

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

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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, simultaneous 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 two chemical elicitors, and mechanical damage treatments on the quantitative secondary chemistry of *Pinus pinaster* juveniles (namely on non-volatile resin in the stem and total phenolics in the needles). The real herbivory involved phloem wounding by *Hylobius abietis* and defoliation by *Brachyderes lusitanicus* (two pine weevils); the chemical elicitors to simulate herbivory induction were 40 mM methyl jasmonate (MJ) and 20 µM benzothiadiazole (BTH); and the mechanical treatments included 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 show the same effect on stem resin. Contrastingly, we did not find significant changes in the concentration of resin in the stem or phenolics in the needles after needle clipping and *B. lusitanicus* feeding. Mechanical damage followed by the application of extracts from the insects *B. lusitanicus* and *H. abietis* on the injured tissues 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. Results from this paper support future methodological approaches using chemical elicitors and mechanical damage as simulated herbivory treatments for the experimental induction of conifer defences.

Keywords Induced defences · Benzothiadiazole · Methyl jasmonate · Needle clipping · Phloem wounding · Conifers · Salicylic acid

Introduction

During recent decades several chemical elicitors and mechanical treatments mimicking insect herbivory have been used in manipulative studies of plant immunity and induced defensive responses, both in herbaceous plants (reviewed by Chen 2008) and, to a lesser extent, in woody plants (reviewed by Eyles et al. 2009). Particularly in trees, experimental mechanical damage, such as phloem wounding (e.g. Lombardero et al. 2006; Knebel et al. 2008) or leaf clipping (e.g. Baraza et al. 2004) have been reported to induce plant responses increasing the concentration of

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resistance traits. For example, Lombardero et al. (2006) observed that a wounding treatment using a bark chipper to remove two strips of bark and phloem significantly increased the resin flow of *Pinus resinosa* trees. Baraza et al. (2004) found that a leaf clipping treatment using scissors to simulate ungulate attacks significantly increased phenolic compounds in leaves of *Quercus pyrenaica*. Artificial activation of wound signalling pathways through the exogenous application of methyl jasmonate (MJ) has also been successfully used for studying the damage-associated changes in primary and secondary metabolism in several tree species (e.g. Franceschi et al. 2002; Martin et al. 2002, 2003; Hudgins et al. 2003; Huber et al. 2005; Miller et al. 2005; Krokene et al. 2008; Holopainen et al. 2009; Sampedro et al. 2011a, b). The use of other chemical elicitors of plant defensive responses, such as ethylene (Hudgins and Franceschi 2004) or benzothiadiazole (BTH, a synthetic chemical analogous of salicylic acid; Skłodowska et al. 2010) has been comparatively less studied in woody plants, however.

The simultaneous comparison of mechanical, chemical and biotic induction of chemical defences in the same experimental approach can be useful for enhancing our understanding of the ecology and physiology of plant induced defences, particularly for distinguishing the effects of defensive elicitation from those derived from tissue damage or loss. Recent advances have shown that both the signalling from injured tissues and the biotic recognition of herbivore-associated molecules could play an essential role in plant defensive induction (Mithöfer and Boland 2008; Heil 2009; Hilker and Meiners 2010). It is known that simulated herbivory treatments such as MJ and mechanical wounding are able to induce defensive responses that provide effective resistance in juvenile pine trees (Heijari et al. 2005; Sampedro et al. 2011b). To be representative of real herbivory, however, the effects of chemical and mechanical elicitors should be within the same quantitative range as those elicited by real herbivory. Simultaneous quantitative comparisons between chemical defensive responses induced by real and simulated herbivory are relatively scarce, particularly in conifer trees. For example, in one of the few available examples in conifers, Miller et al. (2005) found that wounding by the white pine weevil and exogenous application of MJ induced similar terpenoid defensive responses in the stems of Sitka spruce trees.

In this study, we compared the effect of several real and simulated herbivory treatments potentially used in experimental studies of induced defences on the quantitative secondary chemistry of *P. pinaster* juveniles, specifically the concentration of resin in the stem and total polyphenolics in the needles. Conifer trees usually accumulate large amounts of terpene and phenolic compounds in their

tissues, and resistance against herbivores has been shown to be proportional to the global concentration of these chemicals (e.g. Franceschi et al. 2005; Keeling and Bohlmann 2006 and references therein). Accordingly, conifer resistance against herbivores is assumed to be based on ‘quantitative defences’ more than on qualitative changes in the profiles of secondary chemicals (sensu Feeny 1976). The specific questions of this study were: (1) whether needle clipping and phloem wounding could elicit similar quantitative responses to those caused by real herbivores, i.e., to check whether specific defence responses to insect herbivores differ from stress responses due to mechanical damage; (2) to compare the effect of known plant hormones involved in plant defensive responses with the responses observed after real herbivory and mechanical damage.

Materials and methods

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

P. pinaster seedlings from the coastal population of Galicia (NW Spain) were individually grown in 1 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°C at day and 18°C at night) and daily irrigation. On 20 July 2009, when seedlings were 12 months old, we manipulated the defensive chemistry of the plants with the following treatments: (a) MJ, plants were sprayed over the foliage to run off with a solution of 40 mM methyl jasmonate (MJ; Sigma-Aldrich, #39270-7) in deionized water with ethanol 2.5% (v:v); (b) BTH, plants were sprayed with a solution of 20 µM BTH (Syngenta Bion® 50 WG wettable powder, 50% active ingredient) in deionized water with ethanol 2.5% (v:v); (c) HYL, two adults of the large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) were confined into a fine-mesh cage covering the whole above-ground part of each living seedling; (d) BL, one adult of *Brachyderes lusitanicus* (Coleoptera: Curculionidae) was confined as above; (e) mechanical wounding (MW), three 3 mm diameter wounds were made through the phloem until the xylem along the stem simulating the wounds made by the large pine weevil; (f) clipping (CLIP), about 25% of needle length was removed in all the needles with laboratory scissors; and (g) control, untreated plants.

Exogenous application of MJ and BTH were considered as chemical induction treatments. The concentration of MJ

was determined according to plant size and previous studies with Maritime pine seedlings (Moreira et al. 2009; Sampedro et al. 2011a); concentration of BTH was previously checked to be within the effective range but below those leading to evident toxicity (Moreira et al. unpublished data).

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

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

On 5 August 2009, two weeks after the application of treatments, all the pine juveniles of four randomly chosen blocks were harvested, separated into stems and needles and transported to the lab into ice coolers. Stem samples were frozen and preserved at -30°C for analysis of resin and needle samples were oven dried (45°C to constant weight) and then manually grounded in a mortar with liquid N₂ for analysis of total phenolics. Plants of the remaining four blocks were harvested on 20 August 2009, 4 weeks after the application of treatments.

On 20 January 2011, we conducted a second additional experiment (using one-year-old pine juveniles and the same greenhouse conditions as above) aimed to separate specific defence responses to insect herbivores from stress responses due to mechanical damage. To this end, we

compared the effect of mechanical damage treatments with and without the application of insects extracts over the wounds, in order to identify whether recognition of herbivore associated molecular patterns was in part responsible for the elicitation (Mithöfer and Boland 2008). We prepared extracts from *H. abietis* and *B. lusitanicus* with 20 g of weevils in 100 ml of insect ringer solution. To prepare the extract, weevils were previously anesthetized at 0°C for 24 h, deep frozen with liquid nitrogen, finely crushed in a mortar with the saline solution and then extracts filtered through GFF filter paper. We manipulated the plants with the following treatments: (1) MW as above followed by the application of a drop of *H. abietis* extract with a fine brush on the wound; (2) clipping of the needles followed by the application of *B. lusitanicus* extract on the needles with a fine brush; (3) MW with the application of the saline solution as control; (4) clipping with application of the saline solution as control. Mechanical damage (MW and clipping) was identical to that applied in the initial experiment described above. This experiment was established following a randomized complete-block design with eight replicated blocks. 2 weeks after the application of treatments, all the pine juveniles were harvested and manipulated as above.

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

The effects of the induction treatment, sampling time, block (nested within sampling time) and the interaction between the induction treatment and the sampling time were analyzed with a linear model using the PROC-MIXED procedure of the SAS System. In the second experiment to separate specific defensive responses from stress responses, we also used the PROC-MIXED procedure with the induction treatments and blocks as the main factors. When the induction treatment effect was

significant differences among means were tested for significance using the LSMEAN statement.

Results

Comparison between real and simulated herbivory treatments

Concentration of stem resin and leaf total phenolics was significantly affected by the induction treatments (Table 1). Sampling date significantly affected the concentration of non-volatile resin in the stem but not that of total phenolics in the needles (Table 1). Concentration of stem resin was significantly greater 2 weeks after the application of induction treatments than 4 weeks after (31.07 ± 1.69 vs. 25.00 ± 1.45), but differences among treatments remained unchanged (not significant treatment \times sampling date interaction, Table 1).

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

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

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

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 (non-volatile resin in the stem

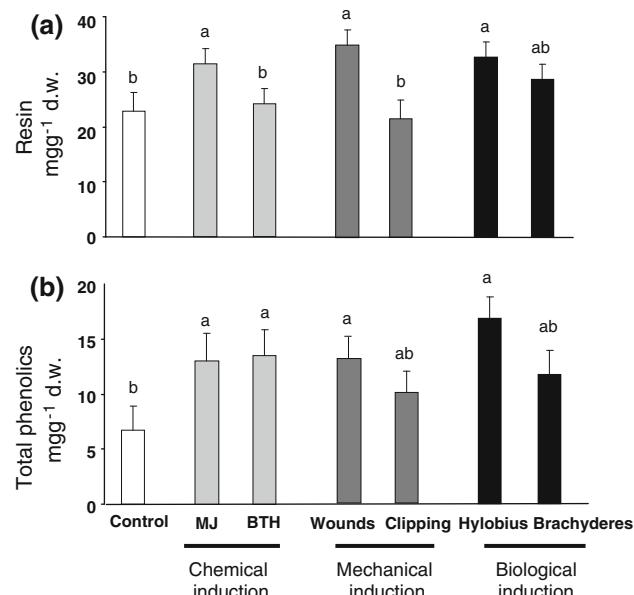


Fig. 1 Concentration of non-volatile resin in the stem (a), and total phenolics (expressed as tannic acid equivalents) in the needles (b) in 1-year-old *P. pinaster* juveniles after the application of several chemical (light grey bars), mechanical (dark grey bars) or biological (black bars) treatments of defensive induction. MJ 40 mM Methyl jasmonat, BTH 20 μ M benzothiadiazole, an analogous of methyl-salicilate; 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 SEM ($N = 8$) of two independent sampling dates. Different letters indicate significant differences between treatments ($P < 0.05$)

Comparison between specific defence and stress responses

We did not observe significant differences in the induction of chemical defences between treatments based on only mechanical damage and treatments based on mechanical damage plus the application of herbivore extracts (Fig. 2). Specifically, the response of stem resin and needle phenolics was not different between the wounding treatment

measured gravimetrically and total polyphenolics in the needles quantified by the Folin–Ciocalteu method) of 1-year-old *Pinus pinaster* juveniles

Effect	df (effect, error)	Resin in the stem		Total phenolics in the needles	
		F	P	F	P
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 \times time	6, 32	0.98	0.455	1.44	0.232

Significant P values are marked in bold type

df degrees of freedom

and the wounding plus *H. abietis* treatment (Fig. 2a, b). Similarly, no differences were observed between the clipping treatment and the clipping plus *B. lusitanicus* treatment (Fig. 2c, d). Concentrations of chemical defences were similar to those observed in the experiment 1 for MW and clipping.

Discussion

Results indicate that chemical induction with 40 mM MJ, mechanical wounding and real herbivory by *H. abietis* produced quantitatively similar results, increasing the concentration of resin in the stem and total phenolics in the needles of 1-year-old *P. pinaster* juveniles by similar magnitudes. These results agree with previous observations reporting that the exogenous application of MJ (Franceschi et al. 2002; Martin et al. 2002, 2003; Hudgins et al. 2003; Heijari et al. 2005; Huber et al. 2005; Miller et al. 2005; Moreira et al. 2009; Sampedro et al. 2011a, b) and mechanical wounds in the phloem (Tomlin et al. 1998; Lombardero et al. 2006; Knebel et al. 2008) induce large chemical defensive responses in young conifer trees. The effect of mechanical wounding and real phloem herbivory on needle phenolics however has not been commonly

determined in previous studies. Moreover, results presented here suggest that the responses to phloem damage could involve leaf protection too, indicating a systemic signalling of the damage involving distal responses. The systemic effect of induction treatments between different stem parts of conifer juveniles was previously reported by Moreira et al. (2009), who observed that exogenous application of MJ on a basal branch induced defensive responses in the upper main stem.

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

In our second experiment, we observed that mechanical damage with further application of insect extracts from *B. lusitanicus* and *H. abietis* on the wounds did not increase the responses in comparison with mechanical damage alone. The fact that such a response is elicited simply by the mechanical damage indicates low specificity of the response, and it is consistent with a quick response to damage with fatal consequences irrespective of the identity of the enemy. These results agree well with the findings of our first experiment in which, as previously described, chemical induction with mechanical wounding and real phloem herbivory by *H. abietis* produced quantitatively similar results, increasing chemical defences by equivalent magnitudes.

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

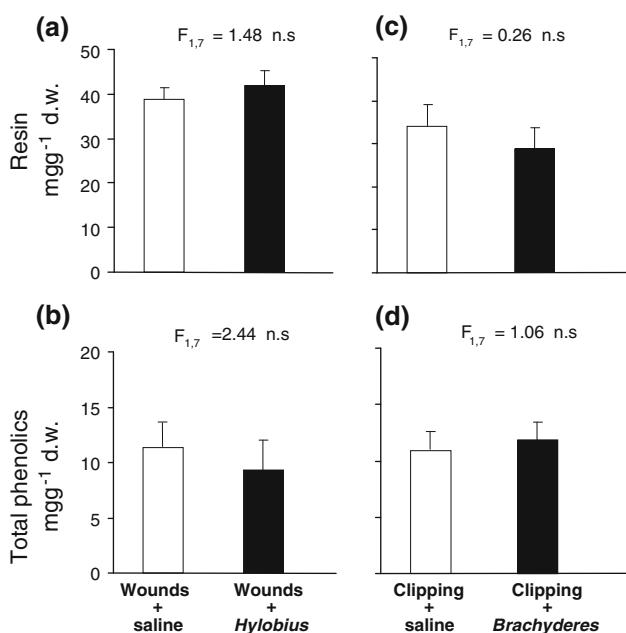


Fig. 2 Concentration of non-volatile resin in the stem (a, c), and total phenolics (expressed as tannic acid equivalents) in the needles (b, d) in 1-year-old *P. pinaster* juveniles after the application of treatments of experimental elicitation of defences. *Wounds* mechanical wounding of the phloem, *Clipping* top needle clipping. *Hylobius* and *Brachyderes* extract consisted of insect tissues extracted in a saline solution. Data are the mean values \pm SEM ($N = 8$)

The aim of this study was to compare the effect of several real and simulated herbivory treatments of induced defences on the quantitative secondary chemistry of *P. pinaster* juveniles, measurable by simple quantifications. Both the gravimetric estimation of non-volatile resin and the quantification of total phenolics using the Folin–Ciocalteu-method are simple and accurate approximations for estimating gross quantitative changes of two main resistance traits in pine trees (Sampedro et al. 2011a, b). However, with our methodology, we cannot discard significant changes in the profiles of phenolics or/and terpenes after the induction treatments. In this sense, qualitative changes in the terpene and phenolic profiles have been previously reported in response to herbivore attack (e.g. Iason et al. 2011), mechanical damage (e.g. Tomlin et al. 2000; Ratto et al. 2009) and exogenous application of chemical elicitors (e.g. Martin et al. 2002, 2003; Huber et al. 2005). Although changes in the profile of these chemicals could lead to different anti-herbivore properties (e.g. Salminen and Karonen 2011), a change in the global concentration is likely indicating functional changes in herbivore resistance (Franceschi et al. 2005). Further work should address whether the changes in the profile of phenolics and terpenes occur when different simulated herbivory treatments are applied, and what biological interpretation (direct defence, indirect defence or other kind of biotic interactions) can be derived from the profile changes of secondary metabolites.

A fair amount of evidence already exists that the use of simulated herbivory treatments induces defensive responses in a vast number of conifer species (e.g. Franceschi et al. 2002; Martin et al. 2002, 2003; Hudgins et al. 2003; Huber et al. 2005; Miller et al. 2005; Lombardero et al. 2006; Knebel et al. 2008; Krokene et al. 2008; Sampedro et al. 2011a). However, comparisons between treatments of real and simulated herbivory in young conifers remain scarce. Results presented in this paper may help with the interpretation of previous research using needle clipping for eliciting induced responses in conifers, and as well for supporting future methodological approaches using simulated herbivory treatments for the induction of defences, owing to the fact that real herbivory is usually less uniform and more laborious than simulated herbivory.

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