Resistance and response of *Pinus pinaster* seedlings to *Hylobius abietis* after induction with methyl jasmonate

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Abstract

Experimental induction of plant chemical defences with methyl jasmonate (MeJa) is a valuable tool for understanding the ecology of plant defensive responses. However few studies have examined whether MeJa-induced defences in conifers are effective against insect herbivores. We studied, in 17 half-sib *Pinus pinaster* families, (i) the effect of MeJa application on plant growth and on the induction of diterpenoid resin in different sections of the stem; (ii) whether MeJa-induced defences increase the resistance of living pine juveniles against the large pine weevil *Hylobius abietis* in an *in vivo* bioassay, and (iii) the induction of resin content after weevil wounding. Resin concentration was greater in the upper section of the stem compared with basal sections in both MeJa-induced and non-induced seedlings. Sixty days after MeJa application treated plants showed 40% greater resin content all along the stem, but reduced height growth compared to control plants. MeJa-induction was effective against the pine weevil, as induced seedlings were 30% less damaged than control plants. Wounding activity by *H. abietis* produced a strong local defensive response after 48 h, where resin concentration was double that observed in the basal and apical sections not exposed to the insects.

Key-words: induced resistance, in vivo bioassays, resin content, herbivory, diterpenes, conifer resistance

Introduction

In response to chemical, physical or biotic stimuli, constitutive defences can be reinforced by induced defences, newly synthesized or mobilized to the site where a plant is injured. This form of phenotypic plasticity has been suggested to be cost-saving,

as resources useful for growth are employed in defences only when needed (reviewed by Karban & Myers 1989). However, triggering of induced defences requires an activation time after identifying the biological enemy. Once triggered, defences will continue until the biotic challenge disappears, at which point they will usually revert back to their initial state (decay time *sensu* Gómez et al. 2009).

Several phytohormones involved in plant defence signalling and induced defence triggering are increasingly used as experimental chemical elicitors for studying plant defensive responses. Particularly, the exogenous application of methyl jasmonate (MeJa) is known to activate a wide variety of resistance traits in several annual plant species (e.g. Baldwin 1998; Kessler et al. 2004). In conifers, although the information is more limited, MeJa has been also reported to induce plant responses similar to those caused by insect herbivory through up-regulating gene expression, enzyme activity and accumulation of terpenoid defences (reviewed by Bohlmann 2008).

Recently, Moreira et al. (2009), using *cafeteria* bioassays in Petri plates, reported that the large pine weevil *Hylobius abietis* fed significantly less on cut twigs of MeJa treated *Pinus pinaster* seedlings than on twigs of untreated plants. This pine weevil is a generalist phloem and bark feeder that causes important damage in young conifer stands all around Europe, including *P. pinaster* (Zas et al. 2006), a model Mediterranean species for genetic and ecological studies in south-west Europe (González-Martínez et al. 2004). In field conditions, induced plant responses to the feeding activity of this insect may be crucial to counteract the insect attack, and thus the results of *in vitro* bioassays may not strictly reflect field resistance. In the present work, we explored whether exogenous application of MeJa is also effective in reducing pine weevil damage to living *P. pinaster* seedlings using whole-plant *in vivo* bioassays. We were also interested in whether the weevil damage during the bioassay could induce

defensive responses in pine seedlings, and so we analyzed the resin content in the plants before and after the exposure to the weevil.

Material and methods

Seeds of 17 open-pollinated *P. pinaster* families, selected from the Atlantic coast population in Galicia (NW Spain), were individually sown in 2 L containers filled with peat and perlite (1:1 v:v), fertilized with 12 g of a slow release fertilizer (Multicote® N:P:K 15:15:15), and grown in a greenhouse with controlled temperature (25/18 ºC day/night). In June 2007, when pine seedlings were 16 months old and about 116 ± 1.4 cm tall (mean ± s.e.), half of the seedlings were treated with a suspension of 100 mM MeJa (Sigma-Aldrich, #39270-7) in deionised water with 0.1% (v/v) Tween-20®. The remaining seedlings (control plants) were treated with the carrier solution. Both treatments were sprayed over the foliage to run off (about 3.1 ± 0.2 ml per plant) in two separate rooms of the greenhouse, where the seedlings remained for 24 h. Then all seedlings were positioned according to a randomized complete block design.

Sixty days after the application of the treatments, just before the bioassay, tree height was measured again, and twelve randomly selected plants (N = 6 treated with MeJa and N = 6 from the control treatment) were destructively sampled to analyze the resin content in the stem prior to the bioassay. Needles were carefully separated from the stem, and 10 cm sections of the basal, intermediate and apical sections of the stem were sampled, immediately frozen, and preserved at -30ºC until analysis.

With six seedlings of each family (N = 3 MeJa-induced and N = 3 control) we established an *in vivo* bioassay to evaluate the actual resistance against *H. abietis*. Total number of seedlings for the bioassay was N = 102 (3 blocks × 2 treatments × 17 half-sibs). Two pre-weighed pine weevils were confined into fine-mesh cages fixed onto the

intermediate section of the stem of each living seedling (see details in Online Resources 1). Insects had been caught in the field one week before the experiment and starved in Petri dishes with a moist filter paper for 24 h prior to the bioassay. After 48 h of exposure to weevil feeding, insects were removed from the cages and all the seedlings immediately harvested. Bark and phloem consumed by the weevil was measured with a millimetric grid as the debarked area of the stem. The stem was then divided into basal, intermediate and apical sections and sampled as above for resin analysis.

The concentration of diterpenes in the stem, a main resistance trait against insects in conifers (Bohlmann 2008), was determined gravimetrically after two cycles of quantitative extraction with n-hexane, following Moreira et al. (2009) (see detailed methodology in Online Resource 2). The non-volatile resin residue is composed mainly of diterpene resin acids that remain as an oxidized residue after volatilization of the lighter fraction of the oleoresin (mono and sesquiterpenes). Results obtained following this simple procedure were well correlated to the concentration of the diterpenoid fraction in the stem ($r = 0.9214$; $P < 0.0001$) as analyzed by GC-MS according to Arrabal et al. (2005).

The effects of induction (MeJa), Family (F) and MeJa × F on plant height and on the damage caused by the weevil in the *in vivo* bioassay were analyzed with a mixed model with Block as a random factor, using the initial plant height and the weevil weight as covariates, respectively. The resin content in the different stem sections of the plants harvested before the bioassay was analyzed with MeJa, stem Section and Section × MeJa as main factors. Stem section was considered a within-subject factor, as measures were taken in the same experimental units (Littell et al. 2006). For analyzing the resin content in the plants after the bioassay we included the Family effect to account for the genetic variance and reduce the error term. Block (random), MeJa,

Family and their interactions were considered between-subject factors, and stem Section, Section × Family and Section × MeJa within subject factors. All analyses were performed using the Proc Mixed procedure of SAS. Data are presented as means ± standard errors.

Results and Discussion

The analysis of the plants harvested just before the bioassays showed that treatment with MeJa induced a 40% increase in the resin content of stems (Fig. 1a). The resin concentration was significantly greater towards the upper section of the stem, and the induction was similar in the three studied stem sections (no significant MeJa × Section interaction) (Fig. 1a).

Results from the *in vivo* bioassay showed that MeJa-induction was also effective in increasing the resistance to the large pine weevil. The damage by the weevil after the 48 h bioassay was 21% smaller in those plants previously treated with MeJa, with already activated induced defences, than in control plants (Fig. 2a). We did not detect significant differences in damage by the insect among pine families ($F_{16,65} = 0.74$, $p = 0.746$) nor in MeJa × Family interaction ($F_{16,65} = 0.76$, $p = 0.727$). Although MeJa is well known to elicit anatomical and chemical defences in conifers (reviewed by Eyles et al. 2009), few studies have focused on whether MeJa-induced responses are effective in improving the resistance against insect herbivores or fungal pathogens. Our results agree with the limited information available for the genus *Pinus* (Gould et al. 2008; Heijari et al. 2005; Moreira et al. 2009). Of particular relevance, Moreira et al. (2009) reported that application of MeJa significantly increases the resin content in the stems of *P. pinaster* seedlings and reduces the area debarked by this weevil in *in vitro* bioassays.

146 Here we demonstrate that the defensive responses elicited by MeJa also effectively
147 protect living seedlings of this Mediterranean pine.

148 These results could lead to the assumption that the application of MeJa could be
149 considered as a potential tool to protect seedlings against these insects (Holopainen et
150 al. 2009). However, as commonly observed (Gould et al. 2008; Heijari et al. 2005), the
151 improvement of seedling resistance through MeJa application was not cost-free, and the
152 application of MeJa significantly reduced the pine height growth (Fig. 2b), which may
153 affect field performance and future fitness, as has been observed in other plant species
154 (Baldwin 1998). Nevertheless, in a previous experiment with the same species (Moreira
155 et al. 2009) we did not detect significant effect of MeJa on plant growth, although this
156 was in all likelihood because MeJa was applied at the beginning of the growing season
157 when growth rates were markedly lower than in the present paper. Seedling phenology,
158 thus, appears crucial for measuring vegetative costs of MeJa-induced defences.

159 Another key finding was that the exposure of living seedlings to weevil feeding
160 altered the pattern of resin defences along the stem (Fig. 1b). The greater resin
161 concentration was now found in the section exposed to the insect, with values double
162 those observed before the bioassay, suggesting a strong local response to the weevil
163 damage just after 48 h. Up-regulation of defensive gene expression just a few hours
164 after exposure to the white pine weevil *Pissodes strobi* was reported for Sitka spruce
165 (Ralph et al. 2006) and altered resin acid concentration in bark and xylem of *P.*
166 *sylvestris* seedlings was found after four days of insect feeding (Heijari et al. 2005). Our
167 results indicate that short term responses induced by insect feeding also involved large
168 quantitative functional changes in resistance traits. Although this rapid response was
169 restricted to the stem section exposed to the insects, we do not discard further systemic
170 extensions to other parts of the plant after a longer period than 48 h. The existence of

such a local response to insect wounding should be considered when interpreting the results of feeding tests with living plants. Results from *in vivo* bioassays could reflect purely constitutive resistance, or indeed constitutive plus induced resistance if the time that insects are allowed to feed is longer than the activation time (Gómez et al. 2009) for triggering induced defences.

After insect feeding, we found a significant interaction Section × MeJa (Fig. 1b) because the effect of MeJa on the resin content, although still significant in the apical and basal sections of the stem, was not significant in the section of the stem exposed to the insect. The resin content in the intermediate section of the stem was actually slightly greater in control than in MeJa-treated plants, probably due the greater feeding supported by the control plants. We did not observe, however, a significant correlation between the feeding scar area and the resin content after insect damage ($r = 0.029$, $p > 0.05$).This result suggests that other MeJa-inducible defences could be also important in limiting insect feeding.

Further work should clarify if local herbivory by this harmful weevil in Maritime pine trees produces a systemic induction of defences, and aim to identify other induced defences involved in resistance to this insect, and determine the decay time of those responses.

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FIGURE CAPTIONS

Figure 1. Resin concentration in the basal, intermediate and apical sections of the stem in control and MeJa-induced *P. pinaster* seedlings destructively sampled (a) before an *in vivo* bioassay and (b) after exposing the pine seedlings to wounding by the large pine weevil *H. abietis* during a 48 hours bioassay. F values and associate probability levels of the main factors are also shown. Means ± s.e. are presented, with N = 6 in panel (a), and N = 51 in panel (b). Asterisks indicate significant differences between MeJa-induced and control plants within each stem section. Different letters indicate significant differences between plant sections (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$).

Figure 2. Effect of MeJa induction of *P. pinaster* seedlings from 17 half-sibs on (a) actual resistance to the large pine weevil *H. abietis* in an *in vivo* bioassay, and on (b) seedling height growth, 60 days after treatment application. F values and associate probability levels of the main factors MeJa, Family and MeJa × Family are also shown. Means ± s.e.m. (N = 51).

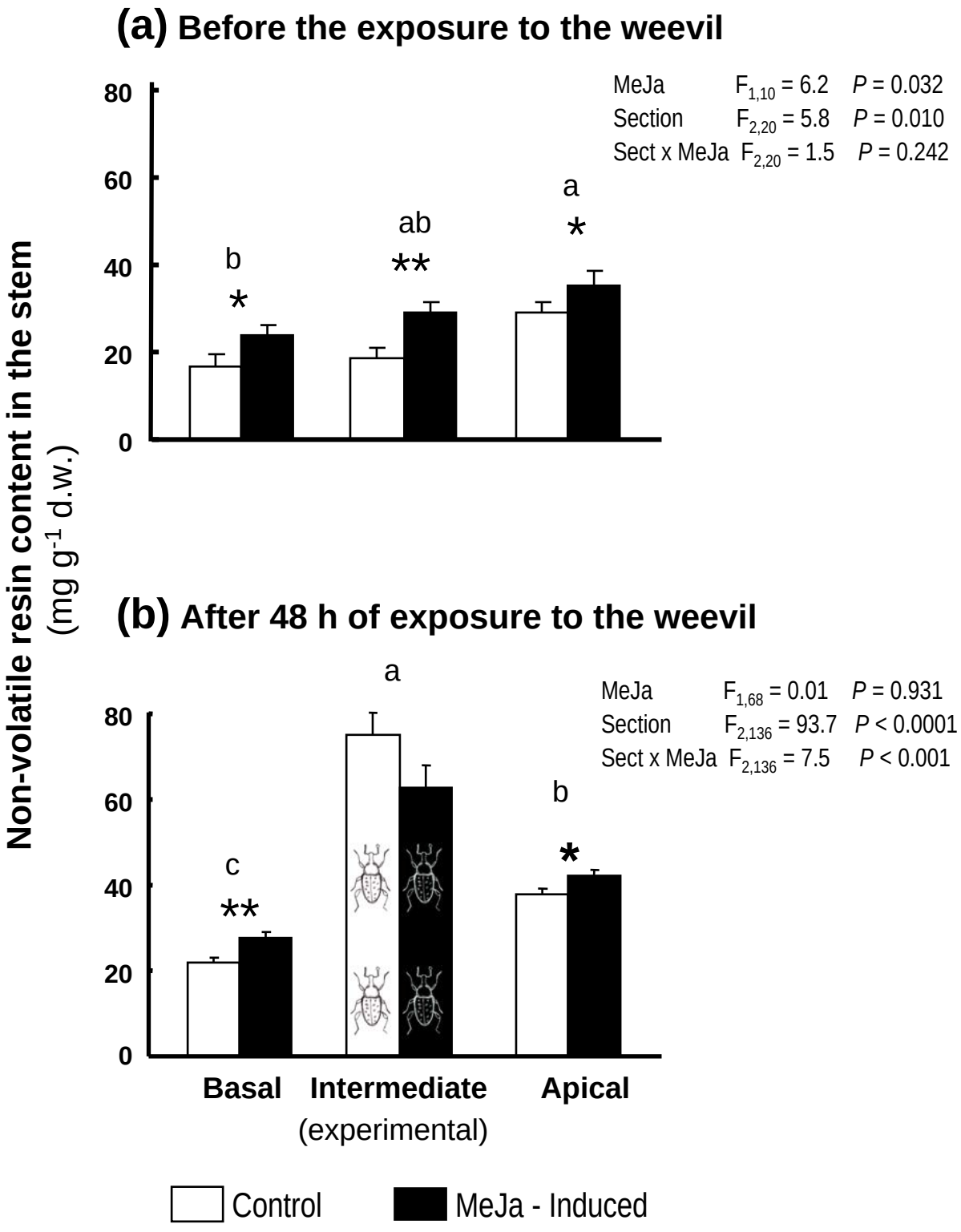

Figure 1. Sampedro et al. VEGE2370_REV1

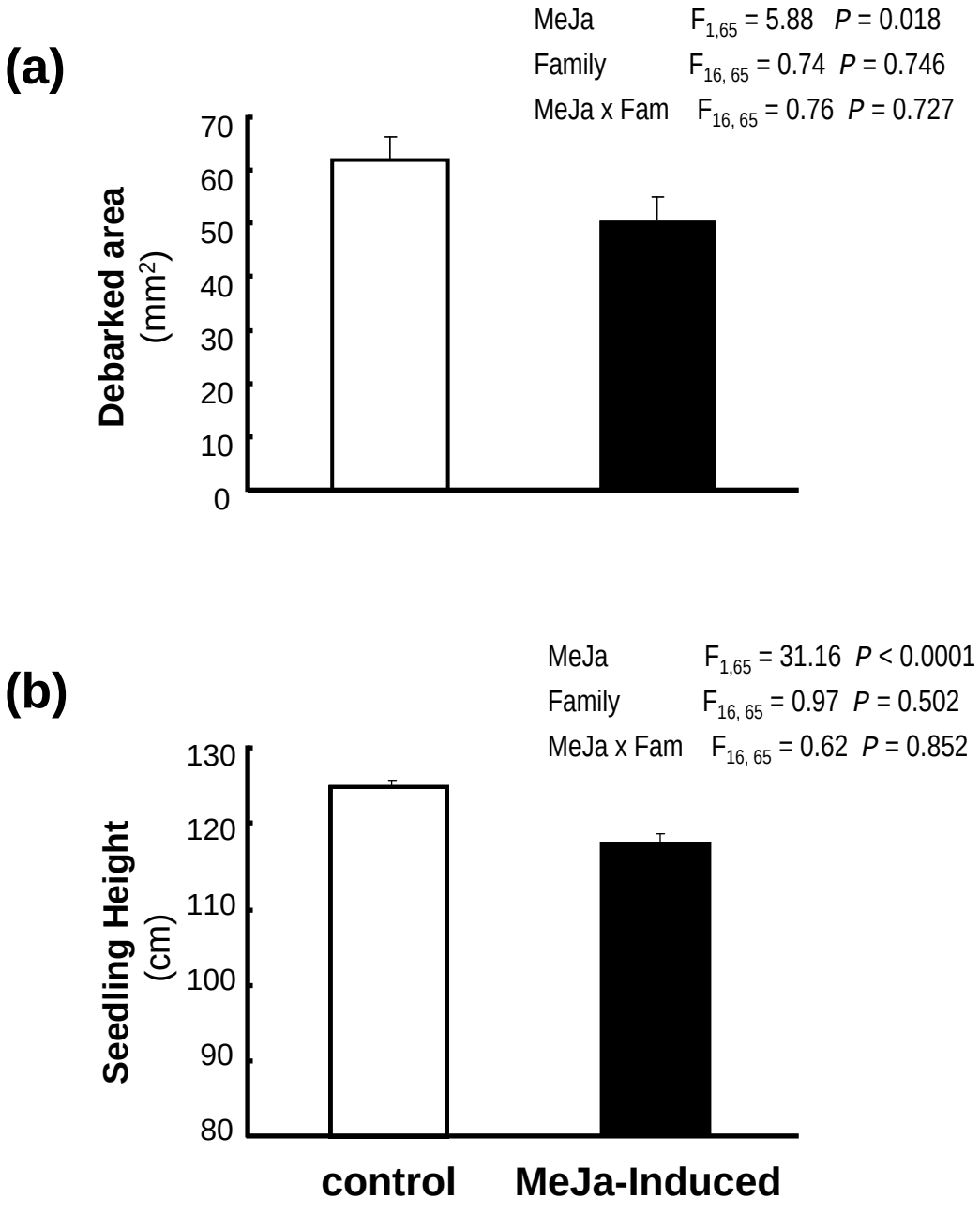

Figure 2. Sampedro et al. VEGE2370_REV1

Online Resource 1. Schematic representation of the experimental approach used for the *in vivo* bioassays with sixteen months-old *P. pinaster* seedlings.

Article: VEGE2370_REV1. Resistance and response of *Pinus pinaster* seedlings to *Hylobius abietis* after induction with methyl jasmonate

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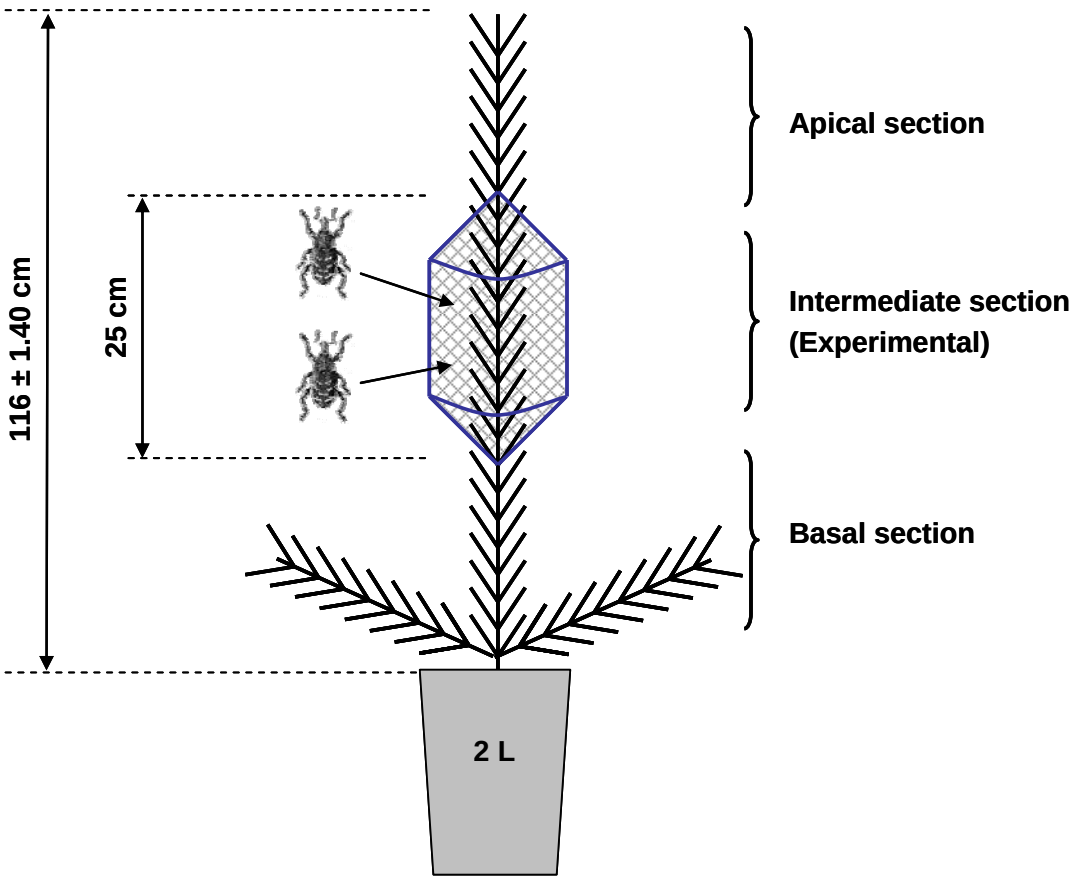

Schematic representation of the experimental approach used for the *in vivo* bioassays with sixteen months-old *P. pinaster* seedlings.

Online Resource 2. Detailed methodology for gravimetric resin determination.

Article: VEGE2370_REV1. Resistance and response of *Pinus pinaster* seedlings to *Hylobius abietis* after induction with methyl jasmonate

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The concentration of diterpenes in the stem was determined gravimetrically after quantitative extraction with n-hexane, following the procedure proposed by Wainhouse et al. (1998) and modified by Moreira et al. (2009). Stem specimens, approximately weighing 5 g fresh weight, were cut into pieces about 5 mm long and transferred into pre-weighed test tubes. Resin compounds were extracted with 5 ml of n-hexane in an ultrasonic bath (15 min at 20 ºC), and then for 24 h at room temperature under the fume hood. The extract was poured through a Whatman GF/B glass microfiber filter into a second pre-weighed test tube, plant material on the filter was carefully recovered back into the first test tube and the whole extraction step was immediately repeated. The solvent in the test tubes was evaporated to dryness under the fume hood at room temperature (usually 2-3 days), and the non-volatile resinous residue weighed in a precision scale (0.0001 g). The plant tissue recovered after the second extraction was oven dried at 80 ºC and weighed, and the resin concentration was expressed as mg of non-volatile resin g-1 stem dried weight (d.w.). The second extraction usually yielded less than 10% of the total resin mass. A third extraction, performed in a subset of plants,

yielded less of the 2% of the total resin mass, and so for all subsequent measurements it was deemed sufficient only to consider the first two extractions.

The non-volatile resin residue is composed mainly by diterpene resin acids that remain as an oxidized residue after volatilization of the lighter fraction of the oleoresin (mono and sesquiterpenes). We calibrated this gravimetric method by analyzing the mono-, sesqui- and diterpene content in the stem by GC-MS according Arrabal et al. (2005). Gravimetric determination of non-volatile resin following this procedure was well correlated to the concentration of the diterpenoid fraction in the stem ($r = 0.9214$; $P < 0.0001$; $N = 20$).

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