



## Defensive responses of *Pinus pinaster* seedlings to exogenous application of methyl jasmonate: Concentration effect and systemic response

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### ABSTRACT

Methyl jasmonate (MeJa) is a plant chemical elicitor which has been used to artificially induce chemical defensive responses and resistance against herbivores in many plant species in recent decades. In this paper, we studied the effect of exogenous MeJa application at different concentrations (0, 5, 50, and 100 mM) on growth, chemical defenses and resistance against the large pine weevil *Hylobius abietis* L in one year old *Pinus pinaster* Ait. seedlings. We also investigated whether the local application of MeJa on a basal branch would distally elicit defensive responses or growth reductions on the opposite branch and on the upper main stem. Exogenous application of MeJa induced resin accumulation in the stems, and enhanced resistance against *H. abietis*. The resin content in plants treated with 100 mM was nearly two-fold greater, and the pine weevil consumed 80% less phloem than in control plants. However, concentration of total phenolics in needles was lower in plants treated with the higher MeJa dose, probably because of a local toxic effect. We did not detect any effect of MeJa application on plant growth traits. In addition, the application of MeJa on the experimental branches did not significantly affect the resin content, nor growth rates of the opposite untreated control branches. However, the application of MeJa on the experimental branches significantly increased the resin content in the stem and total phenolics in the needles in the apical stem section of the seedling. These results suggest that the systemic effect of the MeJa induction is restricted to the most valuable parts of the seedlings, and should be considered in further studies aimed to understand the systemic resistance of conifers.

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### 1. Introduction

To defend themselves against insect attacks, conifers have evolved elaborate constitutive and inducible defense mechanisms. Constitutive defenses are permanent structural or chemical compounds that occur regardless of the presence of damage and are determined by genetics, environment and former individual life history (Rosner and Hannrup, 2004; Franceschi et al., 2005). In the family Pinaceae, oleoresin stored in the preformed resin canal system and phenolic compounds seem to play the most important role in the constitutive defense system (Strom et al., 2002; Hudgins and Franceschi, 2004; Franceschi et al., 2005). On the other hand, inducible defenses are activated by plants following a tissue attack or wounding and are determined by the nature of the damage as well as the environment and the genetics of the individual (Karban and Baldwin, 1997; Franceschi et al., 2005). Inducible defenses include the increasing synthesis of antifeedant secondary compounds, such as some monoterpenes, alkaloids and phenolics

(Klepzig et al., 1995), the formation of traumatic resin ducts in the xylem (Alfaro, 1995; Tomlin et al., 1998), swelling and proliferation of polyphenolic parenchyma cells in the bark (Franceschi et al., 2000; Krokene et al., 2003), and the formation of a wound reaction zone (Oven and Torelli, 1994; Solla et al., 2002).

In recent decades, several plant chemical elicitors such as jasmonic acid (Ja) and its methyl ester, methyl jasmonate (MeJa), methyl salicylate, and ethylene have been used to induce defensive responses equivalent to insect or pathogen attacks (e.g. Thaler, 2002; Hudgins and Franceschi, 2004; Broekaert et al., 2006; Erbilgin et al., 2006). All these substances are endogenous plant phytohormones which are known to be involved in triggering induced defense responses after insect attacks (Farmer et al., 2003; Halitschke and Baldwin, 2004). At the beginning of the 1990s, several researchers demonstrated that the exogenous application of MeJa on annual plants induced the synthesis of plant defensive compounds such as alkaloids (Gundlach et al., 1992). Some years later, similar experiments were carried out in gymnosperms, observing that MeJa also induced plant chemical and anatomical defense responses such as the formation of traumatic resin ducts or the accumulation of mono- and diterpenes, and increased phenolic synthesis (e.g. Franceschi et al., 2002; Martin et al., 2002). In

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the family Pinaceae, the application of MeJa has also been shown to induce effective resistance against insect herbivores in *Pinus sylvestris* (Heijari et al., 2005), and against pathogenic fungi in *Picea abies* (Kozłowski et al., 1999; Franceschi et al., 2002).

The exogenous application of MeJa can also influence other plant functions such as vegetative growth, ontogenic development and photosynthetic rate. In conifers, some studies have demonstrated that exogenous application of MeJa drastically reduced seedling growth rate and photosynthetic activity (Heijari et al., 2005; Gould et al., 2008). For instance, Heijari et al. (2005) observed that a 100 mM MeJa treatment significantly decreased seedling height and diameter growth and net photosynthesis when compared to a control or to 10 mM MeJa treated plants of *P. sylvestris*.

Although there is extensive evidence that exogenous MeJa application induces defenses and reduces growth rates in many species of the Pinaceae (Hudgins et al., 2004; Heijari et al., 2005), we lack information about MeJa effects in maritime pine (*Pinus pinaster* Ait.). This species is of vital importance to the forest economy of south-west Europe, where it occupies nearly 4 million ha, and has become a model species for genetic and ecological studies in the Mediterranean region (Gonzalez Martinez et al., 2004). In particular, understanding the genetics and ecology of the expression of resistance traits against pathogens and insects is receiving increasing attention. In this context, the exogenous applications of MeJa provide a very valuable homogeneous, non-wounding technique to characterize induced defensive mechanisms against herbivores.

In addition, little is known about the distal effects of proximal MeJa application in conifer plants. Previous studies demonstrated that MeJa could elicit a response in the untreated tissues above or below the treated area of mature trees (Franceschi et al., 2002; Hudgins et al., 2003; Krekling et al., 2004). For example, Franceschi et al. (2002) observed that MeJa induced the formation of traumatic resin ducts some distance away from its application site. This systemic effect of MeJa in defense responses among different regions of conifer plants would be very important for understanding systemic resistance.

The aim of this paper was to determine if the exogenous application of MeJa at different concentrations induces measurable chemical defensive reactions and growth reductions in *P. pinaster* seedlings, and whether these defensive responses could be effective against the model phloem-feeder insect *Hylobius abietis* L. (Coleoptera:Curculionidae). Finally, we investigated if the local application of MeJa on a basal branch would distally elicit defensive responses or growth reductions on the opposite branch and on the upper main stem.

## 2. Material and methods

### 2.1. Plant material

The study material consisted of 16 *P. pinaster* open-pollinated families of plus trees randomly selected in a first generation seed orchard (Sergude, 42.82° N, 8.45° W) which provides high genetic quality seed for reforestation in the Atlantic Coast of Galicia, NW Spain. All these plus trees are superior trees phenotypically selected within the Atlantic-Coast provenance for superior growth, stem form, and branch characteristics.

*P. pinaster* seedlings were individually sown in 2 L. containers in February 2006. After sowing, the seedlings were fertilized with 6 g of a slow release fertilizer (Multicote® N:P:K 15:15:15), and grown in a greenhouse with controlled temperature (around 25 °C during daytime and 18 °C at night) and daily irrigation. Experimental MeJa treatments were applied to one year-old seedlings, when they were about 60 cm tall.

### 2.2. Experiment I. Effect of MeJa concentration on the whole seedling

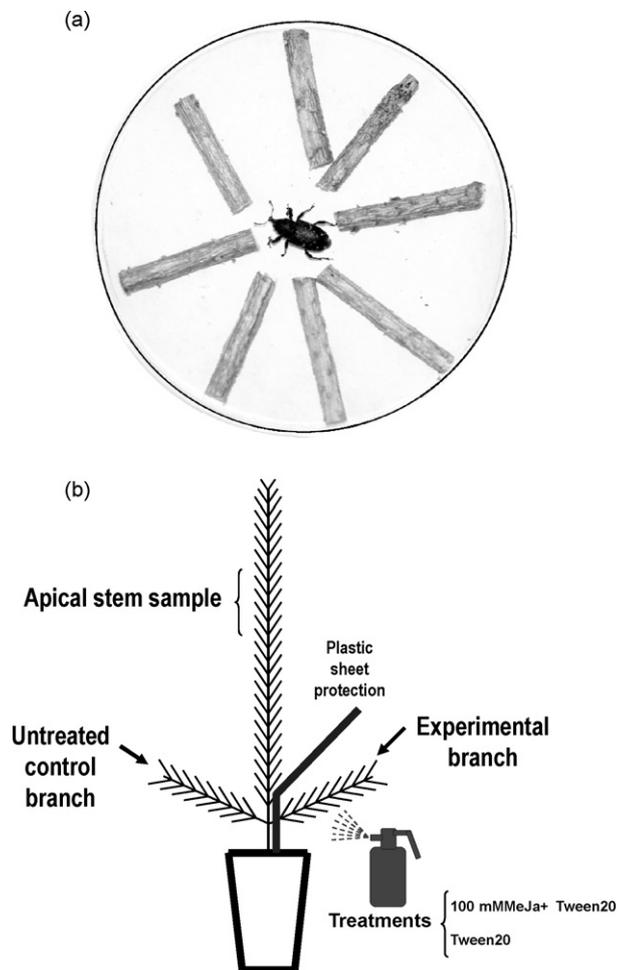
On March 14, 2007, suspensions of 5, 50, and 100 mM MeJa (Sigma–Aldrich, #39,270-7) in deionised water with 0.1% (v/v) Tween-20® were applied to seedlings of eight families with a hand sprayer to run off. The control seedlings were treated with a solution of deionised water and 0.1% Tween-20. The application of MeJa treatments was performed in a different room to the control treatment. The seedlings treated with MeJa remained in that separate space for 24 h to allow the volatilization of the excess MeJa before being placed in the greenhouse with the controls. A randomized complete block design was used in the experiment, with the families acting as the block units. There were no replicates of each pine family in each MeJa concentration treatment, but the same genetic plant material was presented in each treatment ( $n=8$ ). The total number of seedlings was 32 plants (four MeJa concentration treatments  $\times$  8 families).

Seedling height and stem base diameter were measured at the beginning, before the application of the experimental treatments, and at the end of the experiment. Sixty days after MeJa application the seedlings were measured and harvested. To determine the effects of MeJa concentration on defensive responses, samples of the needles and the stem were separated and immediately preserved at  $-30^{\circ}\text{C}$ . We determined the concentration of total phenolic compounds in the needles and the gravimetric resin acid content in the lowest part of the stem. Shoot and root biomass (oven dried at  $65^{\circ}\text{C}$ ) were also determined.

A non-choice feeding test was used to evaluate the effect of the application of MeJa on the effective resistance against the large pine weevil *H. abietis*, a phloem feeder that causes important damages in conifer plantations after clear cutting in Europe (Langstrom and Day, 2004). Immediately after harvesting, the needles were carefully removed from the stem with a surgery knife. The fresh basal stem of each seedling was transversally cut up into several 2.5 cm long pieces, and the diameter of each piece (mean of two opposite measures) registered. One piece of each family was deposited on moistened filter paper in 11 cm diameter plastic Petri dishes, and offered to one weevil for 48 h in darkness (eight pieces of the same MeJa treatment per plate) (Fig. 1a). After this time, we measured the area of gnawed feeding scars of each stem piece in  $\text{mm}^2$ . The bark area consumed was estimated by measuring the length of the scars in four longitudinal transects of each twig. Previous results indicated that the resulting wounding area estimate predicts reasonably well ( $r^2=0.76$ ,  $p<0.001$ ,  $n=108$ ) the real wounding area determined with the aid of a transparent millimetre grid. The total number of Petri dishes were five replicates  $\times$  4 MeJa concentration treatments = 20 dishes. The experiment was conducted with adult pine weevils collected during May 2007 at Cuspedriños (San Xurxo de Sacos Forest, Galicia, Spain, 42.30° N; 8.30° W; altitude 550 m a.s.l.) (Moreira et al., 2008).

### 2.3. Experiment II. Systemic effect of MeJa after application on a basal lateral branch

On March 15 2007, one basal branch (“experimental branch”) of *P. pinaster* seedlings belonging to the remaining eight families was treated with 100 mM MeJa (MeJa + TW20) or with the manipulative control, Tween-20 alone (TW20) (Fig. 1b). Treatment applications to the branch were applied by spraying carefully the branch, while protecting the rest of the plant from contamination by means of a 1  $\text{m}^2$  plastic sheet. A randomized complete block design was used in the experiment with the families acting as the blocks, and the same genetic plant material represented in each treatment. The total number of seedlings was 2 treatments  $\times$  8 families = 16 plants. In order to determine whether the application of 100 mM MeJa



**Fig. 1.** (a) Petri dish example of the *in vitro* bioassay with *H. abietis*, (b) scheme of the experimental procedure in the Experiment II aimed to analyse the systemic effect of MeJa after application on a basal lateral branch.

could involve systemic or distal effects on vegetative growth or defenses, besides the “experimental branch” we included in the design another untreated opposite basal branch as “control branch”, and the “upper apical stem” of the seedling as repeated measures (Fig. 1b).

Plants were harvested, processed, and plant relative growth (height and diameter) and resin content were determined in the experimental branch, in the control branch, and in the upper stem as in Experiment I. We also determined the concentration of total phenolic compounds in the needles of the upper apical stem section.

#### 2.4. Chemical analyses

Concentration of resin compounds in the stem was determined gravimetrically after extraction with hexane. Just after harvesting, a fresh 10 cm long piece of the stem of each plant was sampled, immediately frozen and preserved at  $-30^{\circ}\text{C}$  until analysed. Frozen specimens, approximately weighing 5 g fresh weight, were cut up into small transversal pieces (of about 5 mm), transferred into preweighed test tubes, and extracted with 3 mL of hexane in an ultrasonic bath (15 min at  $20^{\circ}\text{C}$ ), and then for 24 h at room temperature under the fume hood. The extract was filtered through a GFF filter into a second preweighed test tube, and the vegetable material recovered back into the first test tube. The solvent in the second test tube was evaporated to dryness under the fume hood at room temperature, which took about five days, and the resinous residue

was determined gravimetrically with a precision scale (0.0001 g). The whole extraction step was immediately repeated, so the total resin content was the sum of the resinous residues extracted in the two consecutive extractions. The second extraction usually yielded less of the 10% of the total resin mass. After the second extraction the vegetable tissue was oven dried at  $80^{\circ}\text{C}$  and weighed. The resin concentration was expressed in mg of resinous residue  $\text{g}^{-1}$  stem dry weight (d.w.).

The concentration of total phenolic compounds in the needles was determined colorimetrically using the Folin-Ciocalteu reagent and tannic acid as standard (Scalbert, 1992; Covelo and Gallardo, 2001). A sample of fresh needles was immediately oven dried at  $45^{\circ}\text{C}$  and then manually grounded in a mortar with liquid nitrogen. Total phenolics were determined according Glyphis and Puttick (1988) in 0.3 g of needles, by extraction with 50% aqueous methanol with an ultrasonic bath for 15 min, following centrifugation and subsequent dilution of the methanolic extract. Total phenolics were determined colorimetrically in a Biorad 650 microplate reader at 740 nm as tannic acid equivalents, in a d.w. basis.

#### 2.5. Statistical analyses

The effects of MeJa concentration on plant growth and defense traits in Experiment I were analysed by means of the mixed model  $Y_{ij} = \mu + MJ_i + G_j + COV_{ij} + \varepsilon_{ij}$ , where  $\mu$  is the general mean,  $MJ_i$ , and  $G_j$  are the main effects of MeJa concentration treatment  $i$  ( $i=1-4$ ) and family  $j$  ( $j=1-8$ ),  $COV_{ij}$  is the effect of a covariate, and  $\varepsilon_{ij}$  is the experimental error. The family factor (G) was considered a random effect. Height and diameter were analysed using the initial measurement as covariate. Final basal diameter was used as covariate in the analysis of resin and phenolic concentrations.

The bioassay with *H. abietis* in Experiment I was analysed by means of a mixed model. We used the individual data in the analysis to allow the use of the diameter of the stem twig as a covariate, which is known to account for much variability (Thorsen et al., 2001). The Petri dishes, nested within the MeJa treatments, were also included in the model as a random factor in order to account for the dependence of observations within each Petri dish (Littell et al., 2006).

In Experiment II, the effects of the elicitor treatment (application of TW20 or MeJa + TW20) on the relative growth and resin concentration in the control and the experimental branches, and the resin of the stem were analysed using a repeated measures mixed model with plant part as a within-subject factor and treatment as a between-subject factor. Family and family by part interaction were also included in the model as random effects. Using this whole model, we performed subsequent specific contrast analyses testing for differences between the two treatments in each plant part, or between the experimental and control branches within each treatment. Degrees of freedom were corrected by the Kenward-Roger method, as recommended by Littell et al. (2006).

All analyses were performed using the MIXED procedure of SAS (Littell et al., 2006). When main effects were significant, differences among treatment means were tested for significance using the LSMEAN statement. Data are shown as least square means  $\pm$  S.E.

### 3. Results

#### 3.1. Experiment I. effect of MeJa concentration

Exogenous application of MeJa induced resin accumulation in the stems of the *P. pinaster* seedlings (Table 1). The resin concentration in the 100 mM MeJa treated seedlings nearly doubled that in the control seedlings (Fig. 2a). Seedlings treated with 5 and 50 mM MeJa presented intermediate values, but no significant differences

**Table 1**

Resume of the mixed model analyses showing the effects of the MeJa treatments on *Pinus pinaster* growth, pine chemical defenses, and *Hylobius abietis* gnawed bark area in *in vitro* bioassays.

Trait/source of variation	DF	DFerror	F-ratio	<i>p</i> > F
<b>Total height</b>				
MeJa treatments	3	20	0.71	0.557
Covariate (initial height)	1	20	120.48	<0.001
<b>Diameter</b>				
MeJa treatments	3	20	0.20	0.892
Covariate (initial diameter)	1	20	73.46	<0.001
<b>Total dry weight</b>				
MeJa treatments	3	20	0.75	0.537
Covariate (initial diameter)	1	20	10.67	0.004
<b>Root dry weight</b>				
MeJa treatments	3	20	2.46	0.093
Covariate (initial diameter)	1	20	9.22	0.007
<b>Resin content</b>				
MeJa treatments	3	20	10.72	<0.001
Covariate (final diameter)	1	20	15.28	<0.001
<b>Total phenolics</b>				
MeJa treatments	3	21	3.13	0.047
Covariate (final diameter) <sup>a</sup>				
<b>Gnawed area</b>				
MeJa treatments	3	16	3.26	0.049
Covariate (twig diameter)	1	139	97.66	<0.001

<sup>a</sup> The covariate was not significant and was removed from the analysis.

were observed with the control (Fig. 2a). Total phenolics concentration in the needles was also affected by the MeJa treatments (Table 1). Total phenolic concentration was slightly greater in the 5 mM MeJa treated seedlings than in the other MeJa treatments, with the lowest values observed in the plants treated with the highest MeJa concentration (Fig. 2b).

Plant growth traits (height and diameter) were not affected by the MeJa treatments (Table 1). Neither total nor root biomass were affected by MeJa treatments (Table 1).

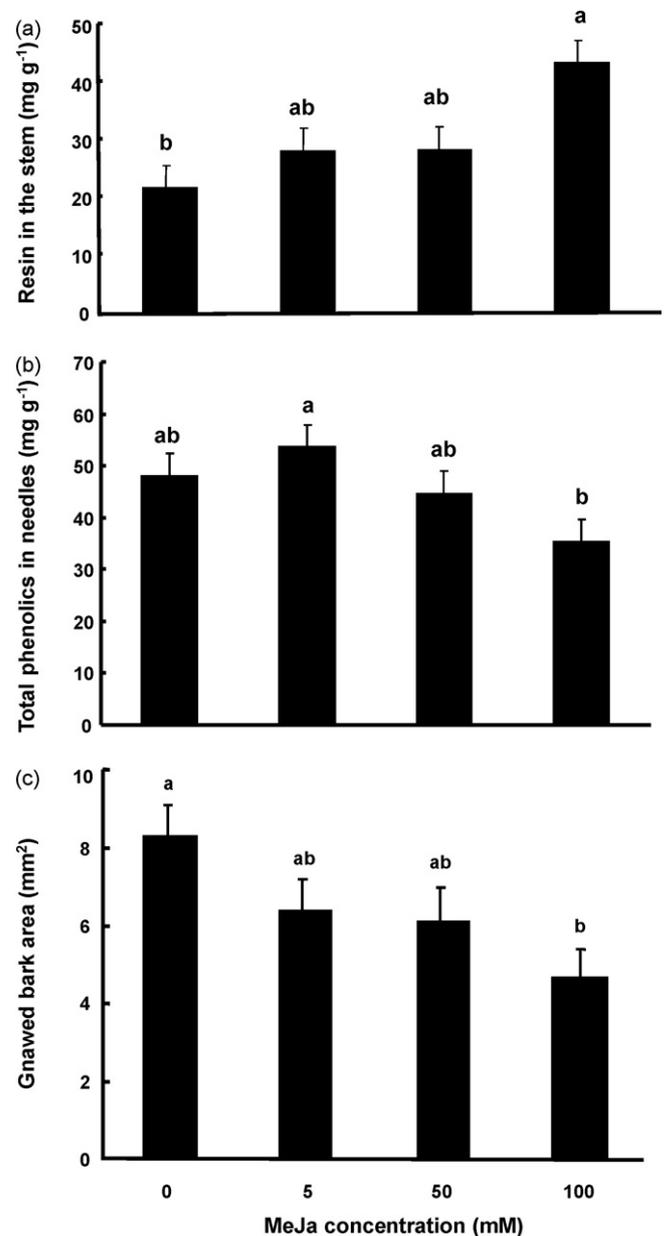
Application of increasing concentrations of MeJa significantly reduced the amount of phloem gnawed by *H. abietis* in *in vitro* bioassays (Table 1). The pine weevils consumed 80% less phloem in the 100 mM MeJa treated seedlings than in the control seedlings (Fig. 2c). As with resin content, 5 and 50 mM MeJa treatments produced intermediate values.

### 3.2. Experiment II. systemic effect of MeJa

Sixty days after the application of MeJa + TW20 on the experimental branches, we did not detect significant changes in their resin content compared with the application of TW20 ( $F_{1,7.8} = 1.47$ ,  $p = 0.249$ ; Fig. 3b). However, MeJa significantly inhibited the secondary growth of the treated branches ( $F_{1,6.53} = 31.59$ ,  $p = 0.001$ ; Fig. 3a), even though we only detected a marginal effect on primary growth ( $F_{1,6.79} = 5.28$ ,  $p = 0.056$ ; data not shown).

The application of MeJa + TW20 on the experimental branches did not affect the resin content ( $F_{1,11.7} = 0.53$ ,  $p = 0.53$ ; Fig. 3d), nor the secondary ( $F_{1,12.1} = 1.30$ ,  $p = 0.276$ ; Fig. 3c) or primary growth ( $F_{1,13.5} = 0.31$ ,  $p = 0.587$ ; data not shown) of the opposite untreated control branches.

Even though the application of MeJa + TW20 did not increase resin content in experimental branches compared with the application of TW20 alone, we found that application of MeJa + TW20 to the experimental branches actually increased resin content in those branches compared with the control branches (specific contrast in the repeated measures model,  $F_{1,10.3} = 9.00$ ,  $p = 0.013$ ). However, this effect was not enough to differentiate TW20 and MeJa + TW20 experimental branches, because resin concentration in the TW20



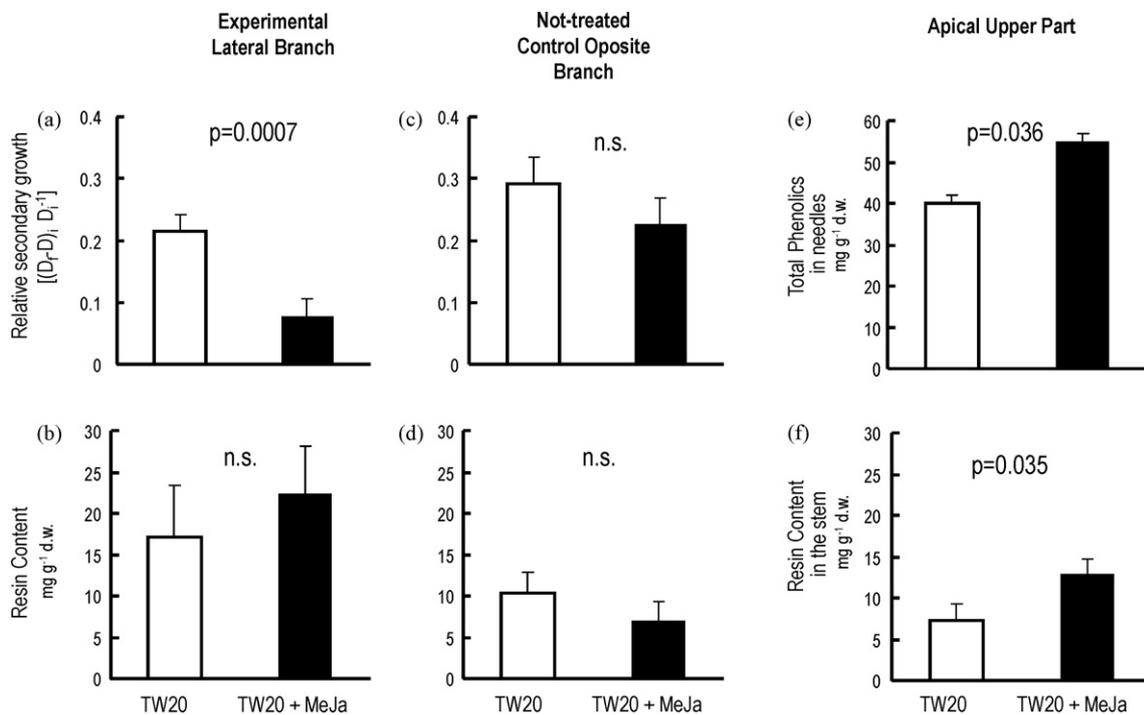
**Fig. 2.** (a) Concentration of total resin in the stem, (b) Concentration of total phenolics in the needles, and (c) gnawed bark area by the large pine weevil *H. abietis*, in one year-old *P. pinaster* seedlings two months after treatment with different MeJa concentrations. LS means  $\pm$  s.e.m. ( $N = 8$ ). Different letters indicate significant differences between treatments ( $p < 0.05$ ).

experimental branch was also higher than in the control branch, although not significantly so ( $F_{1,9.86} = 0.92$ ,  $p = 0.358$ ).

Nevertheless, the application of MeJa on the experimental branches significantly stimulated defensive responses in the apical upper stem section of the seedling. Both stem resin content ( $F_{1,8.26} = 7.89$ ,  $p = 0.022$ ; Fig. 3f) and concentration of total phenolics in the needles ( $F_{1,7} = 0.036$ ; Fig. 3e) were significantly greater in seedlings receiving MeJa + TW20 than in seedlings receiving TW20 alone.

## 4. Discussion

This study shows that the application of MeJa to *P. pinaster* seedlings induces chemical defensive responses and effective resistance against the generalist insect herbivore *H. abietis*. These results



**Fig. 3.** Growth and defensive response in three parts of *P. pinaster* seedlings two months after application of Tween20 (TW20) or a suspension of methyl jasmonate + Tween20 (MeJa + TW20) to a basal experimental branch. Figures show the values observed in the experimental branch (a and b), and in an opposite not-treated control branch of the same tree (c and d). Panel e and f shows defensive response in needles and in the stem in the upper apical part of the seedlings, distanced more than 30 cm from the treated branch.  $D_1$  = initial diameter;  $D_f$  = final diameter. LS means  $\pm$  SE; n.s.: not significant.

are consistent with the role of MeJa in defensive responses that have been extensively studied in other conifers. MeJa have been reported to induce anatomical and chemical changes such as the formation of traumatic resin ducts in the xylem and the increasing synthesis of phenolic and alkaloid compounds in many conifer species (Martin et al., 2002; Hudgins et al., 2003; Hudgins et al., 2004; Zeneli et al., 2006). Hudgins et al. (2004) observed that 100 mM MeJa application increased significantly the resin accumulation on the stem of several conifer species. Zeneli et al. (2006) reported that 100 mM MeJa application in *P. abies* plants provoked an increased accumulation of terpenes and resin flow in the sapwood with respect to 0, 5, 25 and 50 mM MeJa treatments. MeJa has also been reported to induce resistance against herbivore insects and pathogens (Franceschi et al., 2002; Heijari et al., 2005; Zeneli et al., 2006). Similar to our results, Heijari et al. (2005) observed that *H. abietis* consumed 62% less bark in 100 mM MeJa treated *P. sylvestris* seedlings than in control seedlings. Franceschi et al. (2002) found that lesion lengths caused by *C. polonica* in *P. abies* were less than half in the MeJa treated trees than in the untreated control trees.

The response to the MeJa treatments in the phenolic content in the needles was not as clear as that observed for the resin content in the stem. Although total phenolic concentration in the 5 mM MeJa treated plants was slightly greater than in control plants, the phenolic concentration significantly dropped in seedlings treated with the highest MeJa concentration. In most cases of our study, seedlings treated with 100 mM MeJa showed some signs of stress or toxicity in the form of chlorotic or death needles that might alter total phenolics concentration in the needles. The phytotoxicity of MeJa in high concentration has been previously reported by Heijari et al. (2005). These authors found that 56% *P. sylvestris* seedlings treated with a solution of 100 mM MeJa died over a period of 3.5 months, whereas mortality was only 1.6% in the control and 10 mM treated seedlings.

In Experiment I, applying directly MeJa treatments on the whole plant, needle total phenolic concentration was significantly lower in the 100 mM MeJa treated plants. However, in Experiment II when we applied 100 mM MeJa on a lateral branch, needle total phenolics concentration in the upper part of the stem was 25% greater in MeJa treated seedlings than in the control ones. This observation agrees with the idea that MeJa in high concentrations could locally depress total phenolics concentration in the needles due to local toxicity.

The exogenous application of MeJa to conifer seedlings was responsible for a decline in growth in many conifer species (Heijari et al., 2005; Gould et al., 2008). Heijari et al. (2005) observed that height and diameter growth, as well as shoot and root biomass were significantly reduced at the 100 mM MeJa concentration. In the same way, Gould et al. (2008) observed that growth rate was reduced in the week following MeJa treatment. In the present study, however, no growth trait of the whole plant was significantly affected by MeJa concentration, although we did observe a significant decrease of secondary growth in the experimental branches treated with MeJa in Experiment II. The most plausible explanation for the lack of a growth response to the MeJa application in Experiment I was the low absolute diameter and height increments between the application of the treatments and the final assessments, probably because most of the yearly growth was completed before treatment applications.

## 5. Systemic effect of MeJa

The application of MeJa on a basal branch induced defensive responses in the stem, but not in the untreated opposite branch. The systemic effect of MeJa between different regions of conifer plants was previously reported by other authors in older plants (Franceschi et al., 2002; Hudgins et al., 2003). Franceschi et al. (2002) observed that anatomical defensive responses in adult Norway spruce such as the formation of traumatic resin ducts are also found some distance

from the application zone, indicating that MeJa is transported axially away from the application site. The greater the distance from the application area, the smaller the traumatic resin ducts were, indicating the existence of a dose-response phenomenon. In the same way, Hudgins et al. (2003) observed in several species of the Pinaceae family the formation of traumatic resin ducts 5 cm above the MeJa treatment site, although in lower numbers. We found that concentration of resin and total phenolic were significantly greater in the upper stem of seedlings with a basal branch treated with MeJa than in the control seedlings. Conversely, there was not a systemic effect due to MeJa application between the experimental and control branches. According to the Optimal Defense Theory (Hamilton et al., 2001; Pavia et al., 2002) seedlings receiving information of herbivore or biotic risk could respond by preferentially allocating resources to better guard the most valuable tissues and plant parts. This theory would be consistent with our results, in that pine seedlings could allocate their resources, including the induced phenolics and terpenoid compounds, to the most valuable tissues, i.e. the upper part of the stem, which supports primary growth and most of the photosynthetic tissues. On the other hand, allocation of chemical defenses or resources to basal old branches would be not a priority. This could not explain only the lack of a systemic effect of MeJa on the opposite basal branch, but also the absence of increased resin reserves and depressed growth observed in the experimental branches. Nevertheless, the lack of a local response to MeJa in the experimental branches could also be explained by the relative increase in the resin content in the branches treated with TW20, due to the simple manipulation of the branches and/or to a response to TW20. Although not significantly different, the resin content of the experimental branches treated with TW20 was higher than that in the respective control branches (white bars in Fig. 3b and d), preventing the detection of significant differences between experimental branches treated with MeJa and with TW20 (Fig. 3b). The induction of defenses with TW20 in conifer seedlings has been found before. For example, Miller et al. (2005) demonstrated that the application of 0.1% TW20 on *Picea sitchensis* Bong seedlings increased di- and sesquiterpene concentration with respect to the control. This result is of practical relevance because TW20 is commonly used as MeJa control, and should be considered in further experiments.

The apparent effect of TW20 could also explain the relatively higher resin concentration values in Experiment I ( $20\text{--}45\text{ mg g}^{-1}$ ) than in Experiment II ( $7\text{--}22\text{ mg g}^{-1}$ ). If the application of TW20 induced defensive responses, the resin values of the control plants of Experiment I, where treatments were applied to the whole plant, should be larger than those in the stem of Experiment II, where treatments were locally applied to a lateral branch. In fact, the differences were much lower when the resin values of the control plants of Experiment I ( $22.8 \pm 3.3$ ) are compared to those in the experimental branches treated with TW20 in Experiment II ( $17.13 \pm 6.2$ ).

This is the first work reporting the effect of MeJa on growth, defensive responses and herbivory resistance in *P. pinaster*. Despite the reduced sample size used and the difficulties associated with the use of seedlings, we were able to detect a significant effect of the MeJa application on the resin content in the stems, consequently reducing the bark gnawed by *H. abietis*. In addition, we also demonstrated that the application of MeJa on a basal branch induced defensive responses in the upper stem. This result may have relevance for forthcoming studies aimed at understanding systemic resistance in conifers.

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