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Exploiting jasmonate-induced responses for field protection of conifer seedlings against a major forest pest, *Hylobius abietis*

Running title: Jasmonate-induced defense against a forest pest

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Supplemental material

Appendix A. Details of methyl jasmonate treatments and field trials, including photographs of the experimental sites and the treated seedlings. (Table A1, Figure A1-A2).

Appendix B. Supplementary results: Specific contrasts testing the effect of single and double application of 25 mM methyl jasmonate. (Table B1).

Appendix C. Supplementary results: Effect of methyl jasmonate treatments on chemical defenses in the needles, on seedling growth at different times and on weevil damage during the second growing season. (Figure C1-C4).

Abstract

Herbivore damage commonly initiates an increased synthesis of chemical defensive compounds in attacked plants. Such induced defences are a vital part of plant defence systems, but when herbivore pressure is high, as frequently occurs in man-made ecosystems such as agricultural and forest plantations, plants may suffer considerable damage before adequate induced defences build up. To prepare the plants for such conditions their induced defence may be artificially triggered by simulated herbivory, e.g. by application of a chemical elicitor. This method is already widely employed in agriculture but within forestry systems it has so far been restricted to promising laboratory results. The pine weevil, *Hylobius abietis*, causes damage by feeding on the bark of young conifer plants and it is one of the main threats to successful regeneration in the Palaearctic region. Here we present results from a large scale field experiment where we triggered the induced defences of conifer seedlings using exogenous application of the chemical elicitor methyl jasmonate. To enhance the generality of the results different species were planted under extremely different environmental conditions; Maritime pine and Monterrey pine in Spain, and Scots pine and Norway spruce in Sweden. Weevil damage, chemical defences, and seedling growth were studied during the two growing periods following planting. In general, treated plants showed increased quantitative defences, and were less attacked, less wounded, less girdled and showed lower mortality rates than their untreated counterparts. Effects were mostly dose dependent, although some interactive effects with tree species were observed. The treatment initially caused a growth reduction but it was later compensated by the benefit, in terms of growth, of being less damaged. The measures that are currently taken to protect forest plantations against this harmful pest all around Europe have enormous economic costs and cause important environmental hazards. Elicitation of inducible defences in seedlings in the nursery appears to be a cost-effective and environmentally-friendly alternative to these measures. To our knowledge, this is the first field study that explores the applicability of chemical elicitors of induced defences as a way to protect forest plantations against biotic threats.

Keywords conifer seedlings; forest regeneration; growth costs; *Hylobius abietis*; induced defence; methyl jasmonate (MJ); *Picea abies*; pine weevil; *Pinus pinaster*; *Pinus radiata*; *Pinus sylvestris*; priming; reforestation; seedling protection.

Highlights

> Methyl jasmonate emerges as an attractive alternative to protect conifers against *H. abietis*

> MeJa treated seedlings were less attacked, less wounded, and showed higher survival

> Protection was long-lasting and remained effective during two growing seasons

> Results were consistent across species and environmental conditions

> Initial growth reductions were largely compensated by growth benefits due to reduced damage

1. Introduction

In common with most plants, conifers defend against herbivores with a combination of physical and chemical mechanisms. Some defences are permanently expressed, irrespective of whether the plants are actually suffering damage (constitutive defenses), while others are enhanced after the recognition of damage (induced defenses) (Franceschi *et al.*, 2005; Eyles *et al.*, 2010). Induced defenses are assumed to have evolved as a cost saving strategy in which the costs of producing resistance mechanisms are only incurred when defenses are actually needed, i.e. after the damage or the risk of damage has been recognized (Sampedro *et al.*, 2011a). Constitutive defenses inhibit initial attacks but are frequently insufficient to deter the attack or to avoid the proliferation of the damage. In such cases, induced resistance, including increased synthesis of chemical defensive compounds already existing in healthy plants, synthesis of new chemical defenses, and the formation of new physical structures can be vital for the plant to survive the attack (e.g. Zas *et al.*, 2011; Zhao *et al.*, 2011b; Schiebe *et al.*, 2012).

In recent decades considerable progress has been made towards an increased understanding of the physiological mechanisms and metabolic pathways involved in the recognition, signaling and triggering of plant induced defenses against biotic stressors (Heil, 2009; Erb *et al.*, 2012). Different plant phytohormones such as jasmonates, ethylene and salicylic acid are now known to be involved in the activation of induced defensive responses in a wide array of different plant species (e.g. Creelman and Mullet, 1995; Halitschke and Baldwin, 2005). In particular, jasmonate signaling is thought to be involved in triggering defenses against herbivores and necrotrophic pathogens in several plant taxa (Glazebrook, 2005).

Due to the conserved relevance of phytohormones in plant defense, the use of mutants or transgenic plants with over or under expression of these compounds has become a very common and highly efficient research tool for investigating induced resistance in plants, as has the exogenous application of phytohormones as elicitors of plant immune responses (Gase and Baldwin, 2012). In particular, methyl jasmonate (MJ), i.e. the methyl ester of jasmonic acid, has been widely used as a chemical elicitor to simulate herbivory (Koo and Howe, 2009), with the exogenous application of MJ provoking responses similar to those occasioned by insect feeding (Franceschi *et al.*, 2002; Rohwer and Erwin, 2008). In conifers, the exogenous application of MJ sprayed to aboveground tissues is known to have a large impact on the synthesis of both terpenoids and phenolics (Zulak *et al.*, 2009), two of the main chemical defenses of conifers against insect herbivores (Franceschi *et al.*, 2005). Increased total amounts and/or alterations of the profile of these compounds have been reported following MJ application both in young seedlings (e.g. Martín *et al.*, 2002; Heijari *et al.*, 2005; Moreira *et al.*, 2009; Erbilgin and Colgan, 2012) and adult trees (e.g. Erbilgin *et al.*, 2006; Heijari *et al.*, 2008; Erbilgin and Colgan, 2012), and for many different conifer species (Hudgins *et al.*, 2004) from boreal conifers such as *Pinus sylvestris* (Heijari *et al.*, 2005; Heijari *et al.*, 2008) and *Picea abies* (Erbilgin *et al.*, 2006; Zhao *et al.*, 2011b; Schiebe *et al.*, 2012) to Mediterranean pines such as *Pinus pinaster* (Moreira *et al.*, 2009; Sampedro *et al.*, 2011a) and *Pinus radiata* (Gould *et al.*, 2008; Gould *et al.*, 2009; Moreira *et al.*, 2012b). Anatomical long-lasting responses such as the proliferation of traumatic resin canals are also well documented (Huber *et al.*, 2005; Krokene *et al.*, 2008).

In keeping with the enhanced defense status, MJ treated conifer seedlings have been repeatedly reported to show increased resistance to a wide array of fungal pathogens and herbivore insects. Spraying *P. radiata* seedlings with a low concentration of MJ (< 5 mM) has been shown, for example, to reduce *Diplodia pinea* infection by 60% (Gould *et al.*, 2009), while spraying or fumigation of *P. abies* with MJ reduced the colonization of *Ceratocystis polonica* (Krokene *et al.*, 2008) and protected seedlings against *Pythium ultimum* (Kozlowski *et al.*, 1999). MJ application has been also shown to be effective against insect herbivores by reducing colonization, oviposition and/or damage levels in several conifer – insect systems (Holopainen *et al.*, 2009; Moreira *et al.*, 2012a). Specifically, significant responses to MJ application reducing insect loading or feeding rates have been reported for different insect feeding guilds, including phloem and bark feeders such as pine weevils (Heijari *et al.*, 2005; Moreira *et al.*, 2009), bark beetles such as *Ips typographus* (Erbilgin *et al.*, 2006), and defoliators such as *Thaumetopoea pityocampa* (Moreira *et al.*, 2013) and diprionid sawflies (Heijari *et al.*, 2008). In some cases, MJ altered the attraction of the insect herbivores to the breeding or feeding sites due to changes in the emission of volatile organic compounds (e.g. Zhao *et al.*, 2011a), while in others, the enhanced physical and chemical defenses within plant tissues seem to be responsible for the reduced damage levels (e.g. Heijari *et al.*, 2005; Moreira *et al.*, 2009). Changes in the emission of volatile organic compounds could also alter the interaction with other trophic levels and be involved in indirect resistance processes (Thaler, 1999). Despite all these examples of positive results of MJ application protecting conifers against biotic stressors, negative results where MJ failed to protect seedlings or mature trees against particular enemies do also exist (Graves *et al.*, 2008; Reglinski *et al.*, 2009; Zhao *et al.*, 2010; Vivas *et al.*, 2012).

The responses of plants to jasmonates are not limited, however, to defense-related processes, but also include alterations of many other physiological traits related to growth and development (Cheong and Yang, 2003). Plants treated with MJ usually show reduced primary and secondary growth rates, either because of reduced photosynthetic activity (as observed by Heijari et al. (2005) after treatment with high doses (100 mM) of MJ) or just as a result of the physiological costs associated with boosting chemical defenses (Sampedro *et al.*, 2011a). This reduction in growth associated with MJ application has been outlined as a critical handicap for the practical applicability of this substance for protecting forest plantations against biotic aggressors (Holopainen *et al.*, 2009). However, not all the growth-related responses to MJ are negative. MJ treated seedlings of *P. pinaster* have been found, for example, to have many more fine roots than control seedlings, and this enhancement of the root system may both help seedling establishment and increase the tolerance to herbivore damage (Moreira *et al.*, 2012c). Additionally, as the effect of MJ on primary growth is usually greater than that on secondary growth (Heijari *et al.*, 2005; Moreira *et al.*, 2013), MJ treatment favors reduced height:diameter relationships, which is something that forest nurseries aim for since it increases seedling growth and survivorship after plantation (Willoughby *et al.*, 2009).

Although our knowledge of the complex responses of conifers to MJ is still limited, there is increasing evidence that MJ application has a clear potential for protecting forest plantations and nursery seedlings against pests and pathogens (Holopainen *et al.*, 2009; Eyles *et al.*, 2010; Moreira *et al.*, 2012a). By artificially triggering the innate resistance capacity, MJ could become an environmental-friendly and cost-effective alternative to the use of the traditional control methods (Rohwer and Erwin, 2008). A particular harmful forest pest that potentially could be controlled by exogenous MJ application is the pine weevil, *Hylobius abietis* (L.), which significantly impacts the regeneration of conifer forests after clear cutting in large areas of Europe and Asia (Långström and Day, 2004). Adult pine weevils feed on the phloem and bark of conifer seedlings of many different species, causing stem girdling and high mortality rates (Örlander and Nilsson, 1999; Day *et al.*, 2004). Volatiles emitted from the stumps of fresh clear-cuts attract massive immigration of adult pine weevils that can cause severe damage on regeneration (Solbreck and Gyldberg, 1979; Örlander *et al.*, 2000). If no protection measures are carried out, weevil damage can cause up to 80% mortality (Petersson and Örlander, 2003). To date no definitive treatment is available, and a combination of different prophylactic measures, including soil scarification, retention of shelter trees, physical protection of the seedlings, delayed planting, and even insecticide treatments, is currently routinely applied (Petersson and Örlander, 2003; Nordlander *et al.*, 2009; Nordlander *et al.*, 2011). Most of these methods are expensive to apply and/or are environmentally hazardous; moreover they are frequently insufficient to reduce the level of damage and mortality to (economically) acceptable levels.

MJ application has been shown to reduce the damage caused by the pine weevil on pine seedlings of different species both *in vitro* (Moreira *et al.*, 2009; Moreira *et al.*, 2013) and *in vivo* bioassays (Heijari *et al.*, 2005; Sampedro *et al.*, 2011b) under controlled conditions in the lab. Whether MJ can also be used to protect seedlings against the pine weevil under real field conditions is, however, yet to be tested. It is well known that a treatment that is highly efficient under controlled conditions in the lab is not always efficient under field conditions, where many interfering factors can potentially modulate its effects (Beckers and Conrath, 2007). Importantly, pine weevils are frequently a serious threat to seedlings not only immediately after planting but also during the second and following years. It is therefore important that the effect of any protecting treatment is long lasting. There are no previous studies where the effects of MJ application have been evaluated after two seasons, although for mature trees it has been shown that the effect of a MJ treatment can last for a long time (Erbilgin *et al.*, 2006; Zhao *et al.*, 2010).

Here, we explore whether increasing resistance traits through MJ application at the nursery stage can be an efficient way to protect seedlings against this harmful forest pest in the field. We performed an exhaustive field experiment with the four most important conifers planted in both northern (Sweden) and southern Europe (Spain). We investigated the effect of concentration and number of applications of MJ on chemical resistance traits, seedling growth and weevil damage during two growing seasons after planting. We aimed to gain insight into the viability of MJ application in the nursery as an environmentally-friendly and cost-effective alternative to the measures currently used to protect forest plantations against the pine weevil. The wide contrasts in ecological conditions between Spain and Sweden, with extreme differences not only in temperature and light conditions but also in forest functioning and insect behavior, should result in a high level of generality of the results of this study.

2. Material and Methods

2.1. Plant material

Four conifer species were used in this study: Maritime pine (*Pinus pinaster* Ait.) and Monterrey pine (*Pinus radiata* D. Don) as representatives of conifers widely planted in southern Europe, and Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.)

Karst.) as the most common conifers in the forests of northern Europe. All four species can be severely damaged by the pine weevil when planted in conifer clear-cuts (Örlander and Nilsson, 1999; Zas *et al.*, 2011).

Seedlings of Maritime pine and Monterrey pine were provided by a commercial Spanish nursery (Norfor Nursery Ltd., Pontevedra, Spain; viverofigueirido@norfor.es). Monterrey pine seedlings were derived from seeds collected in the coast of Asturias (NW Spain) whereas those of Maritime pine came from the Massif des Landes (France). Both provenances are commonly used for reforestation in the area of the Spanish field experiment. Seeds of both species were sown in CETAP40® containers (*P. radiata*, container volume 125 cm^3) and PLASNOR® containers (*P. pinaster*, container volume 150 cm^3) in August 2010, which were kept outdoors and watered and fertilized following conventional nursery protocols.

The two northern species were represented by one-year-old containerized seedlings (container volume 50 cm^3) and were acquired from a Swedish commercial nursery (Sjögränd nursery, Bergvik Skog AB, Uddeholm, Sweden). Seedlings of both species were derived from seeds of central Swedish origin, and thus suitable for the area of the Swedish field experiment. Seeds were sown in March 2010, and seedlings were freeze stored from December 2010 to May 2011, when they were taken outdoors, transplanted into HIKO® trays (container volume 90 cm^3), and then kept on sandy ground and automatically watered daily.

2.2. Methyl jasmonate treatments

Trays of the four species were sprayed with different treatments of methyl jasmonate (MJ) in the spring of 2011. Treatments differed in the concentration of MJ and in the timing of the MJ applications. Methyl jasmonate (Sigma-Aldrich Ref #39924-52-2) was used for preparing 5, 10, and 25 mM MJ emulsions in 2.5% ethanol in deionized water. MJ was first dissolved in the ethanol and water was then added. The solution was shaken vigorously until a uniform milky emulsion was obtained, and then transferred to hand-sprayers, which were also shaken in between spraying each tray.

Treatments were applied twice, roughly 4 and 2 weeks before planting out in the field experiments (30 and 15 days before planting in the case of *P. pinaster* and *P. radiata* and 27 and 13 days before planting in the case of *P. sylvestris* and *P. abies*). At each application date, approximately 10 ml of the MJ emulsions, differing in MJ concentration, was uniformly distributed with a hand-sprayer over the nursery trays, which included 40 seedlings each. Six treatments, differing in the concentration and timing of the MJ applications, were applied to the four species (see Table A1 in Appendix A). The main treatments (T1, T2, T3 and T4) consisted of a control (seedlings sprayed only with the carrier solution) and applications of 5, 10 and 25 mM MJ at both application dates. Single applications of the highest concentration treatment (25 mM MJ) in just one of the two application dates were also conducted (treatments T5 - 4 weeks before planting, and T6 - 2 weeks before planting).

2.3. Field experimental design

Two field experiments were established with the treated seedlings, one in Spain, including *P. pinaster* and *P. radiata*, and the other in Sweden, including *P. sylvestris* and *P. abies*. Both experiments were established in recent conifer clear-cuts, in which pine weevil damage was likely to occur. The two experiments followed a randomized block design with 8 blocks, with each block including 10 plants of each of the six treatments (T1-T6), for both the species of each trial. The 10 plants were planted together in a single row of 10 plants (Swedish trial) or in two contiguous rows of 5 plants each (Spanish trial). Spacing was 1 × 1 m in both experiments.

The Spanish field trial was established on 12-13 May 2011 in Torroña (Pontevedra, NW Spain, 41º 58’ 17’’ N, 8º 51’ 3’’ W, Altitude = 410 m a.s.l.) in a granitic area of sandy soils dominated by pine forest of both Maritime pine and Monterrey pine (see overall view in Appendix A, Figure A1). The experimental site was previously occupied by a mature stand of Maritime pine, which had been clear cut in October-December 2010. One-direction soil ripping was made following the slope of the site just before planting.

The Swedish trial was established on 21 July 2011 at Marma, about 70 km N of Uppsala (Sweden, 60º 29’ 5’’N, 17º 26’ 50’’ E, Altitude = 36 m a.s.l.) (see overall view in Appendix A, Figure A2). The site was located on almost completely flat sand sediment. The previous stand of predominantly Scots pine had been clear cut in December 2009, followed by soil scarification by disc-trenching in July 2010.

In order to have seedlings unaffected by pine weevil feeding, two additional treatments in which seedlings were physically protected against the pine weevil were also included in the experimental design of the two field trials. Extra plants treated twice with the control (treatment T7) or the 25 mM solutions (treatment T8) were established and protected with a plastic shield (Snäppskyddet, Panth-Produkter AB, Östhammar, Sweden) at the time of planting. These two extra treatments were only included in blocks 1-4. In the Spanish trial the efficacy of these barriers was not complete and some seedling damage was observed early on; plants were then further protected by coating the stems with Conniflex®, which is a fine sand (particle size 0.2 mm) embedded in an acrylate dispersion that remains flexible after drying (Nordlander *et al.*, 2009). Conniflex® was applied in March 2012, only in the Spanish trial.

2.4. Assessments

Seedling size (total height and stem basal diameter) was assessed in all planted seedlings in the two experiments just before planting, and seedling size and weevil damage (debarked area) were assessed at the end of the first and second growing seasons after planting (17 October 2011 and 12 December 2012 in the Spanish trial and 27 September 2011 and 11 October 2012 in the Swedish trial). On both dates we also recorded whether or not each seedling had been attacked by the weevil, as a further binary variable. Stem girdling and seedling mortality were also recorded as binary variables in all planted seedlings. A seedling was classified as girdled when there was a continuous feeding scar all around the stem, irrespective of the height of the stem where this scar was found. Dead seedlings without feeding scars were considered to be dead due to other causes.

Because seedling size varied greatly between the two field trials, we used slightly different procedures for weevil damage evaluations. In the Swedish trial, where seedlings were generally smaller, debarked area was estimated by inspecting down to the base of the stem and using graduate millimeter templates as in Nordlander *et al.* (2011), with 0.1 cm^2 being the smallest area recorded. In the Spanish trial, the debarked area during the first growing season was estimated by measuring the length of the scars in four longitudinal transects along the entire stem, as in Moreira *et al.* (2009). The large size of the plants impeded the use of this procedure in the 2012 assessment. On this occasion we used a subjective assessment similar to that used by Zas *et al.* (2006). Each seedling stem was visually divided into 10 equally-sized parts, in each of which weevil damage was recorded using a five-level score (0, 0-25%, 26-50%, 51-75% and 76-100% of the bark surface debarked by the weevils). Debarked area (in cm^2) was estimated from these values by assuming that the stems have a cone shape with basal stem diameter and total seedling height defining the basic cone parameters.

2.5. Sampling and chemical analyses

Twenty seedlings of each of the six main treatments (T1-T6) and species, that were kept in the trays outdoors in the respective nurseries, were sampled for chemical analyses (Table A1) approximately 3 weeks after the field experiments were established (31 May 2011 for *P. pinaster* and *P. radiata* and 12 July 2011 for *P. sylvestris* and *P. abies*), i.e. during the period of intense weevil feeding. Seedlings were thus sampled around 7 and 5 weeks after the first and second MJ applications, respectively. Needles and stems were carefully separated and immediately frozen at -30 ºC. Two main quantitative chemical defensive traits were determined in each of these tissues, the concentration of non-volatile resin and the concentration of total polyphenolics. Chemical analyses were performed at the Misión Biológica de Galicia (Pontevedra, Spain).

Non-volatile resin was extracted with hexane in an ultrasonic bath for 15 min at 20ºC and then for 24 hours at room temperature. After filtering the extract (Whatman GFF, Whatman Int. Ltd, Maidstone, Kent, UK) and repeating the extraction again, the concentration of non-volatile resin was estimated gravimetrically and expressed as mg of non-volatile resin g^{-1} dried weight (d.w.) of the given tissue. The residual material after the extraction of non-volatile resin was then used for total polyphenolics determination. Total polyphenolics were extracted with aqueous methanol (1:1 vol:vol) in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of the methanolic extract. Total polyphenolic content was determined colorimetrically by the Folin-Ciocalteu method in a Biorad 650 microplate reader (Bio-Rad Laboratories Inc., Philadelphia, PA, USA) at 740 nm, using tannic acid as standard, and referred to the vegetal tissue in a d.w. basis (see more details in Moreira *et al.*, 2009). A total of 960 (20 plants × 4 species × 6 treatments × 2 tissues) samples were analyzed (Table A1).

2.6. Statistical analyses

Seedling height, diameter and weevil damage (debarked area) in the field were analyzed independently for each species and year with a two-way mixed model ANOVA in which the effect of MJ treatments was treated as a fixed factor and the blocks and their interaction with the MJ treatments were considered random factors. This allowed us to account for the eventual autocorrelation of the 10 contiguous plants of the same treatment within each block (i.e., the experimental plots), and resulted in the appropriated denominator degrees of freedom for testing the effect of the MJ treatments. Debarked area was log transformed to achieve residual normality in all species and years. Heterogeneous residual variance models were fitted when the Levene test identified significant differences in the residual variance among MJ treatments. Least square means were estimated from the mixed models and used for multiple comparisons among treatments. Specific contrasts testing for significant differences between specific MJ treatments and the control were also performed. All general linear mixed models were fitted with the MIXED procedure of the SAS System (Littell *et al.*, 2006).

Binary variables (i.e. mortality, stem girdling, and whether the seedlings were attacked or not) were analyzed with a generalized mixed model similar to the one described above. The models were fitted with the GLIMMIX procedure of SAS (Littell *et al.*, 2006), assuming a binary residual distribution and a logit link function.

The effect of the application of MJ on the non-volatile resin and total polyphenolics in the stem and needles was analyzed with a repeated measures mixed model in which the MJ treatments, the plant species and their interaction were considered between-subject fixed factors, and the plant tissue (stem or needles) and its interaction with MJ and species as within-subject fixed factors. An unstructured covariance model with independent within-subject residual variance for each tissue type was used.

For all the studied traits (i.e. chemical traits, seedling size and weevil damage) two different analyses were performed. First we tested whether the different MJ concentrations significantly affected these traits analyzing a sub-dataset that included only the treatments T1 (0 mM), T2 (5 mM), T3 (10 mM) and T4 (25 mM), in which MJ was applied twice 4 and 2 weeks before planting (Table A1). We then analyzed whether there were differences among the two single and the double application of MJ, only analyzing the treatments T1 (control), T5 (25 mM applied 4 weeks before planting), T6 (25 mM applied 2 weeks before planting), and T4 (25 mM applied twice 4 and 2 weeks before planting) (Table A1).

3. Results

3.1. Weevil damage at field

Pine weevil pressure was high in the two field trials and lasted for at least two growing seasons (Table 1). During the first year, the weevil fed on between 68 and 85% of the planted seedlings, with a mean debarked area of attacked seedlings ranging from around 1 cm^2 in *P. sylvestris* and *P. abies* in the Swedish trial to around 3 and 5 cm^2 in *P. radiata* and *P. pinaster*, respectively, in the Spanish trial (Table 1). Weevil damage caused stem girdling in 12-22% and 23-30% of the seedlings planted in the Swedish and the Spanish trials respectively (Table 1). Almost all the girdled seedlings of the Swedish trial died, whereas around 70% of the girdled seedlings of the Spanish trial were able to survive by resprouting below the girdling site (Table 2). Accordingly, mortality rates due to weevil damage were greater in the Swedish than in the Spanish trial, especially in *P. pinaster* (Table 2).

During the second growing season, the pine weevil pressure remained high in the Spanish trial, with 73-91% of the seedlings attacked by the weevil and similarly high mean values of debarked area to the first season. Despite this, the percentage of girdled seedlings was much reduced during the second growing season, probably because of the increase in basal stem diameter (Table 1). On the contrary, in the Swedish trial, the damage intensity was largely reduced during the second growing season, but in this case it did continue to provoke stem girdling and seedling mortality in a high percentage of seedlings (Table 1). At the end of the two first growing seasons after planting, overall cumulative mortality due to weevil damage was 16, 24, 23 and 33% in *P. pinaster, P. radiata, P. sylvestris* and *P. abies*, respectively.

MJ application in the nursery effectively reduced the damage caused by the pine weevil during both the first and the second growing seasons after planting (Table 2). During the first season, although MJ application significantly reduced the percentage of attacked seedlings only in *P. pinaster*, it significantly reduced the debarked area of wounded seedlings in all the four studied species (Table 2, Figure 1). The reduction of the debarked area was proportional to the concentration used in the MJ treatments in all species. In the case of the pine species, the damage on seedlings treated twice with the highest concentration of MJ was reduced to less than half of that on control plants, whereas the reduction of damage in spruce was around 38% (Figure 1). The reduction of the debarked area of attacked seedlings was significant only when the 25 mM MJ solution was applied twice, except in *P. pinaster* for which the single early application (4w before planting) also significantly reduced the debarked area during the first growing season compared to control plants (Figure 2, see also Table B1 in Appendix B).

The reduction in weevil damage was translated into a reduction in the percentage of girdled seedlings and mortality rates (Table 2, Figure 1). In control plants the percentage of seedlings that became girdled during the first growing season varied between 22% in *P. sylvestris* and 38% in *P. pinaster,* whereas mortality rates varied between 10% in *P. pinaster* and 24% in *P. abies.* In MJ treated plants these values were strongly reduced in the four species although in the case of stem girdling the effect was only significant for the three pine species, and in the case of mortality only for *P. sylvestris* (Table 2, Figure 1). The effect of MJ on stem girdling and mortality was again dose-dependent and only the highest concentration applied twice led to a statistically significant reduction of these traits in comparison with control plants (Figure 1, Figure 2, Table B1). Following two 25 mM MJ treatments, only around 10% of *P. pinaster*, *P. radiata* and *P. abies* seedlings were girdled, while for *P. sylvestris* girdling was virtually absent; mortality rates were reduced to 3, 7 and 1% in *P. pinaster, P. radiata* and *P. sylvestris*, respectively, but only to 16% in *P. abies.*

During the second growing season, the MJ treated seedlings continued to suffer less new pine weevil damage compared with untreated control seedlings, but the effect was not as clear and consistent as during the first year (Table 2, see also Figure C1 in Appendix C). Weevils still preferred untreated control plants of *P. pinaster* to plants treated twice with 25 mM MJ (Figure C1). The effect of MJ on the mean debarked area of attacked seedlings during the second growing season was significant for the three pines (Table 2), but the reduction of debarked area was only evident for the highest concentration treatment (25 mM) (Figure C1). Consequently, the percentage of girdled seedlings was lower in plants treated twice with 25 mM MJ, although the effect was only statistically significant for *P. sylvestris* (Figure C1). The MJ application at the nursery stage strongly reduced the cumulative mortality rates after two complete growing seasons in the field. This effect was clear for all species and statistically significant for *P. radiata* and *P. sylvestris.* The double application of 25 mM MJ 4 and 2 weeks before planting was the treatment which most strongly reduced mortality rates (Figure 2, Figure C1). Results were especially promising in *P. sylvestris* where the cumulative mortality rates after two growing seasons dropped from 39% in control plants to just 7% (Figure C1). This effect was mainly due to the MJ treatments reducing the percentage of seedlings seriously damaged (Figure 3).

3.2. Growth losses

At the time of planting, i.e. 4 and 2 weeks after the first and second application of MJ in the nursery, the size of the MJ treated plants (total height and stem basal diameter) was significantly lower than that of control plants in all studied species except in spruce, for which the difference in total height was not statistically significant (see Figure C2 in Appendix C). The general trend was that the higher the concentration of MJ applied, the greater the observed reduction in seedling size was observed. The reduction in seedling height after the double application of the highest concentration of MJ (25 mM) was especially large in *P. sylvestris* (43%) and *P. radiata* (35%) and somewhat lower in *P. pinaster* (22%) and *P. abies* (8%) (Figure 4).

Once in the field, the reduction of plant size due to MJ application tended to diminish over time (Figure 4, see also Figure C3 in Appendix C). By the end of the second growing season, height growth losses of MJ-treated seedlings were only significant in *P. radiata* and *P. sylvestris* (Figure C3), and even for these species treated seedlings were just 10 and 15% shorter than control seedlings, compared with the 43 and 35% reduction in size at the time of planting (Figure 4). This decrease in growth losses with age was probably mainly due to the reduction of weevil damage in MJ treated plants. When comparing the growth of control and MJ treated seedlings physically protected against the pine weevil (non-attacked seedlings, treatment 7 and 8), we found that the reduction in height due to MJ remained highly significant in the three pine species two growing seasons after planting (Figure 5). Overall these results suggest that, in unprotected seedlings, the growth benefits of being less damaged compensated the growth loss due to the application of MJ per se.

3.3. Chemical defensive responses

The exogenous application of MJ strongly increased the two studied chemical resistance traits (non-volatile resin and total polyphenolics) but the effect was not the same in all four conifer species (significant MJ × Species interaction) and differed between needles and stems (significant MJ × Tissue and MJ × Tissue × Species interactions) (Table 3). In the case of non-volatile resin, the application of MJ significantly increased its concentration in the four species and the two tissues, and the effect was generally proportional to the concentration used (Figure 6a, see also Figure C4a in Appendix C). Non-volatile resin concentration in the stems of seedlings treated twice with the highest concentration of MJ (25 mM MJ applied 7 and 5 weeks before sampling) was 2.0, 2.7, 1.5 and 2.9 times that of control seedlings for *P. pinaster*, *P. radiata*, *P. sylvestris* and *P. abies*, respectively (Figure 6a). This treatment also more than doubled the non-volatile resin in the needles of the three pine species, but the effect was much lower in the needles of the spruce (Figure C4a). Single applications of 25 mM MJ also significantly increased the concentration of non-volatile resin in the stems but the increments were significantly smaller than after the double application in the four studied species (Figure 2). No significant differences were observed when comparing the effects of the early and late applications, except in the case of *P. radiata*, for which the effect of MJ was stronger when applied 5 weeks before sampling than when applied 7 weeks before sampling (Figure 2).

MJ also significantly increased the concentration of total polyphenolics in both stems and needles (Table 3). In the case of total polyphenolics in the needles, the effect was significant for all four species (Figure C4b), but MJ only significantly increased stem total polyphenolics in *P. pinaster* and *P. radiata* (Figure 6b). Following the double application of

25 mM MJ, concentrations were 1.4 and 2.1 times that of control plants, respectively (Figure 6b), and similar responses were in fact also observed following just a single application of the same concentration (Figure 2). The treatments applying lower concentrations of MJ only significantly increased the total polyphenolics in the stems of *P. radiata* (Figure 6b).

4. Discussion

The results of this study point to a new environmentally-friendly and putatively cost-effective method to protect forest plantations against pests. Application of MJ in the nursery some weeks before planting was effective in reducing weevil damage under real field conditions in all four conifer species, and the protection was long lasting, at least up to two seasons after planting. The reduction in weevil damage appeared to be related to an increase in the chemical resistance of the seedlings. Chemical elicitors are becoming more popular for protecting agricultural crops against pests and diseases (Rohwer and Erwin, 2008; Walters and Fountaine, 2009) but they are still in an experimental phase in forestry and to our knowledge they have never been commercially used for protecting forest plantations or tree seedlings in the nursery. That MJ reduced weevil feeding through an increase in plant defensive traits has been reported before (Heijari *et al.*, 2005; Moreira *et al.*, 2009; Sampedro *et al.*, 2011b), but the important result found here is that this effect remained significantly and quantitatively important under real field conditions. Furthermore, although the practical effectiveness varied depending on the species, the general results were consistent across sites and species, in spite of the huge environmental differences between the two field trials, which represent the northern and southern limits of *H. abietis*' range. This is particularly relevant as climate is known to strongly influence the life cycle of *H. abietis*, the timing of its feeding activity and the amount of damage it causes (Tan *et al.*, 2010; Inward *et al.*, 2012), as well as, of course, the phenology and growth rates of the tree species (e.g. Nobis *et al.*, 2012). By being consistent across such contrasting environmental conditions, our results suggest that the response to the MJ treatments is general, and can be extrapolated to the whole distribution range of *H. abietis.*

The results were especially promising in the three pine species, in which the reduced feeding damage on MJ treated seedlings was translated into a reduced probability of stem girdling and thus improved seedling performance. Mortality was drastically reduced in the case of *P. sylvestris*, dropping from nearly 40% in control plants to less than 7% in MJ treated plants. This reduction is quantitatively of great importance and clearly indicates the potential of MJ as a tool for protecting forest plantations against this insect pest. In the other studied species, the results showed the same trend but the reduction of weevil damage and seedling mortality was relatively smaller, especially in *P. abies*. Further research is needed to fine tune the application procedure in order to optimize its effect in this species.

In contrast with previous studies (Gould *et al.*, 2009), the repeated application of MJ was much more effective in reducing pine weevil damage than single applications. The pattern of response mirrored that observed for chemical defensive traits (see below) but in this case, the effect of the single applications was not statistically significant. Numerous applications of MJ at low concentration rates should thus be further investigated in order to optimize the protecting effect.

4.1. Increase of chemical resistance traits

The observed increase in chemical resistance traits after MJ application was consistent with previous findings reporting the activation of both the phenylpropanoid and terpenoid pathways in different conifer species (Heijari *et al.*, 2005; Moreira *et al.*, 2009; Zhao *et al.*, 2010; Schiebe *et al.*, 2012). The concentration of non-volatile resin, which is highly correlated with the diterpene fraction of the oleoresin (Sampedro *et al.*, 2011b), was increased in all four species and in both the needles and the stems. Previous studies have shown that MJ increased the concentration of total resin acids in the needles and xylem of Scots pine juveniles (Heijari *et al.*, 2005), and in the stems of Maritime pine (Moreira *et al.*, 2009) and Monterrey pine (Moreira *et al.*, 2012b), although in all these cases the minimum concentration of MJ needed to provoke significant changes in the non-volatile resin was much higher (80 or 100 mM) than that used here. In general we found that the increase in non-volatile resin in the stems and needles was proportional to the concentration of MJ applied, and even the lowest concentration (5 mM) was enough to significantly increase the non-volatile resin in the two tissues. These results may have arisen because we applied two consecutive applications of MJ (approximately 7 and 5 weeks before chemical analyses) whereas the previous studies have analysed the effects of just single applications.

Besides the classical segregation of constitutive and induced resistance, an intermediate status may also exist, in which the plant primes defensive mechanisms in response to environmental cues that alert of an increased probability of biotic risk (Frost *et al.*, 2008). Primed plants would be prepared for the biotic risk, and respond faster and more intensively to the biotic stress once it appears (Conrath *et al.*, 2006). The application of low concentration of MJ could be provoking a priming response in our conifer seedlings. Instead of directly increasing the concentration of chemical defensive traits, the first application of MJ at low concentration rates could be provoking physiological changes that allow the seedlings to respond faster and stronger to further applications of MJ. This would explain why the low concentration treatments had a considerably stronger effect than had been previously reported. Our results show, however, that single applications of 25 mM MJ did in fact significantly increase the non-volatile resin in the stems of all species, although not as much as the double application. Repeated applications of MJ at low concentration rates did not provoke stronger defensive responses in Monterrey pine seedlings against the fungus *Diplodia pinea* than single applications of MJ (Gould *et al.*, 2009). In that study, the application of MJ at concentration of just 1 mM was enough to significantly increase the concentration of some monoterpenes in the stems. Similarly low concentration of MJ increased the mono and diterpene fraction in the stems of Norway spruce (Martín *et al.*, 2002). It seems that the sensitivity to MJ may depend on other factors, among which plant ontogeny (Erbilgin and Colgan, 2012), plant tissue and part (Moreira *et al.*, 2012b), plant genotype (Zeneli *et al.*, 2006; Moreira *et al.*, 2013) and phenology (Moreira *et al.*, 2012a) may be especially relevant. It may therefore be significant that in this study we managed young seedlings that are likely to be more sensitive to external application of MJ than older and more lignified saplings or mature trees.

Total polyphenolics were also increased after MJ application, especially in the needles where the MJ effect was significant in all four studied species. Increased polyphenolics after MJ application has been reported before in different conifers (Sampedro *et al.*, 2011a; Schiebe *et al.*, 2012) but the effect is usually not as clear and dose-dependent as that observed for terpenoids (Erbilgin *et al.*, 2006; Moreira *et al.*, 2009). Focusing on the stems, only Maritime pine and Monterrey pine responded to MJ by increasing the total polyphenolics concentration.

The mechanisms of resistance against pine weevils are still not completely understood but different terpenoids and phenolics are known to be involved either in weevil attraction (Nordlander, 1991; Blanch *et al.*, 2012) and/or in deterring weevil feeding (Nordlander, 1991; Borg-Karlson *et al*., 2006), and both non-volatile resin and total polyphenolics, as determined here, have been related to pine weevil resistance (Moreira *et al.*, 2009; Carrillo-Gavilán *et al.*, 2012). The increases of these substances through MJ application may, thus, be related to the reduced pine weevil damage in the field.

4.2. Lasting effect

Planted seedlings frequently face a high risk of being killed by pine weevils for several years after planting (Örlander and Nilsson, 1999). Specifically, in the two field trials of the present study, weevil damage was very intense during the two first seasons after planting, especially in the Spanish trial, where weevil damage was as intense during the second growing season as during the first. Seedlings treated with MJ remained protected during the second growing season as revealed by the reduction in the debarked area of attacked seedlings and/or the reduction of the percentage of girdled seedlings. The response to MJ was, however, not as clear as during the first growing season, and was significant in the three pine species but not in Norway spruce. Previous research with young Norway spruces indicates that the response to MJ in terpenoid-related traits reaches its maximum around 15-25 days after application and then progressively declines from then on (Martín *et al.*, 2002). The decay time of this induced response remains largely unknown, but results from experiments on mature trees indicates that the accumulation of terpenoids after MJ application may last much longer, and differences in terpenoid concentration between MJ and control trees may remain significant more than one year after MJ application (Erbilgin *et al.*, 2006; Zhao *et al.*, 2010). Irrespective of whether the effect of the MJ treatment per se remained significant in our field trials two seasons after the application, pine seedlings are also known to strongly respond to weevil feeding (Heijari *et al.*, 2005; Sampedro *et al.*, 2011b) and these responses may be confounded with the initial responses to MJ application. Nonetheless the results indicate that two seasons after planting the MJ treated seedlings were still being consumed at a lower rate by the weevil, suggesting that the MJ effect remained protecting the seedlings for at least this length of time. The results during the second season differed again depending on the species and field trial. In the Spanish trial, where the damage level remained very high during the second growing season, the surviving MJ treated seedlings were less damaged than the control ones but this was not translated into a lower percentage of girdled seedlings. On the contrary, Scots pine seedlings treated with MJ were less frequently girdled during the second growing season. These differences can be explained again by the huge differences in seedling size during the second growing season between the Spanish and the Swedish seedlings. The Spanish seedlings were much thicker, and thus, it was less likely that the debarked area would entirely surround the stem circumference (Thorsén *et al.*, 2001).

4.3. Growth losses

One of the most frequent limitations for the practical use of MJ in crop protection is the negative effect on growth and reduced plant fitness in the absence of damage (Holopainen *et al.*, 2009; Moreira *et al.*, 2012a). Reduced growth of MJ treated conifer seedlings has been repeatedly observed in several short-term experiments (Heijari *et al.*, 2005; Krokene *et al.*, 2008; Sampedro *et al.*, 2011a). Based on the results of the present work, these growth reductions appear to be, however, a transient effect that tend to diminish with time and became almost negligible after two seasons. Furthermore, even if growth losses remain significant after some years, the application of MJ may still be recommended because of its positive effect on seedling survival. Under favorable conditions, the pine weevil can cause extremely large mortality rates if no protection measures are applied, so it may be justifiable to sacrifice some growth for increased survival (Krokene *et al.*, 2008). Additionally, the reduction of growth rates in MJ treated plants is later compensated to a large extent by the benefits in terms of improved growth as a consequence of being less damaged. Even if seedlings are not killed, weevil damage has been shown to have a negative impact on seedling growth (Sampedro *et al.*, 2009), and so by reducing damage levels, growth losses due to weevil damage were lower in MJ treated plants. Indeed, the net effect of MJ on growth was negligible in the presence of weevil damage, although it remained significant after two seasons if seedlings were physically protected against the weevil.

4.4. Towards practical applications

The pine weevil is among the most harmful handicaps for regenerating conifer forests all around Europe, especially in northern countries where both the huge extensions of continuous conifer forests and the way they are managed - mainly regenerated by planting after clear cutting - favor the maintenance of high population levels of the pine weevil and severe damage on the regenerate (Nordlander *et al.*, 2011). Since the application of insecticides (mainly permethrin) was limited in Europe in the early 2000s, there has been a strong research effort to search for alternative environmental-friendly ways of protecting seedlings (e.g. Zas *et al.*, 2008; Nordlander *et al.*, 2009; Manák *et al.*, 2013). Nowadays a combination of silvicultural measures, insecticides and direct physical seedling protection is applied in northern Europe on a massive scale to limit weevil damage, but all these measures inevitably increase the economic costs of the regeneration process (Petersson and Örlander, 2003; Nordlander *et al.*, 2011). The results of this study suggest that the application of MJ at the nursery stage has the potential to become an environmentally-friendly and cost-effective alternative way to fight against this harmful forest pest. We would expect a similar effect of the treatment when scaling up from a field experiment to a setting where all seedlings are treated, since feeding on seedlings are not essential for the pine weevils but other food sources on the clear-cut are used to a large extent (Wallertz *et al.*, 2006). The defensive response triggered by MJ seemed to be general, being effective at protecting seedlings of different conifer species under very different environmental conditions, from the southern to the northern extremes of the pine weevil distribution. Additionally, given the numerous examples of previous works reporting increased resistance of MJ treated seedlings against other biotic threats (see references in the Introduction), the generality of the responses may be extended to different biotic risks. We can therefore expect that MJ treated seedlings would also have better protection against other pests and pathogens.

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Table 1. Summary data of field performance during the first and second growing seasons of seedlings of four conifer species planted in two clear-cuts, one in Spain (*P. pinaster* and *P. radiata*) and one in Sweden (*P. sylvestris* and *P. abies*), naturally attacked by the pine weevil (*H. abietis*) Seedling growth (mean ± s.e.) and pine weevil damage, including debarked area by weevil feeding (mean ± s.e.), risk of being attacked, and percentage of stem girdling and mortality rates (percentage of planted or surviving seedlings for 1st and 2nd season) are shown. Data are overall means for each site and species; N = 480 seedlings. Presented values are based on data from all seedlings except those with physical protection, i.e. T1-T6 (see Methods for details).

		Spanish trial		Swedish trial	
	Season	*P. pinaster*	*P. radiata*	*P. sylvestris*	*Picea abies*
Mean height [1] (cm)	1st	37.7 ± 0.5	31.1 ± 0.5	16.4 ± 0.2	26.2 ± 0.2
	2nd	102.1 ± 1.2	103.5 ± 1.5	30.7 ± 0.4	35.7 ± 0.4
Mean basal diameter [1] (mm)	1st	6.1 ± 0.06	5.6 ± 0.07	4.2 ± 0.05	4.2 ± 0.04
	2nd	21.8 ± 0.30	20.8 ± 0.35	9.5 ± 0.1	8.1 ± 0.09
Attacked seedlings [2] (%)	1st	79.8	68.3	84.8	78.8
	2nd	91.1	72.9	51.3	29.0
Girdled seedlings [2] (%)	1st	23.1	30.0	11.7	21.7
	2nd	1.4	6.2	12.8	17.3
Mortality due to pine weevil[2] (%)	1st	4.4	10.4	11.7	21.5
	2nd	5.2	15.8	12.6	15.0
Other mortality [2] (%)	1st	4.2	5.0	0.6	3.8
	2nd	0.2	0.5	0.2	0.3
Mean debarked area [3] (cm^2)	1st	4.9 ± 0.3	2.9 ± 0.2	0.8 ± 0.04	1.1 ± 0.05
	2nd	6.2 ± 0.3	3.3 ± 0.2	0.2 ± 0.02	0.5 ± 0.03

[1] Only living seedlings were considered.

[2] Percentage values for the first season were estimated upon the total number of planted seedlings whereas those for the second season were estimated upon the surviving seedlings from the previous season.

[3] Debarked area estimations are not comparable between sites due to differences in methodology (see main text for description).

Table 2. Results of the generalized and linear mixed models showing the effect of the application of methyl jasmonate (0, 5, 10 or 25 mM MJ) on weevil damage and plant growth of seedlings of four conifer species planted in two clear-cuts, one in Spain (*P. pinaster* and *P. radiata*) and one in Sweden (*P. sylvestris* and *P. abies*), naturally attacked by the pine weevil (*H. abietis*). Independent analyses for the first and second growing seasons are shown. Results are based on yearly data so that for the second growing season we are showing the results for new damage during that season, except in the case of mortality for which we show the cumulative mortality after two growing seasons. All treatments were applied twice, 4 and 2 weeks before planting. F ratio and associated probability levels for the main effect of the MJ treatment are shown. Significant p values ($p < 0.05$) are typed in bold. Dash symbols indicate that the generalized mixed model failed to converge.

		Spanish trial				Swedish trial			
		P. pinaster		*P. radiata*		*P. sylvestris*		*Picea abies*	
		$F_{3,21}$	P>F	$F_{3,21}$	P>F	$F_{3,21}$	P>F	$F_{3,21}$	P>F
Height	2011	3.0	0.055	13.2	**<0.001**	40.7	**<0.001**	6.5	**0.003**
	2012	0.2	0.911	4.0	**0.022**	6.2	**0.004**	2.4	0.093
Diameter	2011	7.5	**0.001**	8.8	**0.001**	0.3	0.797	0.1	0.933
	2012	0.1	0.966	4.4	**0.016**	1.4	0.273	0.4	0.735
Probability of being attacked	2011	3.2	**0.046**	1.4	0.286	0.1	0.980	0.6	0.656
	2012	1.9	0.168	0.5	0.723	1.6	0.221	0.3	0.839
Probability of stem girdling	2011	3.4	**0.039**	2.4	0.096	4.1	**0.020**	1.1	0.355
	2012	-	-	1.2	0.353	1.6	0.221	0.8	0.491
Cumulative mortality	2011	-	-	1.2	0.334	4.0	**0.021**	1.0	0.416
	2012	1.1	0.362	1.8	0.174	3.5	**0.034**	1.3	0.289
Debarked area [1]	2011	4.8	**0.011**	3.1	**0.051**	4.8	**0.011**	2.5	0.086
	2012	4.1	**0.019**	5.0	**0.009**	3.4	**0.037**	0.3	0.859

[1] Debarked area was log-transformed to achieve normality. Heterogeneous residual variance models were fitted when needed.

Table 3. Results of the repeated measures mixed model for the statistical analysis of major chemical defenses (non-volatile resin and total polyphenolics) in two plant tissues (stem and needles) of seedlings of four conifer species (*P. pinaster, P. radiata, P. sylvestris* and *P. abies*) treated twice with different concentrations of methyl jasmonate (0, 5, 10 or 25 mM MJ). Plant tissue was considered a within subject factor, whereas species and MJ treatment were considered between subject factors. Degrees of freedom of the numerator (DFnum) and denominator (DFden), F-ratios and associated probability values are shown. Significant p values ($p < 0.05$) are typed in bold. All treatments were applied twice, 7 and 5 weeks before sampling for chemical analyses.

			Non-volatile resin		Total polyphenolics	
Effect	DFnum	DFden	F	P > F	F	P > F
Across subjects						
Species (SP)	3	143	83.2	**<0.001**	56.02	**<0.001**
MJ treatment (MJ)	3	143	105.0	**<0.001**	39.19	**<0.001**
SP x MJ	9	143	3.6	**0.004**	6.59	**<0.001**
Within subjects						
Tissue	1	141	1032.9	**<0.001**	4924.4	**<0.001**
SP x Tissue	3	141	31.1	**<0.001**	114.9	**<0.001**
MJ x Tissue	3	141	0.9	0.459	43.6	**<0.001**
SP x MJ x Tissue	9	141	6.5	**<0.001**	3.73	**<0.001**

Figure legends

Figure 1. Effect of methyl jasmonate application (0, 5, 10 or 25 mM MJ) on the damage caused by the pine weevil (*H. abietis*) during the first season after planting. Four conifer species were planted in two field trials, one in Spain including *P. pinaster* and *P. radiata* (left panels) and the other in Sweden including *P. sylvestris* and *P. abies* (right panels). In both trials seedlings were naturally infested by the pine weevil, *H. abietis*. Damage by the pine weevil is represented by the probability of being attacked, the probability of stem girdling, the impact of weevil damage on seedling mortality and the total debarked area of attacked seedling. All treatments were applied twice, 4 and 2 weeks before planting. Least square means ± s.e.m. are shown (N = 80 seedlings). Different letters above each bar indicate significant differences ($p < 0.05$) among MJ treatments within each species. n.c. denote that the generalized model failed to converge. n.s. = no significance. Note that different y-axis scales are used for the debarked area.

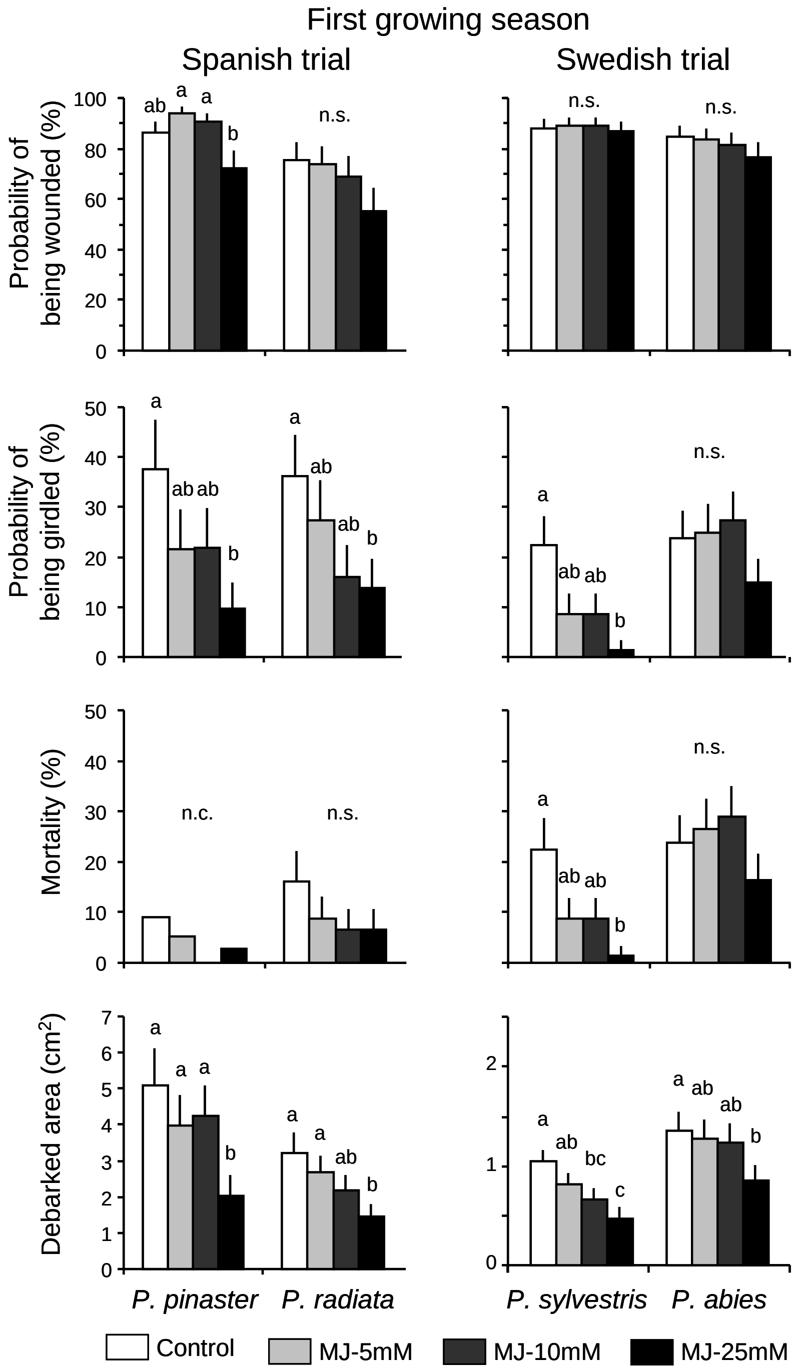
Figure 2. Effect of single (4 or 2 weeks before planting) and repeated (4 + 2 weeks before planting) application of methyl jasmonate on seedlings of four conifer species planted in two clear-cuts, one in Spain (left panels) and one in Sweden (right panels), naturally infested by the pine weevil (*H. abietis*). The effect was measured as the concentration of major chemical defense compounds in the stems (non-volatile resin and total polyphenolics) three weeks after the plantation, the debarked area of attacked seedlings by the pine weevil during the first growing season, and the cumulative mortality after two growing seasons. Least square means ± s.e.m. are shown (N = 20 for chemical traits and N = 80 for weevil damage and mortality). Different letters above each bar indicate significant differences ($p < 0.05$) among MJ treatments within each species. Note that different y-axis scales are used for the debarked area.

Figure 3. Effect of methyl jasmonate application (0, 5, 10 or 25 mM MJ) on the number of attacked and killed *P. sylvestris* seedlings in relation to the amount of debarked area caused by the pine weevil (*H. abietis*) during two growing seasons. Note that MJ treatments shifted the distribution of damage levels to the left and this resulted in reduced mortality rates. All treatments were applied twice 4 and 2 weeks before planting. N = 80 seedlings per treatment.

Figure 4. Recovery of the vegetative costs associated with the methyl jasmonate induced responses measured as loss of height growth of seedlings treated twice with 25 mM MJ in comparison to the control. *P. pinaster* and *P. radiata* were planted in Spain and *P. sylvestris* and *P. abies* were planted in Sweden. Both field trials were naturally infested by the pine weevil (*H. abietis*). Each dot represents the average value of 80 seedlings.

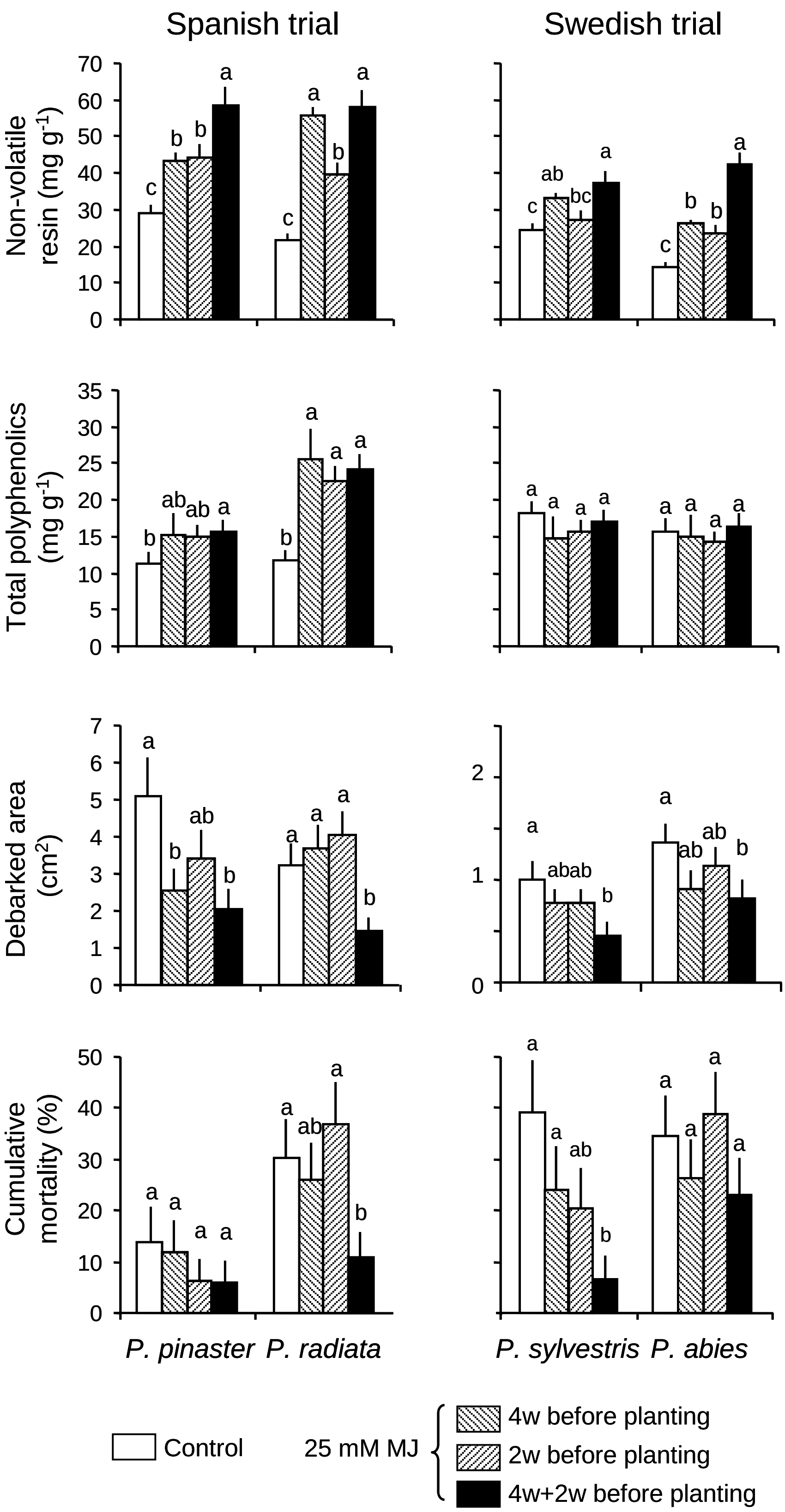
Figure 5. Height of control (white bars) and 25 mM methyl jasmonate treated (black bars) seedlings (double application of 25 mM MJ, 4 and 2 weeks before planting) two seasons after planting of four conifer species in two clear-cut areas in Spain (*P. pinaster* and *P. radiata*) and Sweden (*P. sylvestris* and *P. abies*), with and without physical protection against the pine weevil (*H. abietis*). Only those protected plants that remained non-attacked (or with very low levels of damage) were considered in the analyses. Note that vegetative costs of MJ-associated responses emerged for the three pine species when seedlings were physically protected against pine weevil attack. For unprotected *P. pinaster* and *P. radiata* seedlings, the cost of induced resistance elicited by MJ application was compensated by reduced damage, leading to seedlings of similar height as unprotected control seedlings. For *P. sylvestris,* benefits in form of reduced damage after MJ application did not compensate the reduction of height growth. *Picea abies* showed no reduced growth due to MJ application. Least square means ± s.e. are shown. Asterisks denote significant ($p < 0.05$) difference between control and MJ seedlings, whereas n.s. indicate no significant differences.

Figure 6. Effect of methyl jasmonate application (0, 5, 10 or 25 mM MJ) on seedling defensive chemistry. (A) Concentration of non-volatile resin and (B) total polyphenolics in the stems of four conifer species. All treatments were applied twice, 7 and 5 weeks before sampling for chemical analyses. Least square means ± s.e.m. are shown (N = 20 seedlings). Different letters above each bar indicate significant differences ($p < 0.05$) among MJ treatments within each species.



First growing season
Spanish trial
Swedish trial
Probability of being wounded (%)
Probability of being girdled (%)
Mortality (%)
Debarked area (cm²)
n.s.
n.c.
P. pinaster
P. radiata
P. sylvestris
P. abies
Control
MJ-5mM
MJ-10mM
MJ-25mM

Figure 1.



Spanish trial
Swedish trial
Non-volatile resin (mg g-1)
Total polyphenolics (mg g-1)
Debarked area (cm2)
Cumulative mortality (%)
P. pinaster
P. radiata
P. sylvestris
P. abies
Control
25 mM MJ
4w before planting
2w before planting
4w+2w before planting

Figure 2.

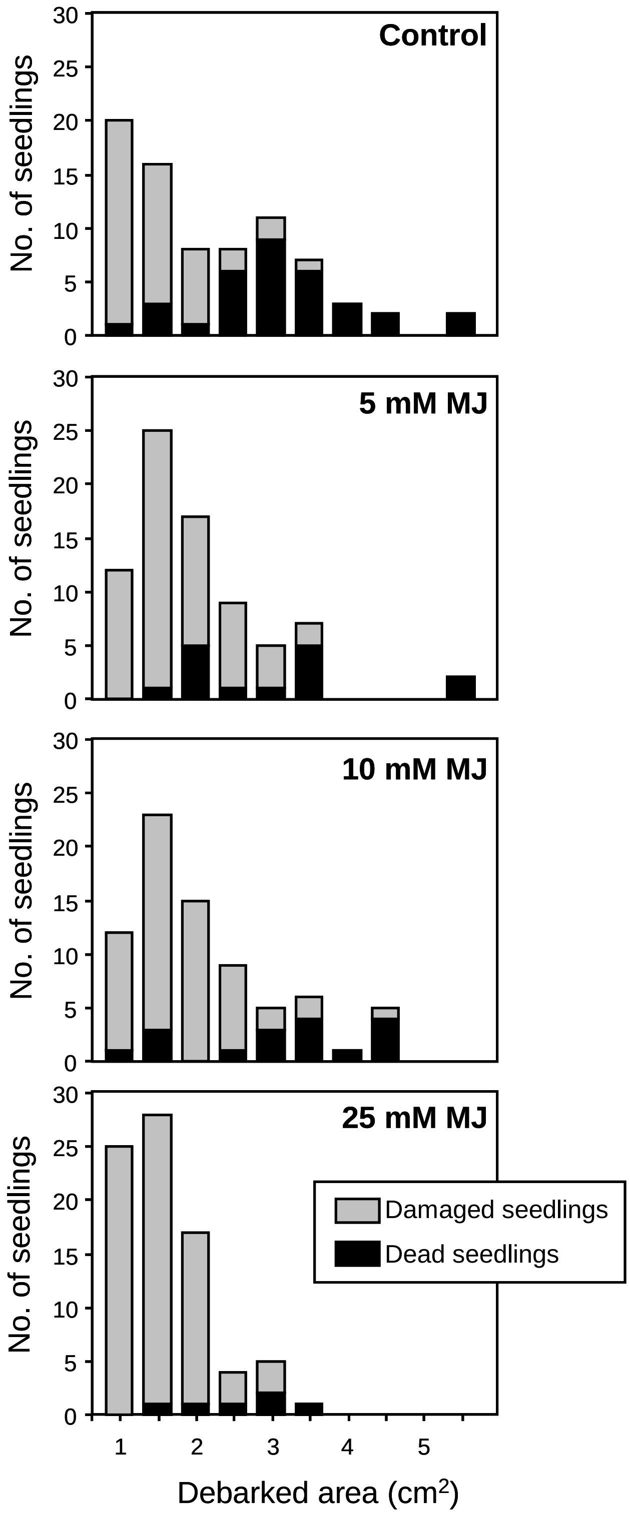
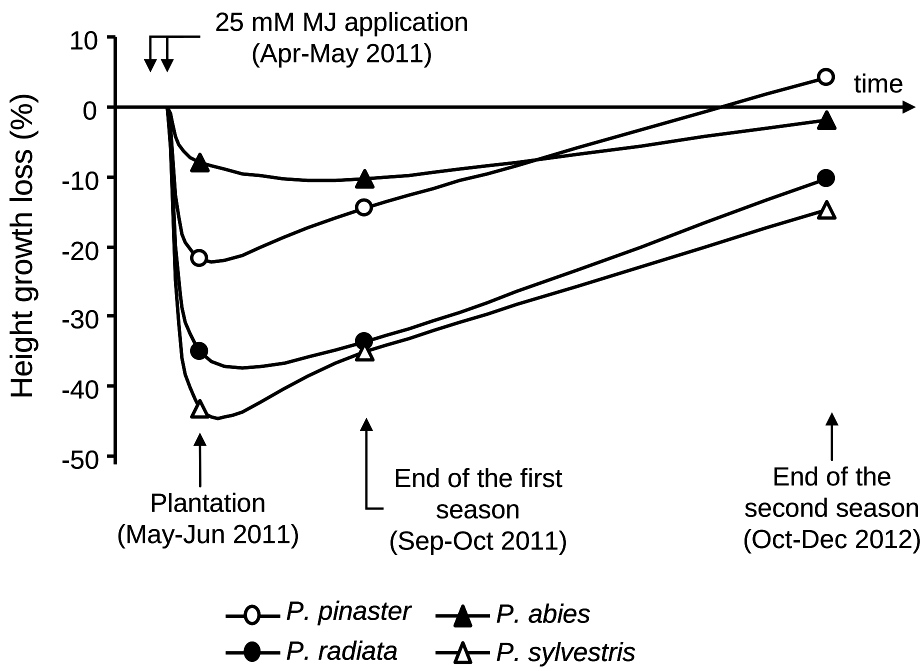

Control
5 mM MJ
10 mM MJ
25 mM MJ
No. of seedlings
0
5
10
15
20
25
30
Damaged seedlings
Dead seedlings
1
2
3
4
5
Debarked area (cm2)

Figure 3.



25 mM MJ application
(Apr-May 2011)
10
0
-10
-20
-30
-40
-50
time
Height growth loss (%)
Plantation
(May-Jun 2011)
End of the first
season
(Sep-Oct 2011)
End of the
second season
(Oct-Dec 2012)
P. pinaster
P. abies
P. radiata
P. sylvestris

Figure 4.

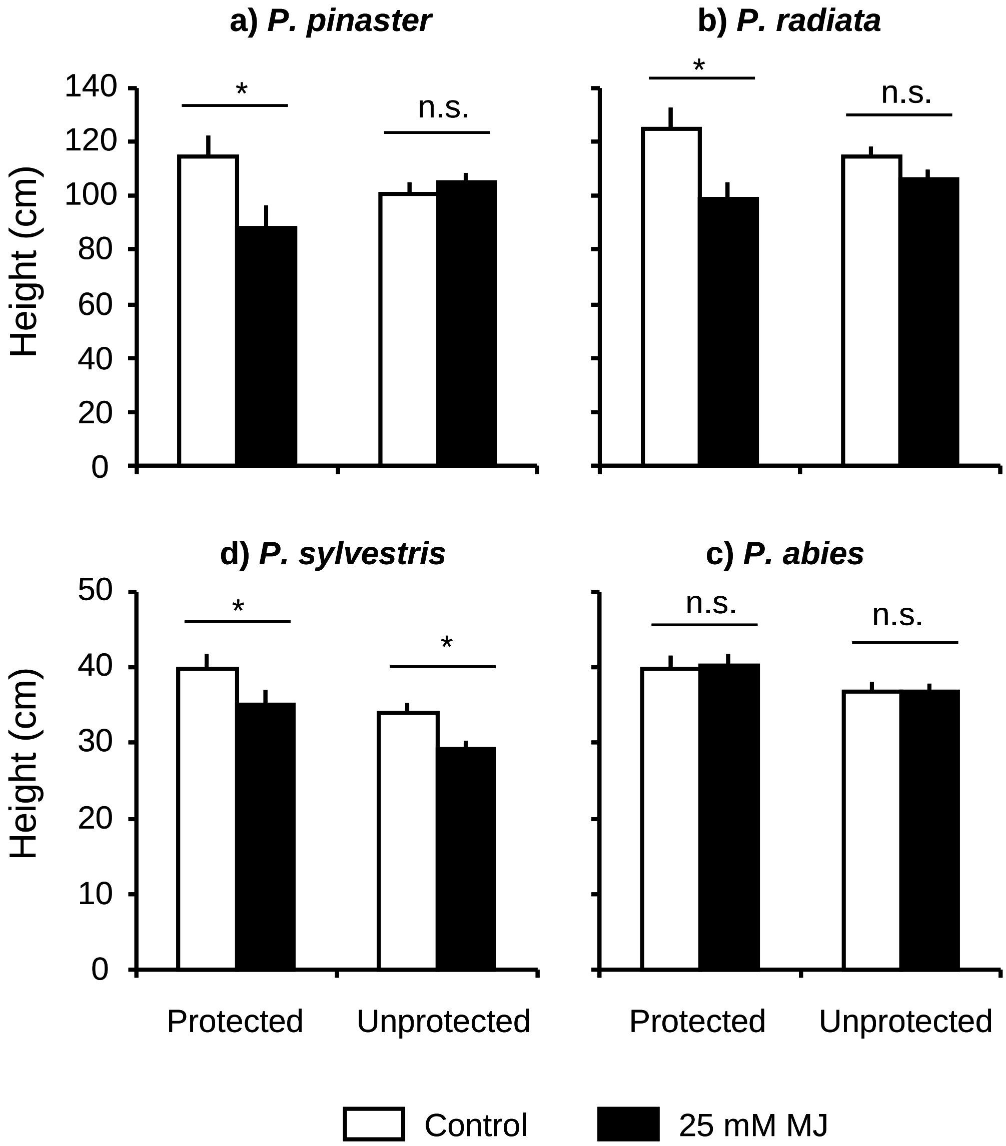

a) P. pinaster
b) P. radiata
d) P. sylvestris
c) P. abies
Height (cm)
140
120
100
80
60
40
20
0
50
40
30
20
10
*
n.s.
Protected
Unprotected
Control
25 mM MJ

Figure 5.

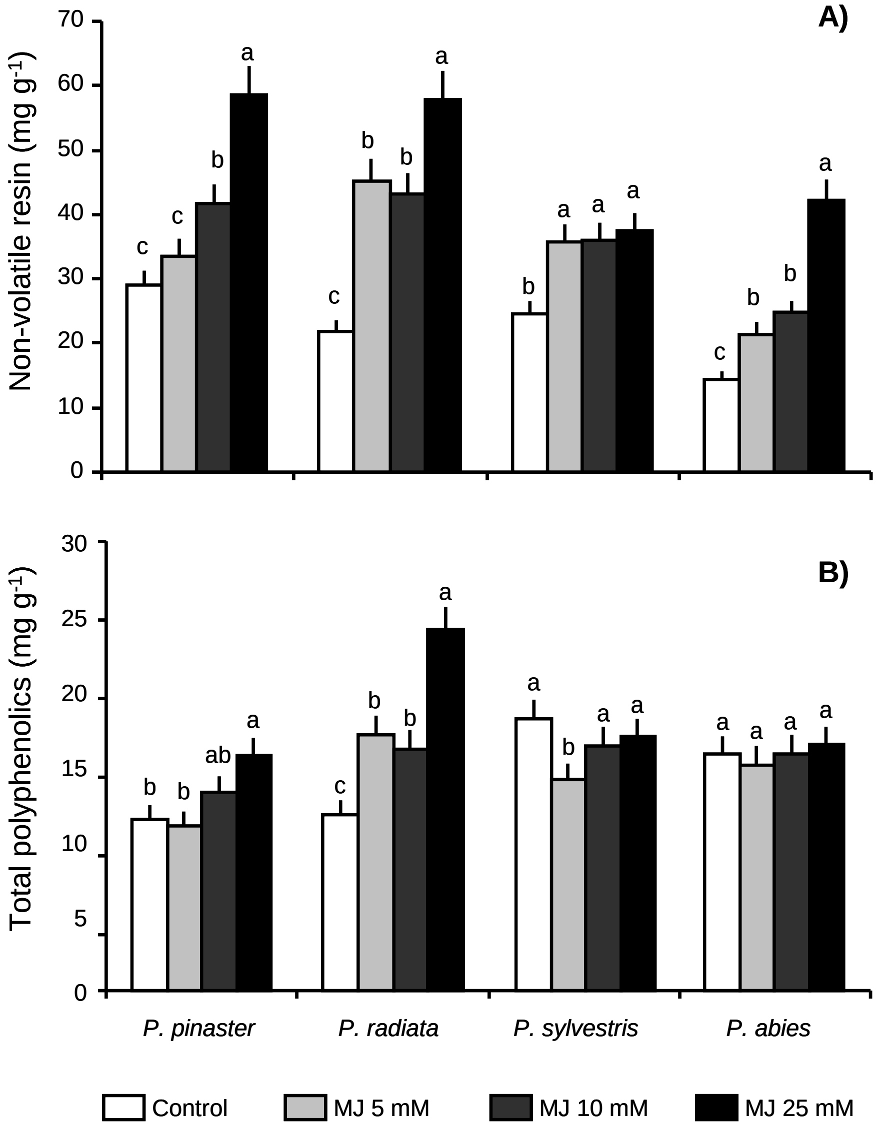

A)
Non-volatile resin (mg g-1)
70
60
50
40
30
20
10
0
c c b a
c b b a
b a a a
c b b a
B)
Total polyphenolics (mg g-1)
30
25
20
15
10
5
0
b b ab a
c b b a
a b a a
a a a a
P. pinaster
P. radiata
P. sylvestris
P. abies
Control
MJ 5 mM
MJ 10 mM
MJ 25 mM

Figure 6.

Tables and figures included in Appendices

Appendix A

Table A1. Details of the methyl jasmonate treatments

Figure A1. Pictures of the Spanish field experiment

Figure A2. Pictures of the Swedish field experiment

Appendix B

Table B1. Specific contrasts testing the effect of a single or double application of 25 mM methyl jasmonate.

Appendix C

Figure C1. Effect of methyl jasmonate application during the second growing season

Figure C2. Effect of methyl jasmonate application on seedling size at the time of planting

Figure C3. Effect of methyl jasmonate application on seedling size at field during the two growing seasons

Figure C4. Effect of methyl jasmonate application on chemical defenses in the needles

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APPENDIX A. Details of the methyl jasmonate treatments and field trials, including photographs of the experimental sites and the treated seedlings.

TABLE A2. Summary of the methyl jasmonate (MJ) treatments included in each experimental site, and total number of seedlings of each species per treatment.

	Treatment code							
	T1	T2	T3	T4	T5	T6	T7	T8
Experimental treatments								
MJ concentration (mM)	0	5	10	25	25	25	0	25
1st application (4 weeks before planting)	×	×	×	×	×		×	×
2nd application (2 weeks before planting)	×	×	×	×		×	×	×
Physical protection							×	×
Sample size								
No. of planted seedlings	80	80	80	80	80	80	40	40
No. of seedlings used for chemical analyses	20	20	20	20	20	20	0	0

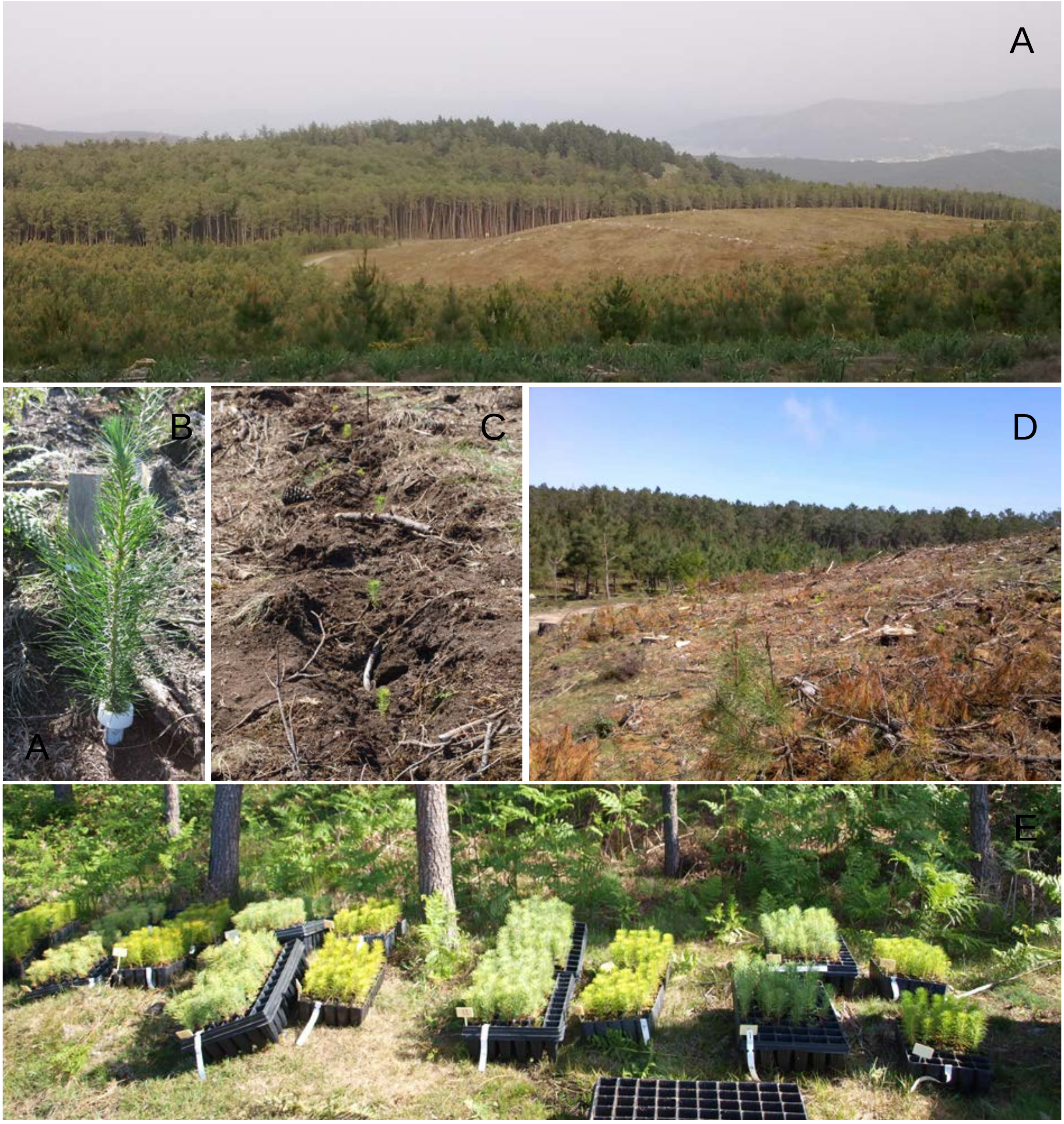

FIG. A1. Overall view and details of the experimental field trial in Spain. (A) Overall view of the clear-cut where the field trial was established, surrounded by mature Maritime pine forest. (B) Detail of a healthy Radiata pine seedling protected with a plastic shield (Snäppskyddet, Panth-Produkter AB, Östhammar, Sweden) one year after planting. (C) Radiata pine seedlings just after planting at field. (D) Details of the clear-cut where the field trial was established. (E) Plant material (*P. pinaster* (olive green seedlings) and *P. radiata* (yellowed green seedlings)) used in the experiment just before planting. Each tray received different MJ treatments.

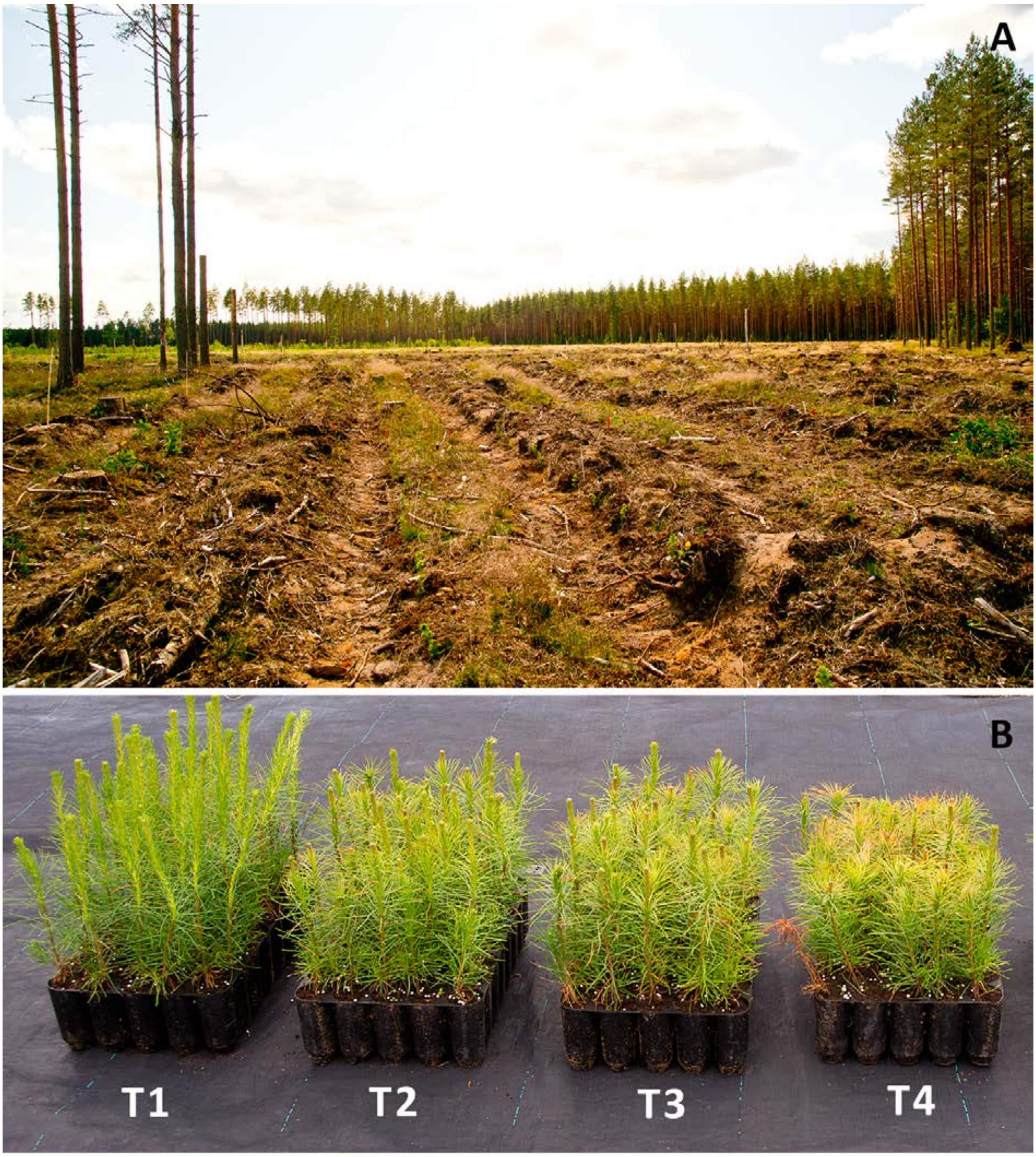

FIG. A2. View of the experimental site in Sweden on the day of planting, 21 June, 2011 (A). Scots pine (*P. sylvestris*) seedlings of the four treatments T1-T4 (see Table A1) just before planting (B).

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APPENDIX B. Supplementary results: Specific contrasts testing the effect of single and double application of 25 mM methyl jasmonate.

TABLE B1. Results of the specific contrasts testing the effect of a single or double application of 25 mM methyl jasmonate (MJ) solution on plant growth and damage by the pine weevil (*H. abietis*) on seedlings of four conifer species planted in two clear-cuts, one in Spain including *P. pinaster* and *P. radiata* and the other in Sweden including *P. sylvestris* and *Picea abies.* P values for the specific contrast testing the differences between each treatment and the control are shown. MJ was applied either 4 weeks (1st application) or 2 weeks (2nd application) before planting, or at both dates. Results are based on yearly data so that for 2012 we are showing the results for new damage in this year, except in the case of mortality which correspond to cumulative mortality after two growing seasons. Significant p-values ($p<0.05$) are typed in bold. Dash symbols indicate that the generalized mixed model failed to converge.

	Spanish trial				Swedish trial			
	P. pinaster		*P. radiata*		*P. sylvestris*		*Picea abies*	
	2011	2012	2011	2012	2011	2012	2011	2012
Height								
Only 1st application	0.074	0.508	0.051	**0.001**	**<0.001**	**0.007**	**0.022**	0.897
Only 2nd application	0.633	0.626	0.541	0.374	**<0.001**	0.317	**0.002**	0.126
Both applications	**0.035**	0.481	**<0.001**	0.052	**<0.001**	**0.005**	**0.002**	0.658
Diameter								
Only 1st application	**0.007**	0.199	**0.050**	**0.000**	0.397	0.124	0.990	0.770
Only 2nd application	0.090	0.465	0.542	0.407	0.631	0.793	0.529	0.685
Both applications	**0.005**	0.732	**<0.001**	**0.014**	0.518	0.261	0.703	0.772
Probability of being attacked								
Only 1st application	0.434	0.094	0.784	0.414	0.148	0.639	0.282	0.688
Only 2nd application	0.603	0.201	0.071	**0.016**	0.488	0.612	0.381	0.331
Both applications	0.147	**0.036**	**0.038**	0.297	0.885	0.587	0.317	0.449
Probability of stem girdling								
Only 1st application	**0.026**	-	0.851	0.737	0.515	0.110	0.326	0.445
Only 2nd application	0.100	-	0.527	0.294	0.194	0.203	0.847	0.873
Both applications	**0.003**	-	**0.026**	0.398	**0.013**	**0.024**	0.283	0.227
Cumulative mortality								
Only 1st application	0.741	0.758	0.715	0.670	0.495	0.158	0.301	0.432
Only 2nd application	0.337	0.163	0.433	0.530	0.184	0.077	0.855	0.712
Both applications	0.201	0.154	0.132	**0.034**	**0.013**	**0.002**	0.387	0.272
Debarked area								
Only 1st application	**0.010**	0.563	0.576	0.723	0.184	0.438	0.075	0.887
Only 2nd application	0.108	0.931	0.318	0.840	0.181	0.232	0.447	0.565
Both applications	**0.002**	0.093	**0.009**	**0.050**	**0.003**	0.386	**0.035**	0.457

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APPENDIX C. Supplementary results: Effect of methyl jasmonate treatments on weevil damage during the second growing season, on seedling growth at different times, and on chemical defences in the needles.

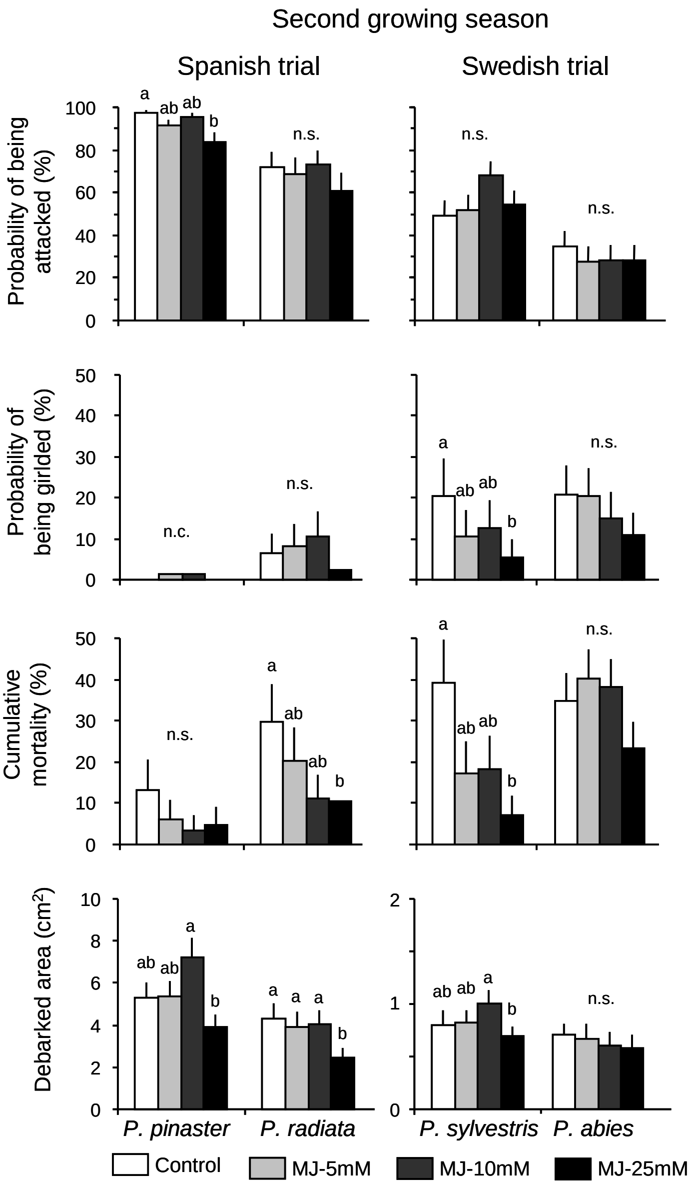

FIGURE C3. Field results for the second growing season. Effect of the methyl jasmonate application (0, 5, 10 and 25 mM MJ) on the probability of being attacked, the probability of stem girdling, mortality rates and new debarked area in attacked seedlings of four conifer species planted in two clear-cuts in Spain (*P. pinaster* and *P. radiata*, left panels) and Sweden (*P. sylvestris* and *P. abies*, right panels) naturally attacked by the pine weevil (*H. abietis*), during the second year after planting. All treatments were applied twice, 4 and 2 weeks before plantation. Least square means ± s.e.m. (N = 80). Different letters above each bar indicate significant differences ($p < 0.05$) among MJ treatments within each species. n.s.: no significant differences were found; n.c.: generalized mixed model failed to converge. Note that different y-axis scales are used for the debarked area.

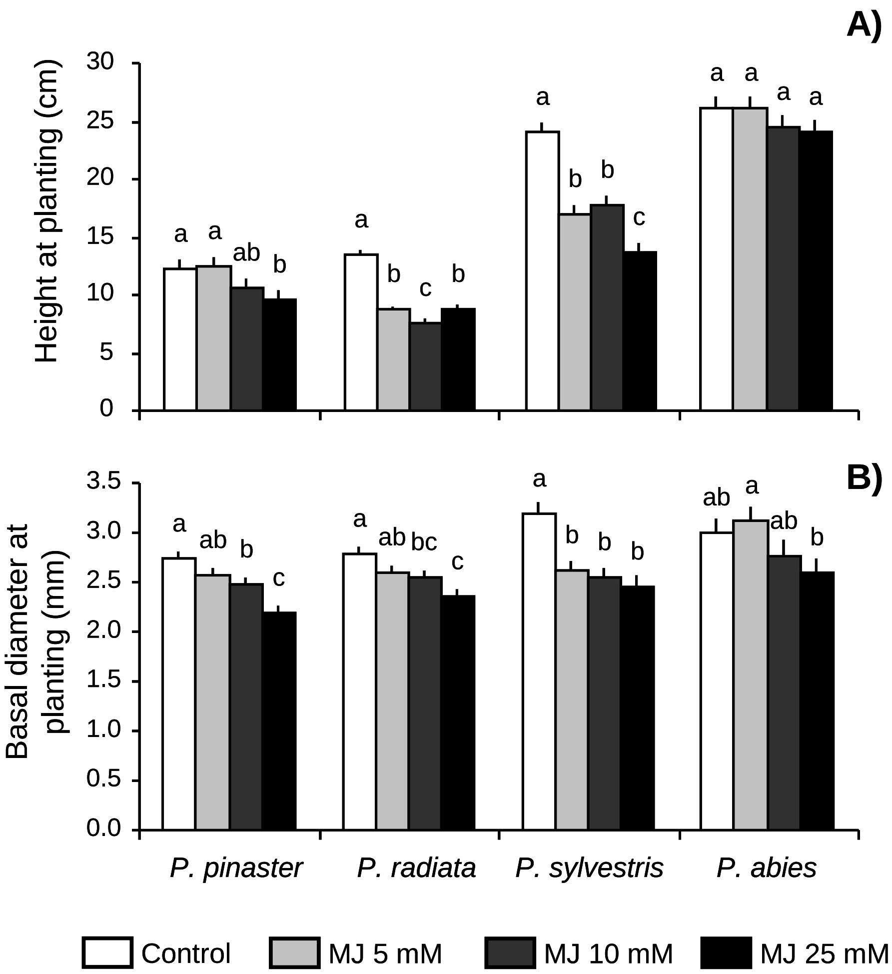

FIGURE C4. Total height (A) and basal stem diameter (B) at the time of planting of seedlings of four conifer species treated with different concentration of methyl jasmonate. All treatments were applied twice 4 and 2 weeks before measurements. Different letters above each bar indicate significant differences ($p < 0.05$) among MJ treatments within each species.

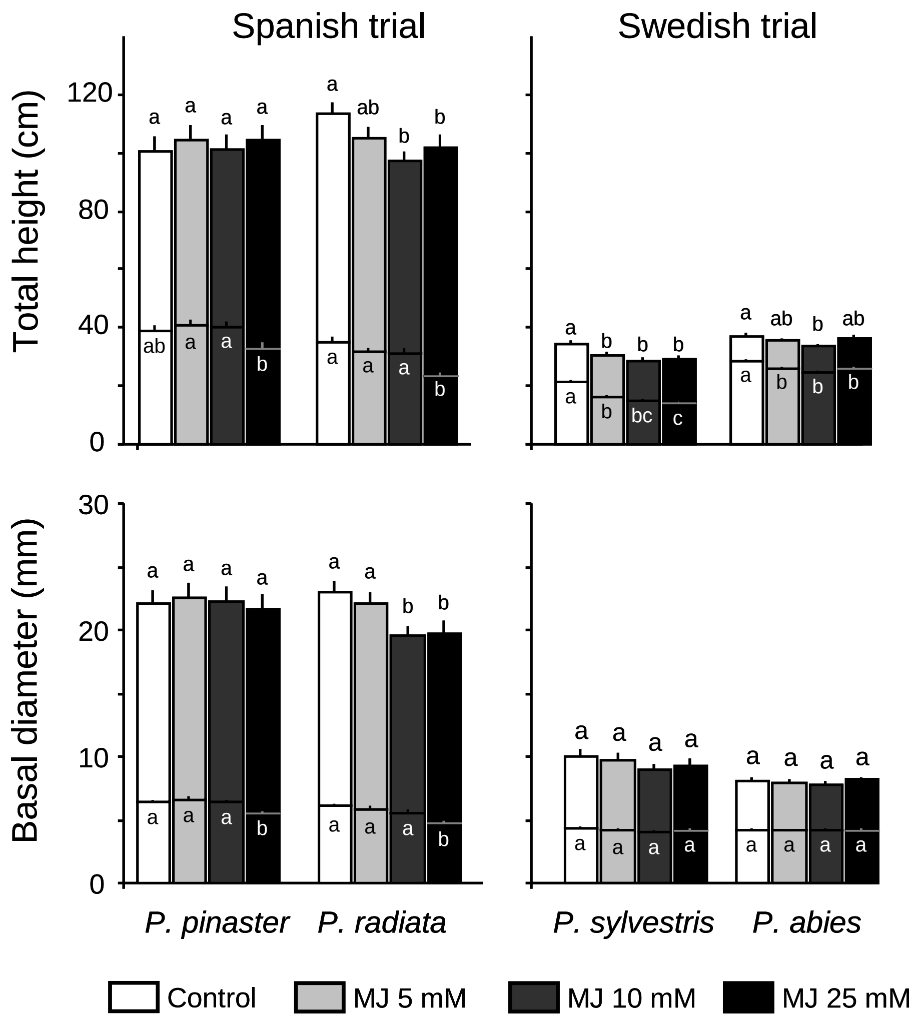

FIGURE C5. Effect of methyl jasmonate application (0 mM, 5 mM, 10 mM, 25 mM MJ) on height and basal diameter of four conifer species planted in two clear-cuts in Spain (*P. pinaster* and *P. radiata*, left panels) and Sweden (*P. sylvestris* and *P. abies*, right panels) naturally infested by the pine weevil (*H. abietis*) after the first (bottom part of the bars) and second (upper part of the bars) growing seasons after planting. All treatments were applied twice, 4 and 2 weeks before plantation. Different letters above each bar indicate significant differences ($p < 0.05$) among MJ treatments within each species and year. Least square means ± s.e.m (N = 80 seedlings).

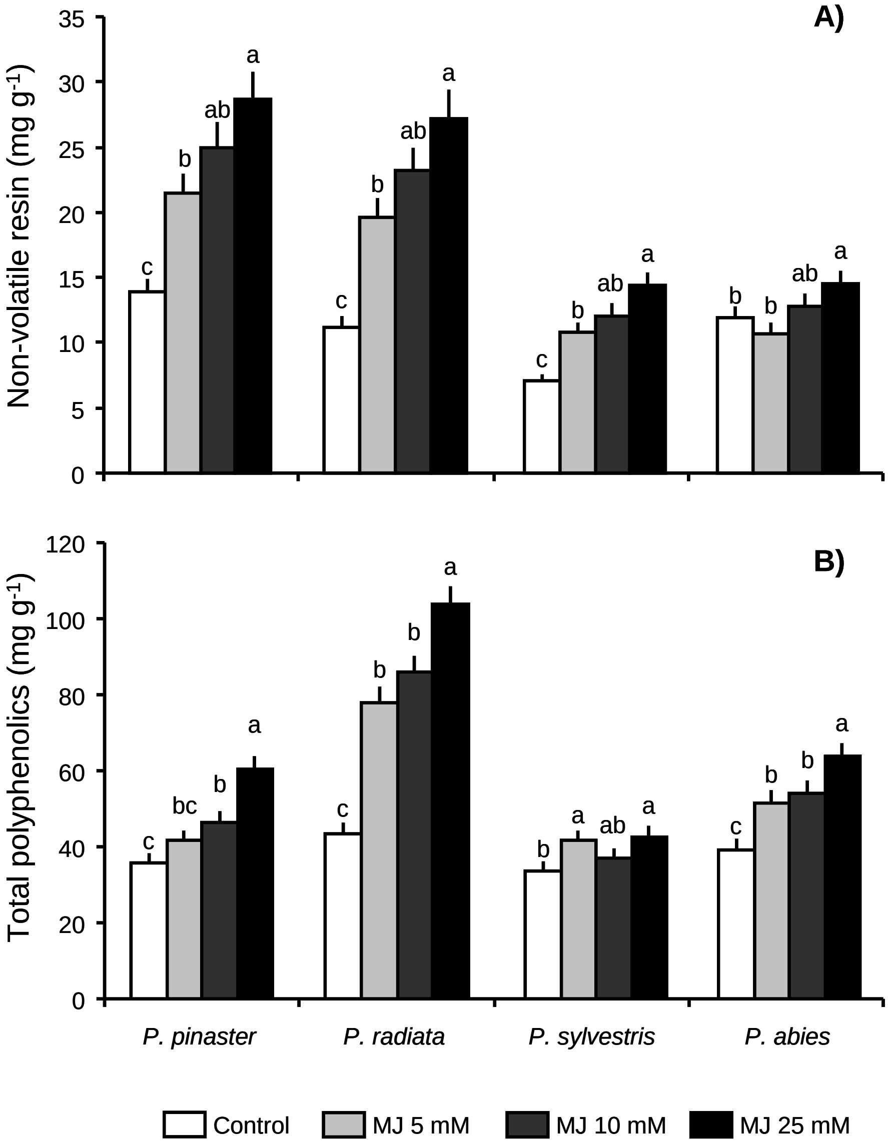

FIGURE C6. Effect of methyl jasmonate application (0, 5, 10 or 25 mM MJ) on major chemical defences in the needles. (A) Concentration of non-volatile resin and (B) total polyphenolics in the needles of seedlings of four conifer species. All treatments were applied twice, 7 and 5 weeks before sampling for chemical analyses. Least square means ± s.e. (N = 80 seedlings). Different letters above each bar indicate significant differences ($p < 0.05$) among MJ treatments within each species.