus* with 20 g of
168 weevils in 100 ml of insect ringer solution. To prepare the extract, weevils were previously

169 anesthetized at 0°C for 24 h, deep frozen with liquid nitrogen, finely crushed in a mortar
170 with the saline solution and then extracts filtered through GFF filter paper. We manipulated
171 the plants with the following treatments: 1) MW as above followed by the application of a
172 drop of *H. abietis* extract with a fine brush on the wound; 2) clipping of the needles
173 followed by the application of *B. lusitanicus* extract on the needles with a fine brush; 3)
174 MW with the application of the saline solution as control; 4) clipping with application of the
175 saline solution as control. Mechanical damage (MW and clipping) was identical to that
176 applied in the initial experiment described above. This experiment was established
177 following a randomized complete-block design with eight replicated blocks. Two weeks
178 after the application of treatments, all the pine juveniles were harvested and manipulated as
179 above.

180 Total phenolics in the needles were determined colorimetrically using the Folin-
181 Ciocalteu reagent in 0.3 g of plant tissue, after extraction with aqueous methanol (1:1
182 vol:vol) in an ultrasonic bath for 15 min, following centrifugation and subsequent dilution
183 of the methanolic extract as in Baraza et al. (2004). Concentration of total phenolics was
184 determined colorimetrically in a Biorad 650 microplate reader at 740 nm and referred to as
185 tannic acid equivalents in the vegetal tissue on a d.w. basis. Concentration of stem resin was
186 estimated gravimetrically as in Moreira et al. (2009) and expressed as mg of resin g⁻¹ stem
187 dried weight (d.w.). Briefly, about 5 g fresh weight of stem material was transferred into
188 preweighed tubes, resin compounds were extracted with 3 mL of hexane (15 min at 20 °C in
189 an ultrasonic bath and then for 24 h at room temperature), the extract was filtered (Whatman
190 GFF) into preweighed tubes, and the whole extraction step repeated again. The solvent in
191 the tubes was evaporated to dryness and the mass of the non-volatile resin residue was
192 determined to the nearest 0.0001 g.

193 The effects of the induction treatment, sampling time, block (nested within sampling
194 time) and the interaction between the induction treatment and the sampling time were
195 analyzed with a linear model using the PROC-MIXED procedure of the SAS System. In the
196 second experiment to separate specific defensive responses from stress responses, we also
197 used the PROC-MIXED procedure with the induction treatments and blocks as the main
198 factors. When the induction treatment effect was significant, differences among means were
199 tested for significance using the LSMEAN statement.

200

201 **Results**

202 **Comparison between real and simulated herbivory treatments**

203 Concentration of stem resin and leaf total phenolics was significantly affected by the
204 induction treatments (Table 1). Sampling date significantly affected the concentration of
205 non-volatile resin in the stem but not that of total phenolics in the needles (Table 1).
206 Concentration of stem resin was significantly greater two weeks after the application of
207 induction treatments than four weeks after (data not shown), but differences among
208 treatments remained unchanged (not significant treatment \times sampling date interaction, Table
209 1).

210 Concentration of stem resin and needle phenolics in plants exposed to real herbivory
211 by *H. abietis* (phloem wounding) were, respectively, 1.4- and 2.4-fold greater than in
212 control plants (Figure 1). However, we did not detect significant increases in these
213 secondary compounds in plants exposed to defoliation by *B. lusitanicus* (Figure 1).

214 Concentration of phenolics in the needles was significantly increased by both MJ
215 and BTH treatments, reaching 1.9 and 2.0-fold greater concentrations than in control plants,
216 respectively (Figure 1b). However, results differed for stem resin concentration, which was

217 significantly increased following the MJ treatment (Figure 1a), but unaffected by BTH.

218 Contrasting results were observed for the two mechanical treatments. Mechanical
219 wounding of the phloem induced a significant increase in the concentration of both stem
220 resin (1.5-fold increase) and phenolics in the needles (1.8-fold), compared with those found
221 in the control plants. However, neither resin nor phenolics were significantly affected by
222 needle clipping (Figure 1).

223

224 **Comparison between specific defence and stress responses**

225 We did not observe significant differences in the induction of chemical defences between
226 treatments based on only mechanical damage and treatments based on mechanical damage
227 plus the application of herbivore extracts (Figure 2). Specifically, the response of stem resin
228 and needle phenolics was not different between the wounding treatment and the wounding
229 plus *H. abietis* treatment (Figures 2a, 2b). Similarly, no differences were observed between
230 the clipping treatment and the clipping plus *B. lusitanicus* treatment (Figures 2c, 2d).
231 Concentrations of chemical defences were similar to those observed in the experiment 1 for
232 MW and clipping.

233

234 **Discussion**

235 Results indicate that chemical induction with 40 mM MJ, mechanical wounding and real
236 herbivory by *H. abietis* produced quantitatively similar results, increasing the concentration
237 of resin in the stem and total phenolics in the needles of one-year-old *P. pinaster* juveniles
238 by similar magnitudes. These results agree with previous observations reporting that the
239 exogenous application of MJ (Heijari et al. 2005; Moreira et al. 2009; Sampedro et al.
240 2011a) and mechanical wounds in the phloem (Tomlin et al. 1998; Knebel et al. 2008)

241 induce large chemical defensive responses in young conifer trees. The effect of mechanical
242 wounding and real phloem herbivory on needle phenolics however, has not been commonly
243 determined in previous studies. Moreover, results presented here suggest that the responses
244 to phloem damage could involve leaf protection too, indicating a systemic signalling of the
245 damage involving distal responses. The systemic effect of induction treatments between
246 different stem parts of conifer juveniles was previously reported by Moreira et al. (2009),
247 who observed that exogenous application of MJ on a basal branch induced defensive
248 responses in the upper main stem.

249 In contrast with mechanical wounding, needle clipping did not induce changes in the
250 concentrations of leaf phenolics or stem resin, and appears to be a poor method of
251 simulating insect herbivory. Criticisms of this kind of simulated herbivory treatment have
252 been outlined before (e.g. Roitto et al. 2009; Heil 2010) and are based on the facts that i)
253 clipping is usually applied only once, whereas real herbivory is normally a continuous
254 treatment that could even increase in intensity over time, and ii) plants could be able to
255 recognize their enemies via the perception of herbivore associated molecular patterns such
256 as elicitors present in the herbivore saliva (Heil 2009; Hilker and Meiners 2010). Although
257 these two factors also apply to mechanical wounding, results presented here suggest that
258 wounding mechanically the bark and phloem did elicit responses that were not
259 quantitatively different from real phloem herbivory, and thus could be helpful in
260 manipulative experiments.

261 In the second experiment we observed that mechanical damage with further
262 application of insect extracts from *B. lusitanicus* and *H. abietis* on the wounds did not
263 increase the responses in comparison with mechanical damage alone. The fact that such a
264 response is elicited simply by the mechanical damage indicates low specificity of the

265 response, and it is consistent with a quick response to damage with fatal consequences
266 irrespective of the identity of the enemy. These results agree well with the findings of our
267 first experiment in which, as previously described, chemical induction with mechanical
268 wounding and real phloem herbivory by *H. abietis* produced quantitatively similar results,
269 increasing chemical defences by equivalent magnitudes. Whether the responses to MJ,
270 wounding and real herbivory differ qualitatively remains to be explored.

271 We also observed that the use of 20 μ M BTH increased the concentration of
272 phenolic compounds in pine needles. Exogenous application of BTH has been commonly
273 found to induce defensive responses and pathogen resistance in several agricultural and
274 herbaceous species (e.g. Gondim et al. 2008; Mbouobda et al. 2010; Sklodowska et al.
275 2010), but to date no information is available about its effects on conifer species. Further
276 studies should address the possible existence of crosstalk between jasmonate and salicylic
277 acid pathways in pine trees, a better characterization of the qualitative response by this
278 bioactive compound and its relationship with different herbivore feeding guilds.

279 A fair amount of evidence already exists that the use of simulated herbivory
280 treatments induces defensive responses in a vast number of conifer species. To our
281 knowledge, however, this is the first work that compares treatments of real herbivory and
282 simulated herbivory by mechanical and chemical inductors in young conifers and reports
283 quantitatively similar induced responses. Results presented in this paper have great
284 relevance for the interpretation of previous research using needle clipping for eliciting
285 induced responses in conifers, and as well for supporting future methodological approaches
286 using simulated herbivory treatments for the induction of defences, owing to the fact that
287 real herbivory is usually less uniform and more laborious than simulated herbivory.

288

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296

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Table 1. Results of the linear model for Experiment 1 with the effects of the block, sampling date and several induction treatments on quantitative defensive chemistry (resin in the stem and total phenolics in the needles) of one-year-old *Pinus pinaster* juveniles. Significant *P* values are marked in bold type.

Effect	DF (effect, error) ¹	Resin in the stem		Total phenolics in the needles	
		F	<i>P</i>	F	<i>P</i>
Block(time)	6, 32	0.42	0.862	1.78	0.138
Time	1, 32	7.42	0.010	1.21	0.280
Induction treatment	6, 32	3.08	0.017	3.16	0.019
Induction × Time	6, 32	0.98	0.455	1.44	0.232

¹ DF: degrees of freedom

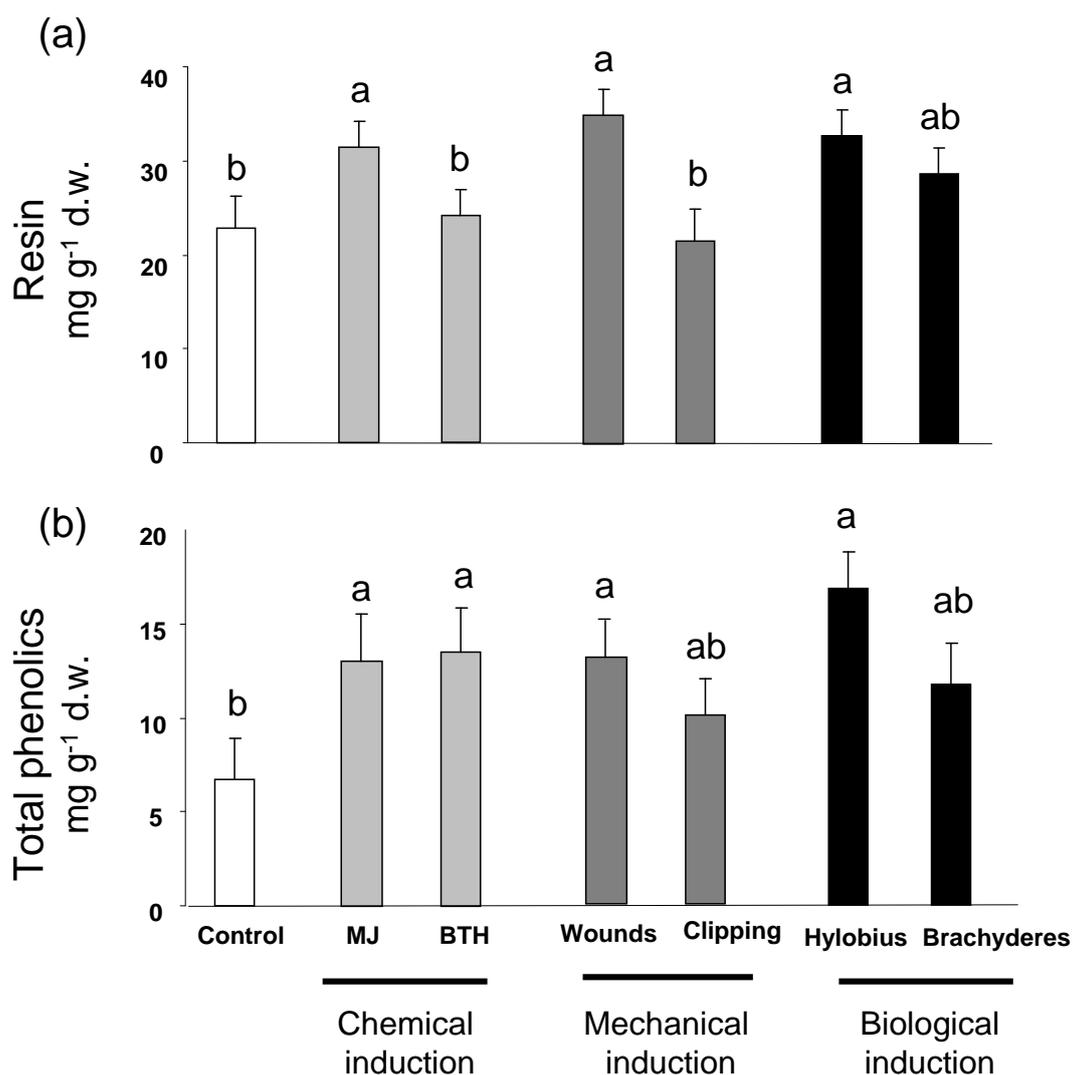
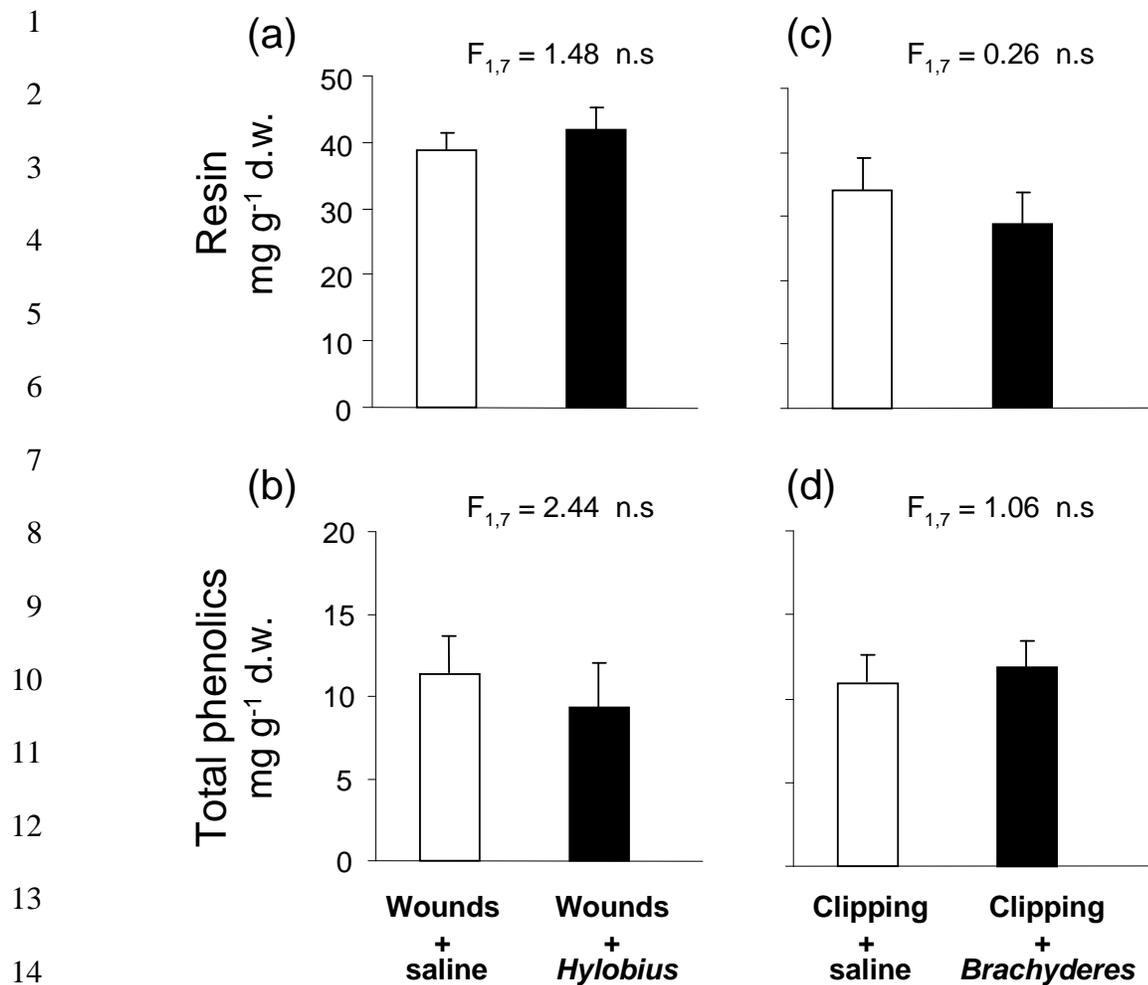


Figure 1. Concentration of resin in the stem (a), and total phenolics (expressed as tannic acid equivalents) in the needles (b) in one year-old *P. pinaster* juveniles after the application of several chemical (light grey bars), mechanical (dark grey bars) or biological (black bars) treatments of defensive induction. MJ = 40 mM Methyl jasmonate; BTH = 20 μ M benzothiadiazole; Wounds = mechanical wounding of the phloem; Clipping = top needle clipping; Hylobius = phloem feeding by the large pine weevil *Hylobius abietis*; Brachyderes = needle defoliation by the weevil *Brachyderes lusitanicus*. Data are the mean values \pm s.e.m. (N = 8) of two independent sampling dates. Different letters indicate significant differences between treatments ($P < 0.05$).



16 Figure 2. Concentration of resin in the stem (a) (c), and total phenolics (expressed as tannic
 17 acid equivalents) in the needles (b) (d) in one year-old *P. pinaster* juveniles after the
 18 application of treatments of experimental elicitation of defences. Wounds = mechanical
 19 wounding of the phloem; Clipping = top needle clipping. *Hylobius* and *Brachyderes* extract
 20 consisted of insect tissues extracted in a saline solution. Data are the mean values \pm s.e.m.
 21 (N = 8).

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