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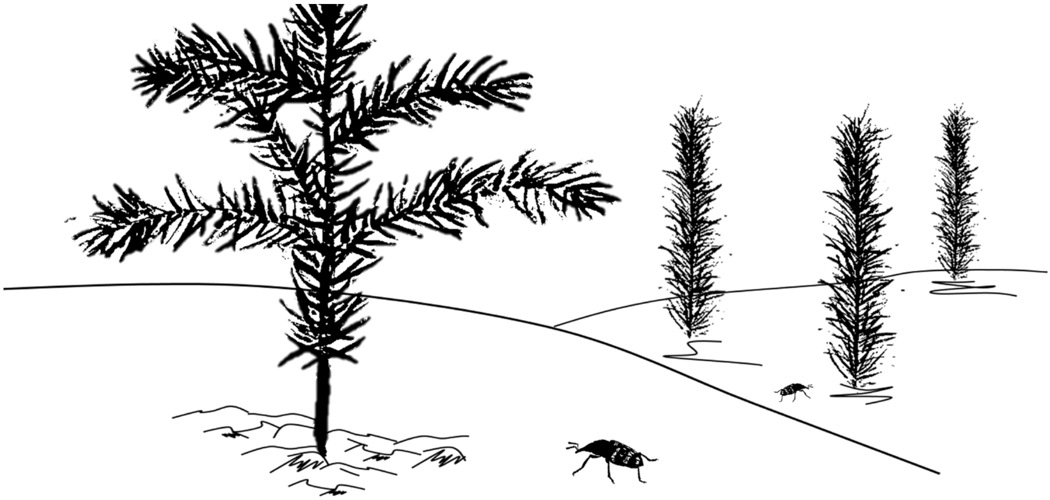
Yayuan Chen



Exploiting plant defenses to protect conifer seedlings against pine weevils

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Cover: Pine weevils in fields planted with Norway spruce and Scots pine seedlings
(Illustration: Yayuan Chen)

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Abstract

Sweden is one of the top five countries having large planted forest areas, and here the forests are maintained by clear-cut regime, which also promotes the pine weevil (*Hylobius abietis*), a major threat to Swedish planted coniferous regeneration. Given interest in developing sustainable forest management practices, efficient non-insecticide alternatives are highly demanded to tackle the pine weevil problem. In this thesis, I investigated several potential measures, based on plant defenses, aimed at reducing pine weevil damage to conifer seedlings. Methyl jasmonate (MeJA) is a plant hormone that can trigger plant induced defense/resistance, and reduce pest insect damage in conifers. I examined the compatibility of MeJA treatment with current plant nursery practices, and explored if inflicting mechanical damage can also trigger induced resistance. I also investigated if the timing of MeJA treatment will affect bark wound healing as a plant tolerance trait. I further examined genetic variation in resistance to weevil damage among different families from the Swedish breeding program, and resistance differences between two types of planting materials, seedlings and cuttings. The results confirmed that seedlings exhibit greater resistance to weevil damage when treated with MeJA, even if treatment is applied the previous growing season. In contrast, the evaluated mechanical damage did not trigger strong induced resistance in seedlings. Moreover, if MeJA treatment occurs after seedlings are wounded, healing of bark wounds can be negatively affected, but not if the treatment occurs before wounding. In addition, I found that seedling's resistance to pine weevil damage is significantly different among families, but the heritabilities of resistance traits were low and may constrain breeding for more resistant seedlings. When examining plant types, cuttings showed higher resistance to pine weevil damage than seedlings from the same family. Further studies should examine the interaction of MeJA-induced seedlings with abiotic and biotic factors under field conditions. The combination of induced resistance with constitutive resistance will also be necessary to fully exploit plant defenses in plant protection.

Keywords: induced defense, induced resistance, mechanical damage, simulated herbivory, wound healing, plant tolerance, cuttings, genetic resistance, genetic variation

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Utnyttjande av växtförsvar för skydd av barrträdsplantor mot snytbaggen

Abstract

Sverige är ett av de fem länder med mest planterad skog och den vanliga skötselmetoden är trakthyggesbruk, vilket gynnar snytbaggen (*Hylobius abietis*) som är ett stort hot mot nyplanterade barrträd. Dagens marknad kräver att råvarorna kommer från ett hållbart brukande av skogen. Således krävs effektiva alternativ till insekticider för att lösa problemet med snytbaggeskador. I avhandlingen undersöker jag flera, potentiella åtgärder som är baserade på växtens eget försvar och syftar till att minska snytbaggens skador på barrträdsplantor. Metyljasmonat (MeJA) är ett växthormon som kan inducera plantans motståndskraften (resistens) och minska skadedjurs angrepp på barrträd. Jag testade kompatibiliteten för MeJA-behandling med plantskolornas nuvarande odlingsmetoder och undersökte om olika typer av mekaniska skador också kan aktivera växtinducerat försvar. Vidare undersökte jag om tidpunkten för MeJA-behandling påverkar läkningen av barkskador, som är en växttoleransegenskap. Slutligen undersökte jag den genetiska variationen av resistens hos olika familjer och eventuella skillnader i resistens mellan plantor och sticklingar från samma familj. Resultaten indikerar att MeJA kan appliceras på plantor redan under föregående växtsäsong i plantskolan och ändå ge tillräckligt skydd mot snytbaggeskador följande växtsäsong. Mekaniska skador påverkade inte märkbart plantornas motståndskraft mot snytbaggen. MeJA-behandling som utfördes efter att plantan skadats minskade läkningshastigheten av skadan signifikant men inte om behandlingen utfördes före skadetillfället. Plantans motståndskraft mot snytbagge skiljer sig signifikant mellan familjer, men ärftligheten hos dessa resistensegenskaper är låg. Sticklingar visade högre motståndskraft än fröplantor från samma familj Ytterligare studier för att undersöka interaktionen mellan MeJA-inducerade plantor och abiotiska och biotiska faktorer under fältförhållanden kommer att vara väsentlig. Kombinationen av inducerat resistens och konstitutivt genetiskt motstånd kommer också att vara nödvändigt för att till fullo utnyttja växtens försvar i skyddet mot skadegörare.

Nyckelord: inducerat försvar, inducerad resistens/motståndskraft, mekanisk skada, simulerad herbivori, växtens tolerans, sårläkning, sticklingar, genetisk resistens/motståndskraft, genetisk variation

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Dedication

To those lively insects out in the nature and every inspiring teacher/supervisor that leads me all the way here.

"I leave no trace of wings in the air, but I am glad that I had my flight"
Rabindranath Tagore

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Chen, Y.**, Bylund, H., Björkman, C., Fedderwitz, F., Puentes, A. (2021). Seasonal timing and recurrence of methyl jasmonate treatment influence pine weevil damage to Norway spruce seedlings. *New Forests* 52, 431–448.

II. **Chen, Y.**, Puentes, A., Björkman, C., Brosset, A., Bylund, H. (2021). Comparing exogenous methods to induce plant-resistance against a bark-feeding insect. *Frontiers in Plant Science* 12: 1504

III. **Chen, Y.**, Björkman, C., Bylund, H., Puentes, A. Wound healing in the bark of Norway spruce seedlings: Effects of methyl jasmonate treatment on a plant tolerance trait (manuscript).

IV. Puentes, A., **Chen, Y.**, Bylund, H., Björklund, N., Högberg, K. A. Genetic variation and differences in resistance to pine weevil (*Hylobius abietis*) damage in Norway spruce (*Picea abies*) cuttings and seedlings (manuscript).

The contribution of Yayuan Chen to the papers included in this thesis was as follows:

I. First author. Performed the field and lab experiments. Developed the research questions and analysed the data. Led the writing with contributions from co-authors.

II. First author. Planed and performed the lab experiment. Developed the research questions and analysed the data. Led the writing with contributions from co-authors.

III. First author. Planed and performed the lab experiment. Developed the research questions and analysed the data. Led the writing with contributions from co-authors.

IV. Second author. Contributed to the experiment design. Performed the field experiment together with Puentes, A. Participated in writing that was led by Puentes, A.

List of figures

Abbreviations

FAO	The Food and Agriculture Organization
JA	Jasmonic acid
KSLA	Swedish: Kungl. Skogs- och Lantbruksakademien English: The Royal Swedish Agricultural Academy
MeJA	Methyl jasmonate
NFL	The Swedish National Forest Inventory
SA	Salicylic acid
UN DESA	United Nations, Department of Economic and Social Affairs

1. Introduction

The world's population reached 7.7 billion in mid-2019, following rises of one billion people since 2007 and two billion since 1994 (UN DESA, 2019). This has not only placed huge demands on food supplies, but also greatly increased needs for fuel, timber and other products of trees for other high-end uses (Hurley et al. 2017). About 420 million ha of forest (as defined in Box 1) has been lost worldwide due to deforestation since 1990, but the area of planted forest (also defined in Box 1) has increased by 123 million ha during this period (FAO 2020). These planted forests usually consist of species that grow rapidly, with favorable properties for timber or other uses (e.g., as sources of fuel, paper and cellulose), as well as providing protection against soil erosion and flooding (Turnbull 1999; Payn et al. 2015).

Thus, it is extremely important to maintain the health of planted forests to ensure desirable outcomes, and counter major threats, including damage caused by insect pests, which can severely reduce the productivity of plantation forests (also defined in Box 1). Insect pests is a term that usually

Box 1. Terms and definitions provided by FAO's Forest Resources Assessment working paper 188.

Forest: Land spanning more than 0.5 hectares with trees higher than 5 meters and a canopy cover of more than 10 percent, or trees able to reach these thresholds in situ. It does not include land that is predominantly under agricultural or urban land use.

Planted forest: Forest predominantly composed of trees established by planting and/or deliberate seedling.

Plantation forest: Planted forest that is intensively managed and meet ALL the following criteria at planting and stand maturity: one or two species, even age class, and regular spacing.

refers to insects that cause economic losses to cultivated plants (Liebhold 2012), including direct losses in dry matter and indirect losses through damage to seeds, wood or other products (García-Lara and Saldivar 2016). Hence, measures must be taken to prevent insect pests causing unacceptable reductions in qualities and quantities of forestry products (Steiro et al. 2020).

1.1 Brief description of Swedish plantation forests

Due to the highly appropriate natural conditions, 58% of the Swedish land area is covered by production forests and a further 11% by other forests (KSLA 2015, NFI 2021). Although this is less than 1% of the world's commercial forest area, forestry plays a major role in the Swedish economy and accounts for 10% of the timber, pulp and paper production in the global market (KSLA 2015, NFI 2021). The Swedish plantation forests are dominated by the coniferous species Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). The ability to provide massive amounts of wood products, is partly attributed to the forest management regime called clear-cutting.

This regime involves growing even-aged stands that are cut when they reach a certain age (60-90 years, depending on site conditions), after notifying the Swedish Forest Agency and receiving permission to cut the mature forest. Proven regeneration methods (planting, sowing or natural regeneration) must be applied within three years after clear-cutting (Skogstyrelsen 2020a). Sweden has the fifth largest area of planted forests (13.7 million ha) in the world, following China, the USA, Russian Federation and Canada according to data published in 2015 (Payn et al. 2015).

Planting with seedlings (young plants grown from seeds in nurseries) is the most efficient regeneration method. However, studies have shown that newly planted seedlings can be stressed by the change in growing condition (Nybakken et al. 2021) and are usually attacked more by insects than naturally regenerated seedlings (Selander et al. 1990). Seedlings are in a very fragile developmental stage, in which they are highly sensitive to damage by insect pests, but they are the only available propagated planting materials on the market (Skogstyrelsen 2020b). Thus, in cases where there are high risks of such damage, seedlings must be effectively protected to ensure successful regeneration and protective measures like physical barriers are attached/applied during their production in nurseries.

Cuttings, another type of propagated planting material, have higher pest resistance than seedlings but their use for large-scale forest regeneration is limited (Gemmel et al. 1991; Hannerz et al. 2002). Cuttings are propagated by cutting off current-year shoots from a mother tree then inserting them in a planting substrate until they form adventitious roots (Hannerz et al. 2002). Scots pine cuttings have poor apparent utility for large scale propagation as they have weak and erratic rooting responses (under conditions tested to date), so most fundamental research in Swedish forestry contexts has focused on Norway spruce cuttings (Högberg et al. 2011).

1.2 The pine weevil problem

Figure 1. A pine weevil (*Hylobius abietis*) feeding on the stem bark of a Norway spruce (*Picea abies*) seedling. Photo: Yayuan Chen.

The pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae, Fig. 1), is a major pest insect that causes large economic losses in forest regeneration. This problem is greatly exacerbated by the clear-cutting in Swedish plantation forests. Adult pine weevils use conifer stumps as breeding substrates and are attracted to clear-cut sites. The larvae develop in the fresh stump roots left on clear-cuts and feed on the root bark for two or three years until they become adult (Nordenhem 1989). Adult weevils can feed on the bark of twigs in the crowns of mature trees, root bark in the humus layer, or stem bark of young seedlings both above and below ground level (Örlander et al. 2000; Nordlander et al. 2005; Wallertz et al. 2006). If seedlings are planted during the first three years after clear-cutting (Fig. 2), their stem bark is a readily available food source for the parental generation and their adult offspring emerging from the soil (Nordenhem 1989). This feeding can cause deformation of seedlings, reduce their growth and even fatally damage them by girdling, i.e., entire ring of bark removed around the stem circumference, which disconnects the phloem above and below the wound (Leather et al. 1999). Mortality rates due to girdling may be up to 100% if seedlings are not protected at all (Eidmann et al. 1991; Von Sydow 1997; Örlander and Nilsson 1999; Nordlander et al. 2011). Replanting, with associated increases in regeneration costs, is needed in places where heavy damage has occurred. Since there are always clear-cuts in the Swedish forests, and pine weevils can not only migrate hundred kilometres to find new clear-cuts (Solbreck 1980) but also maintain a constantly high population, it is essential to take measures to prevent them causing high mortality in plantation forests.

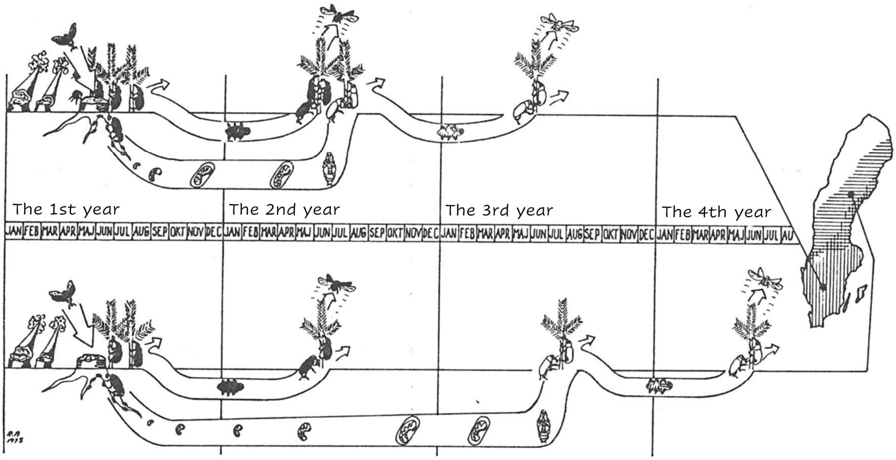

Figure 2. Sketch by Rune Axelsson (Eidmann et al. 1976) showing the life cycle of two generations of the pine weevil (*Hylobius abietis*) in regeneration forests, after clear-cutting in northern and southern parts of Sweden (shaded with horizontal and vertical lines, respectively). Weevils colored in black and white belong to the parental and second generations, respectively.

1.3 Current measures to reduce pine weevil damage

1.3.1 Silvicultural measures

Some silvicultural practices can mitigate the pine weevil damage, but all has its drawbacks. One is to delay planting until the pine weevils have left (>2 years after clear-cut but not longer than 3 years due to the regulation mentioned in section 1.2) (Långström 1982; Örlander and Nilsson 1999). But seedlings planted on old clear-cuts have to compete with more ground vegetation for water and nutrients (Nilsson and Örlander 1995; Örlander et al. 1996). Furthermore, rapid planting is desirable to maximize productivity, and minimize time until the next clear-cutting (Örlander and Nilsson 1999). Reducing the size of each clear-cut, scattering the clear-cuts and leaving shelter trees could also reduce pine weevil damage (Sundkvist 1994; von Sydow and Örlander 1994; Wilson and Day 1996; Nordlander et al. 2003). However, these practices make forest planning more complex, so forest managers prefer greater aggregation of clear-cuts. Various methods of site preparation can also reduce pine weevil damage, but the effects depend on site conditions (Wallertz et al. 2018; López-Villamor et al. 2019). Another option is to use larger seedlings as they less often fatally damaged by pine weevils (Lekander and Söderström 1969; Selander et al. 1990; Selander 1993), but they are more costly and usually produced less frequently in nurseries due to logistic challenges.

1.3.2 Insecticides

Application of insecticides has long been one of the most prevalent methods for reducing damage by insects (Eidmann et al. 1996; Nordlander et al. 2011; Örlander and Nilsson 1999; Von Sydow 1997). They usually have prolonged effects and can be easily applied pre-planting in nurseries as preventive agents or post-planting in stands to kill established pest insect populations (García-Lara and Saldivar 2016; Leather et al. 1999; Tudoran et al. 2019). However, insecticides are being phased out in forest pest insect management due to their negative effects on the environment and human health (Chen et al. 2021).

1.3.3 Physical barriers

Physical barriers are the main alternatives to insecticides for protecting seedlings from pine weevils (Nordlander et al. 2009). There are two main types: shields of plastic, paper or other materials around the stem; and coatings (e.g., sand, wax, latex or other materials) that form a protective layer on the stem bark (Petersson et al. 2004; Nordlander et al. 2009). One coating (of fine sand embedded in fixative, called Conniflex®) that has been promoted as an effective barrier (Nordlander et al. 2009) was developed by colleagues in the Forest Entomology unit, Dept. of Ecology, SLU. However, use of Conniflex is mainly restricted to southern and central Sweden where the pine weevil pressure is extremely high.

Despite improvements obtained from available countermeasures, singly or in combination, there is still strong demand from the forestry sector in Europe for alternative non-insecticide strategies that can meet two main goals. One is to replace insecticides where they were previously used. In Sweden alone more than 70 million seedlings (19% of the total number from plant nurseries) were still treated with insecticides in 2017. The other goal is to provide a non-insecticide option that can be used in areas where currently no plant protection measures are used but protection would substantially improve regeneration results (Nordlander and Hellqvist 2011; Nordlander et al. 2011).

1.4 Potential measures to reduce pine weevil damage: Utilization of plant defenses

Most currently applied protection methods depend in some way on external substances. A key question to address is *How can we maximize plants' potential defenses when they are under attack?* This could generate knowledge that enables development of sustainable plant protection strategies and provides insights into important traits that limit and deter insect pests and/or other herbivores (animals that eat plants).

Plant defenses can be classified into two categories: constitutive defense that is always present, and inducible defense that is either rapidly activated when plants perceive cues indicating potential attackers (induced defense) or activated in subsequent attacks after perception of initial cues (primed defense, is considered to be a special case of induced defense) (Wilkinson et al. 2019; Mageroy et al. 2020). Plant defenses include resistance traits that enable plants to prevent, stop or reduce damage inflicted by insects, and tolerance traits that enable plants to withstand and recover from the damage (Mitchell et al. 2016).

1.4.1 Induced defense (resistance)

Induced defense have known efficacy in reducing damage by aphids, spider mites, thrips and other insect pests to crops such as strawberry, grape, lima bean, soybean and tomato (Omer et al. 2000; Thaler et al. 2001; Choh et al. 2004; Warabieda et al. 2005; Selig et al. 2016). These effects have been achieved without causing physical damage by using the plant hormone methyl jasmonate (MeJA) to artificially trigger defense prior to insect attack (Chen et al. 2021). MeJA is a volatile derivative of jasmonic acid (JA) that higher plants may synthesise when they are wounded or stressed. MeJA mediates plant responses to both abiotic and biotic stresses, especially those involved in induced resistance to insects (McConn et al. 1997; Kahl et al. 2000; Miller et al. 2005). Application of MeJA activates production of defensive compounds and expression of resistance-related genes in plants, thereby reducing insect herbivory (Yu et al. 2019). Thus, it is considered as an effective method to protect plants against various crop pests (Stenberg 2017; Stella de Freitas et al. 2019).

MeJA's potential utility for protecting conifer seedlings from pine weevils is under investigation. MeJA (in solution) triggers production of traumatic resin channels, polyphenolic parenchyma cells (Krokene et al. 2008) in Norway spruce. It also increases total amounts of defensive compounds, including phenolic compounds, terpenes and resin in various coniferous species, including Norway spruce (Hudgins et al. 2004; Hudgins and Franceschi 2004; Heijari et al. 2005; Moreira et al. 2009; Zas et al. 2014). These changes in chemical traits are known to be effective induced defense responses following biotic attack or stem damage (Hudgins et al. 2004; Hudgins and Franceschi 2004; Franceschi et al. 2005). Furthermore, these MeJA-induced changes occur both locally and systemically throughout the plant (Huber et al. 2005; Moreira et al. 2009). MeJA treatment of coniferous seedlings can affect pine weevil feeding behaviour and reduce both overall damage and girdling rates, relative to those in untreated plants (Moreira et al. 2009; Fedderwitz et al. 2016). Moreover, these protective effects have been observed not only in the lab but also the field. For example, results from a previous field study showed that MeJA-mediated protection can decrease the mortality rate of Scots pine seedlings from 40 % to 8 % after two growing seasons (Zas et al. 2014).

Induced defense can be powerful, but the time lag between a plant's perception of a threat and full expression of the induced defense may be detrimental for the plant when the pest pressure is high (Karban 1987). This weakness of induced defense can be artificially circumvented by applying a plant defense activator, which triggers induce defense before exposure to attacks. In most previous studies on the efficacy of MeJA-induced resistance to pine weevil damage, treatments have been applied a few days or weeks before exposing plants to insects. However, if the treatments could be applied several months before seedlings were planted in the field, they could be compatible with seedling production practices in nurseries. In nurseries, seeds are sown in spring and continue growing until late autumn, then they are packed and stored in freezer rooms for a few months until they are planted in the following spring/summer. Thus, there are needs to test whether treatments applied before seedlings are packed for winter storage can sufficiently protect seedlings from pine weevil attacks when seedlings are planted in the field.

Another aspect that must be considered when using MeJA is that the efficacy of defense induction is dose-dependent, and amounts of MeJA required for desired defensive effects vary among species and plants with different properties (e.g., age and size). The MeJA dose is usually manipulated by using either the same concentration but different application frequencies (or volumes), or different concentrations but the same application frequency (or volume). For instance, applying a higher amount of MeJA solution can lead to stronger defensive responses, and older plants can withstand higher concentrations (Zeneli et al. 2006; Heijari et al. 2008), while overdoses can be toxic or even lethal for younger plants (Gould et al. 2009). The combined effects of dose and timing on defense induction efficacy have not been well explored in previous studies but require attention if we want to further develop the use of MeJA-induced defense in practice.

Although application of MeJA is one of the most common methods for triggering inducible defense against herbivory, methods involving damaging plant tissues can also trigger induced defense. Mechanical wounding to simulate herbivory and true insect herbivory have been shown to trigger induced defense-related responses in several plants, e.g., tobacco plant *Nicotiana sylvestris*, Brussels sprouts *Brassica oleracea* var.*gemmifera,* red pine *Pinus resinosa*, and the model plant *Arabidopsis thaliana* (Baldwin 1988; Mattiacci et al. 1994; Lombardero et al. 2006; Herde et al. 2013). Damaging roots can also reportedly increase levels of defense-related compounds, for example in *A. thaliana* and other members of the Brassicacea (Agerbirk et al. 2008). Several studies have also shown that simulated and true herbivory can stimulate chemical defensive responses in conifers (Miller et al. 2005; Moreira et al. 2012a). However, these studies did not include subsequent tests of the insect resistance induced, although assessments of their efficacy (e.g., in insect feeding tests) relative to use of MeJA are warranted partly because machines could be used to damage tissue in seedling producing lines.

Trade-off between induced resistance and plant growth traits

Besides its protective effects, it has been shown that MeJA application can negatively affect plant growth (Heijari et al. 2005; Gould et al. 2009), at least partly, because when defense is triggered by MeJA resources may be allocated to synthesis of defensive compounds and diverted away from growth. However, this growth reduction is not necessarily a hindrance for using MeJA to protect conifer seedlings. In fact, a recent study has shown that this short-term growth reduction could be exploited by plant nurseries to stop growth when seedlings reach a certain height (Fedderwitz et al. 2019). Moreover, in the long term the lower mortality of MeJA-treated seedlings, relative to untreated seedlings, may more than compensate for the temporary reduction in growth rate (Gould et al. 2008; Zas et al. 2014). Thus, monitoring the growth of seedlings subjected to different MeJA treatments is needed to acquire more information of the mechanisms underlying responses to them.

1.4.2 Constitutive genetic resistance

Recently, a Sitka spruce *Picea sitchensis* breeding program identified genotypes with the highest resistance among those tested against a major pest insect, the white pine weevil, *Pissodes strobi* (Alfaro and King 2012). This has allowed the return of Sitka spruce in areas of coastal British Columbia where the damage was previously so severe that planting it was not recommended (Alfaro and Fuentealba 2015). Another study on maritime pine *Pinus pinaster* found genetic variation in several inducible defense traits (Moreira et al. 2012b). Moreover, significant genetic variation in resistance to pine weevils (in terms of both probability of attack and total debarked area) has also been reported in Norway spruce (Zas et al. 2017; Puentes et al. 2018). These encouraging results indicate that exploitation of variation in genetic resistance can contribute to integrated pest management methods against this pest. There are ongoing breeding programs for Norway spruce, Scots pine and other conifer species in Scandinavia and Finland, primarily aiming to enhance growth and wood quality. Thus, screening for genetic families that have high resistance to pine weevil damage and quantifying the heritabilities of resistance traits using currently available genetically improved plants, is the first step towards utilizing genetic materials in pest insect control. Cuttings, as potentially alternative plant material, have stable heritable traits if they are propagated from the same mother tree. Studies with Norway spruce cuttings obtained from open-pollinated progenies in a Swedish seed orchard showed that they had higher resistance to pine weevil damage than seedlings (Hannerz et al. 2002), but these cuttings and seedlings did not have the same origin. Thus, studies of resistance to pine weevil damage in seedlings and cuttings of the same genetic origin are required to improve understanding of the mechanisms underlying differences between seedlings and cuttings.

1.4.3 Plant tolerance

Plants' tolerance traits have been studied less than their resistance traits (Mitchell et al. 2016; Peterson et al. 2017). The most common way of evaluating tolerance is to compare the growth of plants before and after herbivory (Mitchell et al. 2016). However, although growth after herbivory is usually a suitable proxy for tolerance, it is also important to understand other traits that are closely related to plant survival. For woody plants, one of the most important tolerance traits is the ability to heal bark injuries or wounds to overcome damage caused by insects, fire, and trampling (Chano et al. 2015). For younger woody plants, stem bark wounds caused by insect feeding can damage vascular tissue, interrupt nutrient and water transport, and/or facilitate infection by pathogens (Klepzig et al. 1991; Cabane et al. 2012; Bansal et al. 2013; Savatin et al. 2014). Thus, it is essential for plants to close these wounds as fast as possible to minimize the wounds' negative impact on their survival (Cabane et al. 2012). Wound closure in conifers has been examined in some studies, mainly focusing on wound closure rate (Filip et al. 1995; Han et al. 2000; Schneuwly-Bollschweiler and Schneuwly 2012; Jones et al. 2019), but the conifers were mostly more than 20 years old and some of their physiological characteristics differed from those of seedlings, which are usually less than 3 years old. Sparse studies on conifer seedlings have revealed that their early responses to wounding include abundant resin production, and closure occurs from the remaining vascular cambium at the margins of the wound (Chano et al. 2015; Chano et al. 2017). However, the wound healing rates of conifer seedlings and factors that enhance or diminish wound closure have been little explored. As plants require resources to recover from wounds and limited available resources must be used for diverse physiological processes, it is assumed that there is a trade-off between plant resistance and tolerance to herbivory (Herms and Mattson 1992). Moreover, as seedlings treated with MeJA will also be wounded by pine weevils in the field, it is essential to examine how MeJA affects wound healing processes.

2. Aims

The aims of this thesis were to investigate the potential utility of non-pesticide methods based on plant defenses in pest control during forest regeneration and to develop practical strategies for protecting conifer seedlings from damage by pine weevils. In particular, this thesis aimed to understand plant-induced resistance mediated by the plant hormone methyl jasmonate (MeJA) and its consequences for other plant traits, as well as examining the variation in resistance to pine weevil damage in the Swedish breeding population of Norway spruce. The following specific objectives were addressed in different papers including field and lab experiments (Fig. 3):

1). Investigate the factors that affect the efficacy of induced resistance (**paper I and II**).

2). Examine the trade-off between plant resistance and growth (**paper I, II and IV**).

3). Examine the trade-off between plant resistance and tolerance to herbivory (**paper III**).

4) To quantify the extent of genetic variation in resistance to the pine weevil damage (**paper IV**)

The following research questions were addressed respectively in four papers:

- How will seasonal timing and repetition of MeJA treatment in subsequent years, and seedling age, affect pine weevil damage? (**paper I**)
- Can other methods (especially mechanical damage) trigger plant induced resistance to the same extent as MeJA, and how will these methods affect resistance to pine weevil damage and plant growth? (**paper II**)

- How will a tolerance trait (wound healing) be affected by the timing of MeJA treatment and is tolerance potentially correlated with pest resistance (based on the pine weevil damage assessment in **paper IV**)? (**paper III**)
- Is there genetic variation in resistance to pine weevils among families from the Swedish Norway spruce breeding program, and are pine weevil damage levels affected by the type of plant propagation with the same family origin? (**paper IV**).

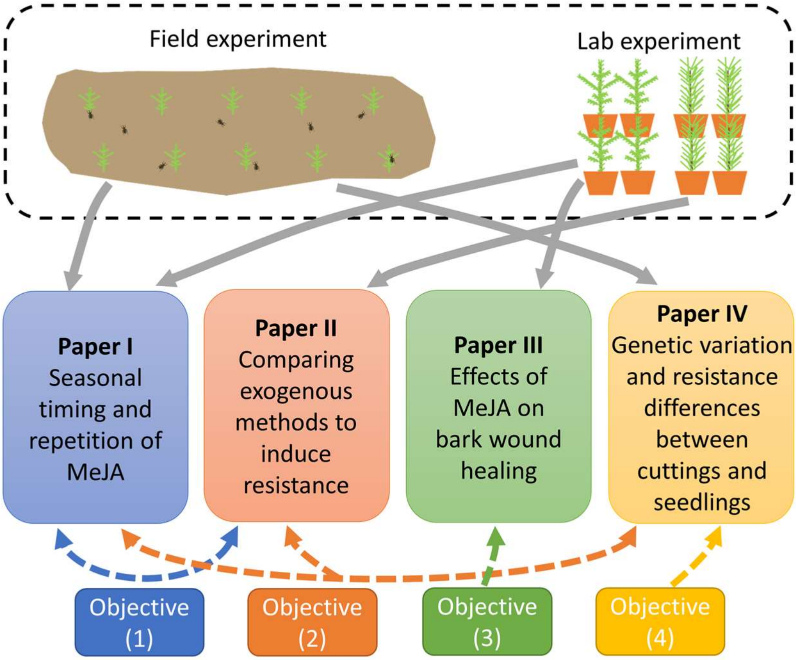

Figure 3. Scheme of conducted experiments and objectives of the thesis, and objectives of the thesis in relation to the four different papers. Illustration: Yayuan Chen

3. Materials and Methods

All the experiments included in this thesis were conducted in Sweden. The lab experiments were conducted in Uppsala, and field experiments in Tierp and Remningstorp. **Paper I** and **II** focused on effects of timing, frequency and defense induction methods on induced resistance to pine weevil damage; **paper III** focused on plant tolerance and effects of MeJA on tolerance traits; **paper IV** focused on genetic variation in resistance among different full-sib families and the difference in resistance between seedlings and cuttings from the same full-sib families.

3.1 Plant and insect materials

3.1.1 Plant materials

Norway spruce and Scots pine were used as plant materials to study plant defenses. Norway spruce seedlings were used in **paper I**, **III** and **IV**; in **paper II** we used Scots pine seedlings; Norway spruce cuttings were also used in **paper IV**. The Norway spruce seedlings used in **paper I** had been treated in another study by our research group (Fedderwitz et al. 2019). Scots pine seedlings in **paper II** were of commercial standard from nurseries. Norway spruce seedlings and cuttings used in **paper III** and **IV** were propagated specifically for testing genetic variation in the Swedish breeding populations.

3.1.2 Insect materials

Pine weevils were used in no-choice feeding tests in **paper I** and **II** to estimate the induced resistance of Norway spruce and Scots pine seedlings, respectively. The weevils were all collected from a sawmill (Balungstrands

Sågverk AB) located in Enviken, Sweden, in June 2017 and May 2020 during the swarming period. In **paper IV**, pine weevil resistance was estimated by assessing feeding damage caused by weevils naturally inhabiting the planting site.

3.2 Methods to trigger induced resistance

The main elicitor used in our studies of induced resistance in conifer seedlings was the plant hormone MeJA (**papers I**, **II** and **III**), but we also studied resistance induced by other elicitors (mostly mechanical damage) to compare their induction efficacy with the MeJA treatments (**paper II**).

3.2.1 Induction with MeJA

MeJA (Sigma-Aldrich 95%, Ref. No. 392707) was diluted in a carrier solution of 2.5% (v:v) ethanol to 10 mM (**paper I**, **II** and **III**) or 15 mM (**paper II**). The MeJA solution was sprayed on conifer seedlings with a Free-Syringe PC 1.5 litre plastic spraying bottle (Jape Products AB, Hässleholm, Sweden) in a fume hood. The nozzle was placed at an appropriate distance for the solution to cover the entire seedling in each pot. Before spraying, the bottle was pumped to reach the 2.5 bar inner air pressure limit so the bottle sprayed the same amount of solution in every unit time. When spraying, the bottle was moved at an even speed so that each seedling was sprayed for one second, then they were sprayed again in the same fashion after turning them 180 degrees. MeJA treated seedlings were left in room temperature for 24h before they were placed with other seedlings that was not treated by MeJA.

3.2.2 Induction with other methods (**paper II**)

Methods that cause damage to plants can also trigger induced defense. Scots pine seedlings were damaged by scalpel, insect-mounting needles and true pine weevil herbivory (Fig. 4, for details see **paper II**). Different amount of bark tissue loss (large versus small wounds on the stem) and location of the damage (on the stem versus on the main root close to the soil surface) were inflicted by scalpel. Insect-mounting needles were used to pierce the stem bark to obtain a different wound pattern from those that the bark tissue was removed by scalpel or true pine weevil herbivory.

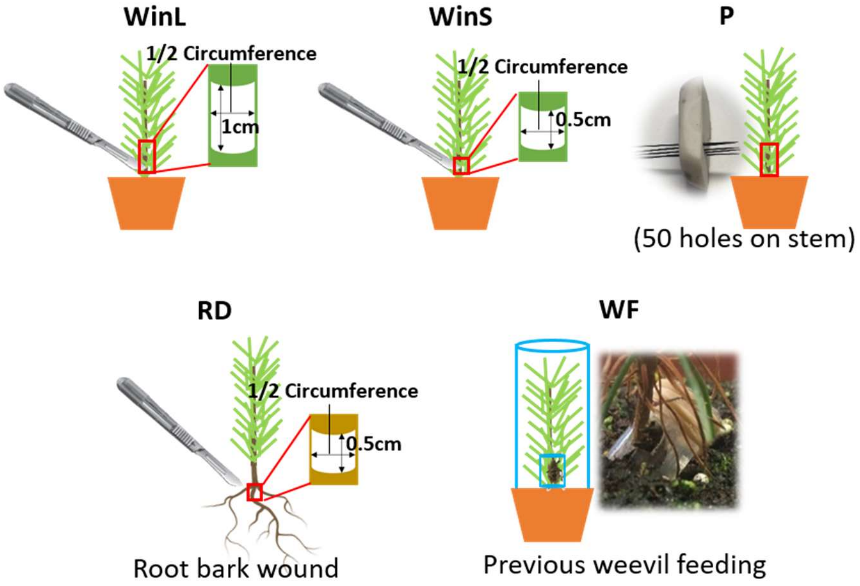

Figure 4. Plant induction methods by inflicting damage on seedlings: large stem window damage (WinL), small stem window damage (WinS), needle-piercing damage to the stem bark (P), root bark damage (RD), and previous weevil feeding damage (WF). Photo and illustration: Yayuan Chen

3.3 Estimation of resistance to pine weevils

Damage severity following seedlings' exposure to pine weevils was used as an inverse indicator of resistance, i.e., treated seedlings that were less damaged than controls were regarded as having higher induced resistance and vice versa. Seedlings were exposed to pine weevils either in the lab (**paper II**, due to limited sample size), in the field (**paper IV**), or in both venues (**paper I**).

3.3.1 Pine weevil exposure in the lab (**paper I** and **II**)

A transparent plastic cylinder (Fig. 5a) was placed in the soil of each potted seedlings, and a pine weevil was allowed to feed freely on the seedling in the cylinder for 24 h. Each seedling was exposed to one female pine weevil. Then the area debarked by pine weevils was recorded to estimate the resistance induced by the applied treatments. In **paper I**, the tested treatments all involved MeJA applications, but at different seasonal timings and with or without repetition of the treatment in the next growing season. In **paper II** the aim was to compare the resistance induction efficacy of MeJA and other treatments involving inflicting damage with different intensities, patterns and locations on seedlings.

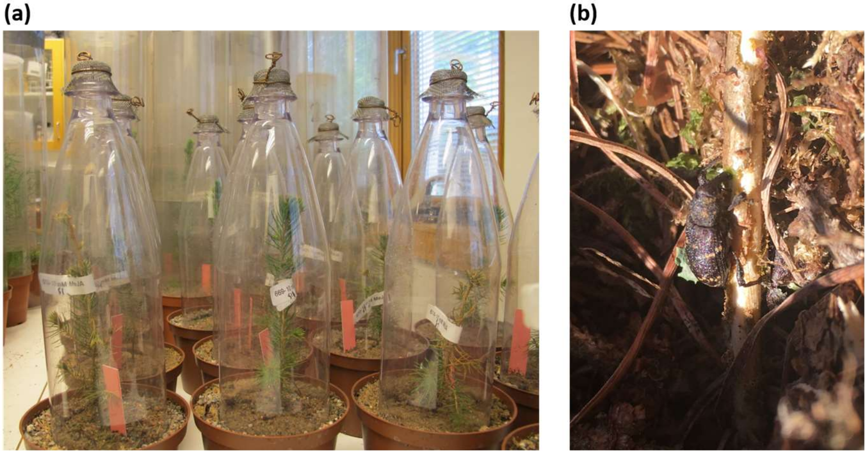

Figure 5. Pictures showing pine weevil (*Hylobius abietis*) exposure: **a**) in the lab using transparent cylinders, each enclosing one female pine weevil on a conifer seedling; **b**) in the field where seedlings were openly exposed to natural pine weevil populations. Photos: Yayuan Chen

3.3.2 Pine weevil exposure in the field (**paper I** and **IV**)

Seedlings were planted in clear-cuts where naturally occurring pine weevils could feed freely on them (Fig 5b). In the field, pine weevil damage was estimated in terms not only of debarked area, but also attack rate (whether or not a plant was attacked by weevils) and girdle rate (whether or not a plant was ring-debarked) and survival (whether or not a plant survived). Several assessments were conducted to observe changes in damage over time as weevil feeding does not occur in a single short period, as in the lab. In the field experiment for **paper I**, located close to Tierp, central Sweden (60°21’ N, 17°26’ E), 330 seedlings were distributed in 33 blocks. Pine weevil damage was assessed in both the planting year September 2017 and following year June 2018. This allowed observation of long-term (two years) effects of the MeJA treatments with different timings and the necessity of repetition of the treatments for effective induction of resistance. Another field experiment was set up for **paper IV**, in Remningstorp, southern Sweden (58°28’ N, 13°34’ E). In total 2050 plants (seedlings and cuttings) were planted. Two assessments were conducted in June and September 2018. No follow-up assessment was conducted in the following year as very few plants were still alive at the time of the second assessment in September 2018. In this experiment we were particularly interested in examining overall levels of genetic variation of seedlings from different full-sib families, and whether the propagation method (seedlings vs cuttings from the same family) affected pine weevil damage.

3.4 Estimation of plant tolerance (**paper III**)

To observe the wound healing of seedlings, two wounds were inflicted at different times: one before and one after MeJA treatment (see **paper III** for details). The wound healing rate was calculated to estimate the seedlings' tolerance to damage and determine whether it was affected by the MeJA treatment and its timing (before or after wounding). Wound healing rate roughly described the extent of newly formed bark per day, it is the amount of healed bark (final measurement of the exposed xylem subtracted from the initial measurement) divided by the number of experimental days.

3.5 Estimation of plant growth

When evaluating plant protection measures, it is important to also consider their potential effects on growth. In all papers, seedlings' heights above ground were measured to estimate their growth under the applied treatments and/or differences in growth between different plant types. In addition, the stem basal diameter of all seedlings used in lab experiments (**paper I, II and III**) were measured, and in the field experiments (**paper I and IV**) we measured diameters of randomly selected individuals representing each combination of treatment and plant type.

4. Results and Discussion

All MeJA treated seedlings showed reduced (yet not always statistically significant) pine weevil damage (debarked area) compared to control seedlings without defense induction. However, the MeJA triggered induced resistance was affected by timing of the treatment and the dose of MeJA applied to seedlings (**paper I and II**). Moreover, some of tested non-MeJA induction methods did not enhance seedlings' resistance to pine weevils, and the induction efficacy depended on how long the seedlings were 'rested' before exposure to pine weevils (**paper II**). The results also showed that MeJA affects the healing of seedlings' wounds, and the effects varied depending on whether MeJA was applied before or after wounding (**paper III**). In addition, growth rates of seedlings subjected to all MeJA treatments were lower than those of non-MeJA treated controls, albeit not significantly lower in some cases (**papers I**, **II** and **III**). Seedlings from different full-sib families showed statistically significant differences in resistance to pine weevils, but the estimated heritabilities of these resistance traits were low. There were differences in pine weevil damage between plant types; cuttings were more resistant to pine weevils than seedlings from the same family, in terms not only of area debarked, but also attack rate, girdle rate, and relative damage (**paper IV**).

4.1 Seasonal timing and repetition of MeJA treatment affect induced resistance (**paper I**)

Our results indicated that the best time to apply MeJA treatment is before winter storage (designated M1), as it offered the best protection to seedlings and an additional application the following year (designated M1+M2) did not improve protection (Fig. 6). A previous study examining MeJA treatment at one and two occasions found that recurrent treatment provided better protection against pine weevils than single treatment (Zas et al. 2014). These results were contrary to our study; however, they used a shorter time lag between their recurrent treatments (4 and 2 weeks before exposure to pine weevils). Similarly, another study found that repetition of MeJA treatment did not provide greater resistance to the pathogen *Diplodia pinea* (Gould et al. 2009). Our results suggest that MeJA application almost a year before seedlings are exposed to pine weevils could provide good protection. To enhance and maintain seedling resistance against pine weevil feeding, the treatment does not need to be recurrent across years. These results indicate that application of MeJA in nurseries could potentially be an effective plant protection approach.

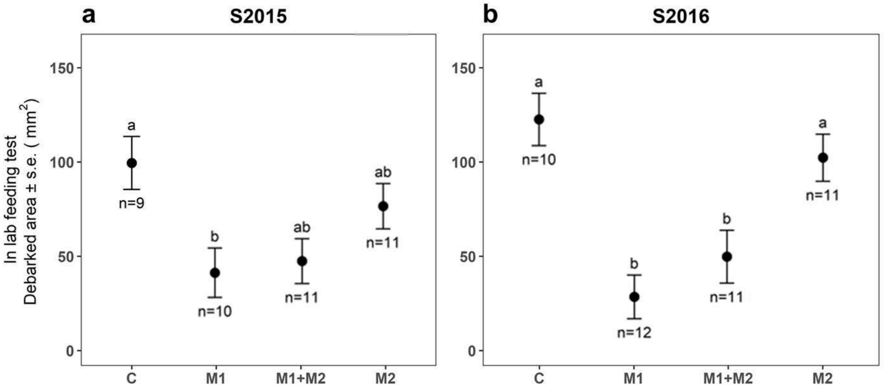

Figure 6. Estimated mean stem area (mm^2) (± standard error) debarked by pine weevils (*Hylobius abietis*) for Norway spruce (*Picea abies*) seedlings belonging to two age cohorts sown in 2015 (S2015; panels **a**) or in 2016 (S2016; panels **b**). Seedlings were treated with methyl jasmonate (MeJA) before winter storage (M1), after (M2), or both before and after (M1 + M2); controls (C) received no MeJA application at all. Different letters indicate significant differences among treatments at the 5% level (Tukey–Kramer tests). Sample sizes (n) varied as some seedlings died during the experiment.

4.2 The dose of MeJA affects induced resistance (**paper I and II**)

Previous studies have shown that the induced resistance triggered by MeJA is positively dose-dependent; increasing the MeJA concentrations in solutions sprayed at a given frequency increases the induced seedlings' chemical responses and reduces subsequent pine weevil damage (Moreira et al. 2009; Zas et al. 2014). Using the same concentration but repeating MeJA treatment also results in higher production of defensive chemical compounds and less pine weevil damage (Zas et al. 2014). The MeJA treatments applied in the thesis resulted in different doses of MeJA. Treatments in **paper I** used a MeJA solution of the same concentration, but different volumes (decreasing in the order M1 + M2 > M1 > M2). In **paper II**, two concentrations of MeJA were used and seedlings assigned to them were sprayed with the same volume of solution. The treatment involving the lowest volume (M2, one spraying) in **paper I** resulted in more (not all statistically significant) pine weevil damage, indicating weaker resistance, than the other two MeJA treatments (Fig. 6). The treatment with the lower concentration of MeJA in **paper II** also led to more (albeit not statistically significant) pine weevil damage than the treatments with the higher concentration (Fig. 7). However, in contrast to other studies, seedlings that were damaged least by pine weevils were not those sprayed with the highest volume of MeJA (M1 + M2) but those sprayed with the second highest volume (M1). This suggests that the timing of MeJA treatment may be more important than the dose for effective protection against the pine weevil.

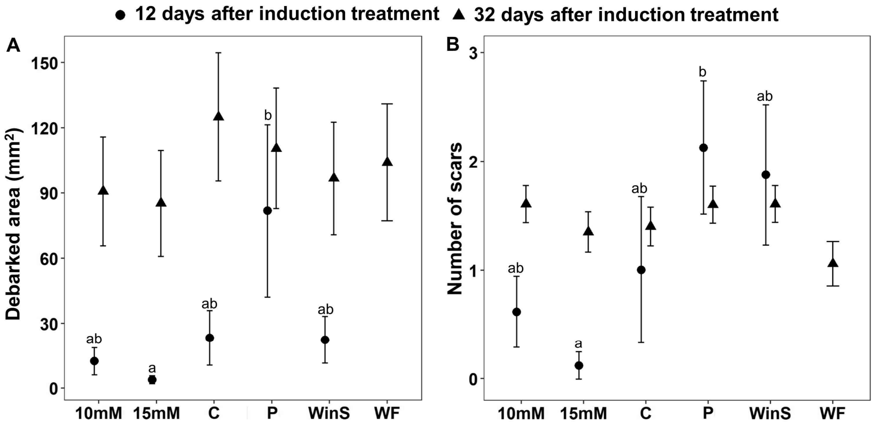

Figure 7. Pine weevil (*Hylobius abietis*) damage to Scots pine (*Pinus sylvestris*) seedlings after receiving different induction treatments [10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF, not included in the weevil exposure test conducted 12 days post-treatment)]. Plants that were exposed to pine weevils 12 days after induction treatments are referred to as early exposure, and those that were exposed 32 days after, are referred to as late exposure. **A)** Estimated mean debarked area (mm^2 ± standard error), and **B)** Estimated mean number of feeding scars (±standard error). Sample size (n) is equal to 8 for all other treatments, except that the

Control group (C) is equal to 7 for the early exposure, and n = 12 for all treatments for the late exposure. Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates.

4.3 Induction methods affect induced resistance (**paper II**)

Seedlings subjected to bark damage treatments did not show significantly greater resistance than MeJA-treated or control seedlings (Figs. 7 and 8), although induced defensive responses have been observed in studies on other conifer plants after both insect herbivory and mechanical wounding (Miller et al. 2005; Moreira et al. 2012c). Our explanation is that defensive responses must exceed a certain threshold to deter pine weevil feeding. Thus, to investigate the protective efficacy of any defense induction method it is essential to expose plants to pest insects after the induction stimulus. Although the induction methods we applied did not show great potential for large-scale implementation to protect conifer seedlings, there might be several reasons why they did not work well.

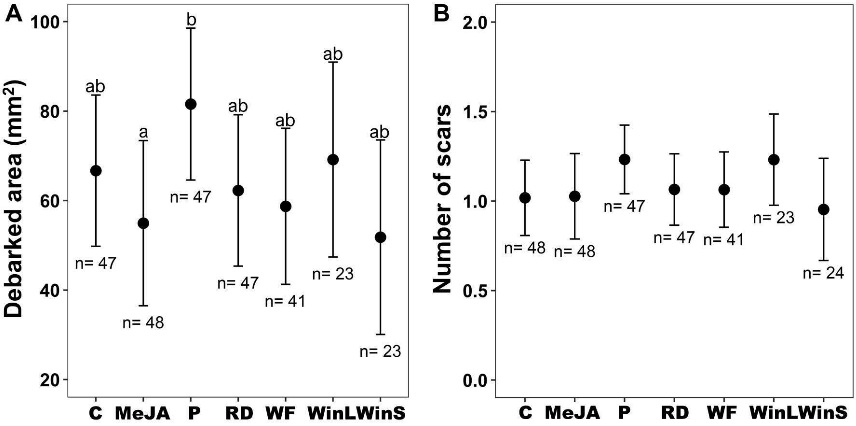

Figure 8. Pine weevil (*Hylobius abietis*) damage to Scots pine (*Pinus sylvestris*) seedlings in experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment) after receiving different induction treatments [Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)]. **A)** Estimated mean debarked area (±95% confidence intervals), and **B)** Estimated mean number of feeding scars (±95% confidence intervals) for each treatment. Sample sizes (n) used in the statistical analyses are also shown. Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates. Note that the y-axis does not start at 0 for **A)**.

First, the needle-piercing treatment we applied increased subsequent feeding by pine weevils to very high levels, relative to all other treatments, possibly because they are attracted by the odours or compounds emitted from recently damaged seedlings (Nordlander 1991). In contrast, the other two stem bark damage treatments (WinS and WinL), which could also potentially induce emission of attractive odours or compounds from recent wounds, did not lead to increased pine weevil damage (Fig. 8a), possibly because patterns of damage also influence weevil feeding behaviour.

The extent of tissue loss might play a role in this. In our study, seedlings subjected to the small window wound (WinS) treatment were damaged by pine weevil feeding to a similar extent to those subjected to the MeJA treatment, or even 25.1% less (although this was a non-significant difference at the $P < 0.05$ level) than those subjected to the larger window wound treatment (WinL) (Fig. 8). Another study found that moderate and severe mechanical stem damage resulted in significant reductions in the photosynthesis rates, needle masses and needle areas of 1-year-old Scots pine seedlings relative to undamaged controls (Bansal et al. 2013). Moreover, a study of tissue loss of tobacco leaves (*Nicotiana tabacum*) found higher levels of resistance traits in plants subjected to whole leaf removal rather than partial leaf removal (Lin and Felton 2020). Similarly, we found that seedlings subjected to the root damage treatment were damaged less by weevils than controls, albeit non-significantly at the $P < 0.05$ level (7% less in terms of debarked area). We also suspect that there was too little tissue loss in the roots to trigger greater resistance to weevils in the aboveground tissues. Evaluation of a broader range of stem damage levels may be needed to elucidate effects of the extent of tissue loss on induced resistance.

We also found that the effect of different induction methods on triggering seedling resistance to pine weevils varied depending on the time since treatment. Plants exposed earlier to pine weevils (12 days after treatment) received less pine weevil feeding compared to those in the late exposure group (32 days after treatment) (Fig. 7). This could indicate that the treatment effects were short-lasting and tended to lose their efficacy gradually. On the other hand, wounds from needle piercing had healed by the late exposure and seedlings then received less pine weevil feeding (11.6% in debarked area albeit non-statistically significant) than control seedlings. This suggests that the cues emitted by freshly damaged bark tissue decrease with time and seedlings became less attractive to pine weevils.

4.4 Side effect of induced resistance

4.4.1 Trade-off between induced resistance and plant growth

All MeJA treatments in the thesis reduced plant growth, albeit not always significantly at the $P < 0.05$ level (**papers I** and **II**). In **paper I,** the negative effect of MeJA became evident shortly after the recent MeJA treatments (M1 + M2 and M2). Over a longer period, the negative effect of MeJA was less prominent for seedlings that had received a lower dose of MeJA (M2 in **paper I** and 10 mM in **paper II**). Thus, significant growth reduction was not always accompanied by greater induced defense, suggesting that resources were diverted away from aboveground growth and presumably invested in defense, but the diversion only resulted in a slight reduction in area debarked relative to seedlings in the control group. After the MeJA-treated plants have recovered from the temporary growth loss, the differences in growth, relative to controls, are expected to gradually diminish (Zas et al. 2014).

Some of the non-MeJA treatments also resulted in different relationships between growth and induced resistance in **paper II**. There were substantial levels of pine weevil feeding following the piercing (P) and large window (WinL) treatments, but the seedlings’ growth was not much impaired, and their diameter even tended to increase more rapidly than controls. Significantly more radial growth of Scots pine seedlings with stem bark damage than undamaged controls was also observed in another study (Bansal et al. 2013). Seedlings with root damage in our study also showed a tendency to grow more in height than controls, and Godfrey et al. (1993) found that a root damage treatment resulted in maize (*Zea mays*) plants having more active photosynthetic tissue and gaining more dry weight than controls without root damage. In conclusion, some non-MeJA induction methods might ‘prime’ seedling defense, which is less costly than fully expressing induced defense (Wilkinson et al. 2019). Indications of this were observed following the late exposure, as seedlings subjected to non-MeJA induction treatments were slightly less debarked by pine weevils than controls (Fig. 8), while their growth increment was not negatively affected. However, in our study we could not discern which of these mechanisms were involved.

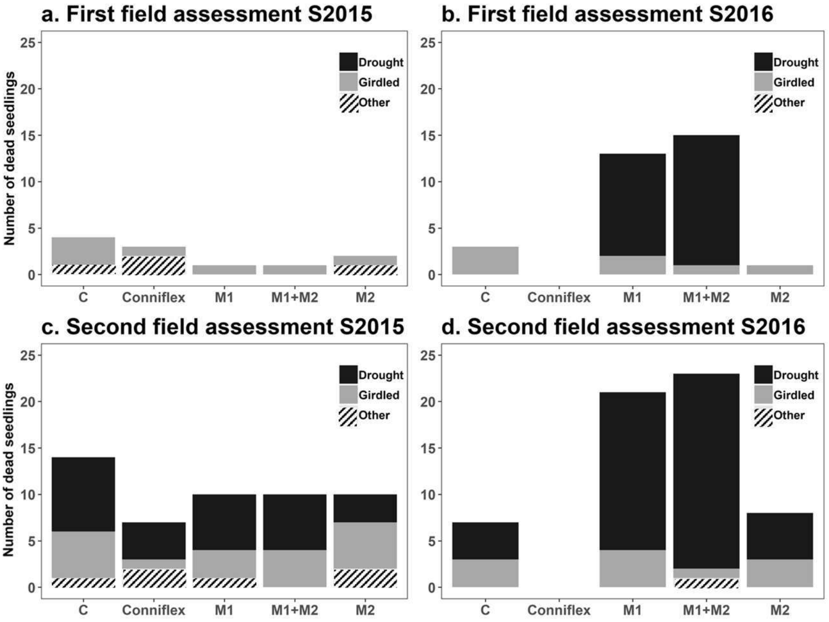

Figure 9. Mortality (number of dead seedlings) of Norway spruce (*Picea abies*) seedlings belonging to two age cohorts [sown in 2015 (S2015) or 2016 (S2016)] during two field assessments of pine weevil (*Hylobius abietis*) damage. A total of 33 seedlings for each treatment and age cohort (S2015: panels **a** and **c**; S2016: panels **b** and **d**) were planted in the field (panels a and b, first assessment of damage in Sept. 2017; panels c and d, second assessment of damage in June 2018). Seedlings were treated with methyl jasmonate (MeJA) before winter storage (M1), after (M2), or both before and after (M1 + M2), controls (C) received no MeJA application at all. Seedlings with the physical barrier Conniflex were included as positive controls. We categorized plants according to several possible causes of death: "Drought" included dead seedlings that appeared to have dried out but showed no signs of girdling; "Girdled" included dead seedlings for which an entire ring of barked had been removed by pine weevil feeding; "Other" included dead seedlings which died due to other unknown reasons.

4.4.2 MeJA induced resistance potentially affects drought tolerance/susceptibility

Seedling mortality due to drought in the field could be affected by MeJA treatment and plant age. In the field experiment in **paper I**, substantial proportions of seedlings suffered from the extreme drought in 2018 (Fig. 9) regardless of their age. MeJA treatment seemed to alleviate drought-related mortality of older seedlings (the S2015 cohort), but exacerbate the drought-related mortality of the younger seedlings (the S2016 cohort) (Fig. 9). In previous studies, varying effects of MeJA treatment on plant drought resistance and associated traits such as root growth have been reported (Heijari et al. 2005; Moreira et al. 2012a; Fedderwitz et al. 2019). Other studies have shown that MeJA application can alleviate drought stress in cauliflower *Brassica oleracea* and soybean *Glycine max* (Wu et al. 2012; Mohamed and Latif 2017). Its effects on drought tolerance/susceptibility in forest regeneration have been little studied as drought has not been considered to cause such high mortality in the past as biotic factors. However, drought could become a major abiotic factor that hampers forest rejuvenation following climate changes.

4.4.3 MeJA induction might antagonize the salicylic acid (SA) pathway

An interesting observation beyond the scope of our research aims is that some Norway spruce seedlings were infested by aphids in the greenhouse (Fig. 10a), and MeJA-treated seedlings had higher aphid infestations than controls. We eliminated the aphid population by hand instead of spraying any solution to avoid contamination by other substances. We speculate that they were members of the *Cinara* genus of the Lachnidae (bark aphid) subfamily, which live without host alternation on conifers, excrete honeydew, and are typically tended by ants (Fischer and Shingleton 2001). Currently 20 species of *Cinara* have been recorded in Sweden according to the Swedish taxonomic database, but we could not identify them to species level. The finding that aphids were more abundant on the MeJA-treated seedlings reminded us of the antagonism between the JA (the free acid form of MeJA) and salicylic acid (SA) pathways (Karasov et al. 2017). SA is a phenolic phytohormone that mediates stress responses to some phloem-feeding insects or biotrophic pathogens (Thaler et al. 2012), and MeJA induction may suppress the SA pathway, thus increasing seedlings' susceptibility to aphids. Aphid feeding does little damage to seedlings, but it could facilitate infection by pathogens in the field.

In **paper IV**, some seedlings were sprayed with MeJA in the field. These seedlings were not included in subsequent data analysis as the high weevil pressure caused rather high overall seedling mortality, and not many MeJA-treated seedlings survived. A few of the MeJA- treated survivors were tended by ants (Fig. 10b) and little pine weevil damage was found on them. The ants in boreal forests mostly feed on honeydew produced by ant-tended aphids (Skinner 1980) and negatively affect other herbivores on the same plants by preying or deterring them. Accordingly, Maňák et al. (2013) found that placing ant-attracting bait on conifer seedlings reduced pine weevil damage. Thus, if there is an antagonistic relationship between JA and SA pathways in conifer seedlings, MeJA-treated seedlings may benefit not only from induced resistance to pine weevils, but also indirect protection via ant-aphid mutualism.

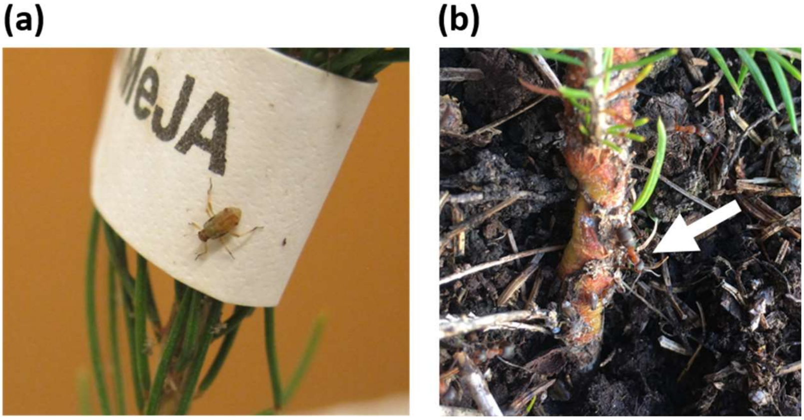

Figure 10. Pictures of **a)** *Cinara* sp. that infested Norway spruce (*Picea abies*) seedlings in the greenhouse in 2018; **b)** Ant (shown by a pointing arrow) patrolling a the Norway spruce seedling to protect aphids infesting the same seedling in the field experiment for **paper IV**. Photos: Yayuan Chen.

4.5 MeJA can affect plant tolerance trait (**paper III**)

Our study examining the closure rate of wounds inflicted at different times on Norway spruce seedlings showed that healing of wounds inflicted before MeJA treatment were negatively affected, and those inflicted after it were unaffected by the treatment (Fig. 11). MeJA is not only involved in resistance induction, as it can also affect plant resource and nutrient relocation, photosynthesis and stem anatomical structures (Gould et al. 2008; Moreira et al. 2012a; López-Villamor et al. 2021). For example, MeJA treatment can reportedly increase fine root biomass of Maritime pine, indicating that it can increase carbon allocation to roots (Moreira et al. 2012a). Temporary reductions in photosystem II capacity and transpiration rate have been observed following MeJA treatment in Monterey pine *Pinus radiata*, although these changes had no detected long-term effects (Gould et al. 2008). Moreover, MeJA can induce traumatic resin duct formation and increase levels of defensive chemical compounds in conifer seedlings (Hudgins et al. 2004; Hudgins and Franceschi 2004). All these responses might indirectly affect wound healing process by reducing available resources. This could explain the negative effect of MeJA on the healing of the first wound. However, MeJA can also trigger plant induced defense and reduce pine weevil feeding. Thus, healing processes in MeJA-treated seedlings could still catch up with those in non-wounded controls as they may have smaller areas to heal.

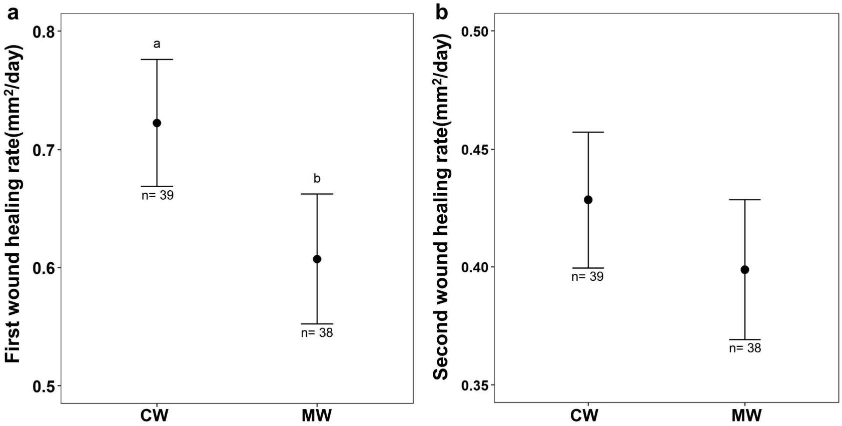

Figure 11. Estimated mean healing rate (mm^2/day) (± 95% confidence intervals) for **a)** the first wound and **b)** the second wound inflicted at two time points on Norway spruce (*Picea abies*) seedlings. The first wound was inflicted before treating seedlings with water (CW) or MeJA (MW), while the second wound was inflicted after treatment. Sample sizes (n) used in the statistical analyses are shown, and letters indicate significantly different means. Note that the y-axis does not start at 0.

Regardless of MeJA treatment, bark wound healing alone can be affected by various factors, for example the season when wounding occurs, temperature, diameter growth and location of the wound. The faster average healing rate of the first wound could be explained by the higher average temperature during the process as it was inflicted in July, while the second wound was inflicted in September. This is consistent with findings that wound healing rates in peach (*Prunus percica*) trees strongly depend on the average temperature after wounding (Biggs 1990). Several previous studies have found that bark wound healing rates are positively related to tree vigor, often expressed in terms of diameter growth (Neely 1988; Jones et al. 2019; Tavankar et al. 2019). Accordingly, in our experiment, the healing rate of the first wound was significantly affected by the plant initial stem basal diameter (a reflection of diameter growth in the previous year). The first wound healed more rapidly in vigorous seedlings than in less vigorous seedlings (indicated by larger initial basal diameter, in accordance with other studies (Boyes et al. 2019; Jones et al. 2019). In addition, another study on Norway spruce found that higher injuries were repaired more quickly than lower ones (Schneuwly-Bollschweiler and Schneuwly 2012). Thus, the locations of the first and second wounds could have also contributed to the differences in healing rates.

Wounding alone did not affect plant growth in our study, but plant growth can be affected by levels of damage they receive (Persson et al. 2005; Metslaid et al. 2013). We did not detect any correlation between plant resistance to, and tolerance, of damage. Similarly, in other studies on woody plants no trade-off between resistance and tolerance to herbivory has been detected (Fineblum and Rausher 1995; Prittinen et al. 2003; Stevens et al. 2007; Cruickshank et al. 2018). Thus, woody plants might be able to have both high resistance and tolerance to herbivory, as resistance and tolerance could be independent of each other (Cruickshank et al. 2018).

4.6 Genetic variation in resistance and type of propagation materials differ in resistance (**paper IV**)

There was significant genetic variation in seedling resistance to pine weevil damage. However, the examined resistance traits (frequency of attack, area debarked, frequency of girdling by pine weevil and plant survival) showed low heritabilities. Low heritabilities of such traits have also been found in other studies (Zas et al. 2005; Zas et al. 2017; Puentes et al. 2018) that have compared similar numbers of families, although higher heritabilities have been detected in studies with substantial numbers of families (King et al. 1997; Moreira et al. 2012a). Thus, according to both our study and previous investigations, the low heritabilities of plant resistance traits might be a constraint for breeding resistant Norway spruce material from existing breeding populations.

We also found significant, but weak, genetic correlations between seedlings' height and total damage pine weevils inflicted on them. As the weevils use olfactory and visual cues to find seedlings (Björklund et al. 2005), they may find taller plants more easily. However, taller and faster growing seedlings can often withstand pine weevil damage better than smaller ones (Selander et al. 1990). Therefore, taller plants are not necessarily strongly disadvantaged.

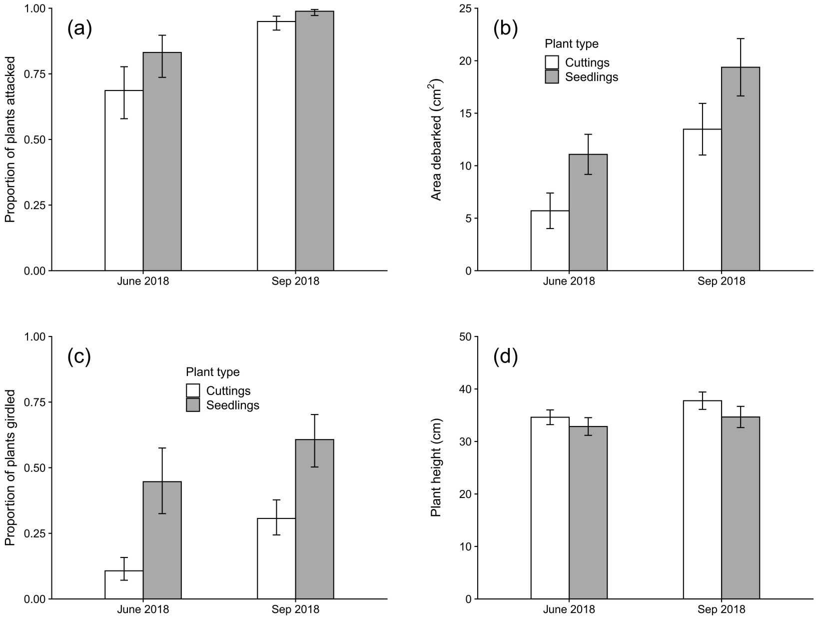

Figure 12. Estimated means (± 95% Confidence Intervals) of various traits for Norway spruce (*Picea abies*) cuttings (white bars) and seedlings (grey bars) belonging to the same full-sib families (n = 11). Trait means are presented for **a)** frequency of attack by the pine weevil (*Hylobius abietis*) (proportion of plants attacked), **b)** stem area debarked (cm^2), **c)** frequency of stem girdling (proportion of plants girdled), and **d)** plant height (cm). All variables were measured at two time points in the season (June and September 2018).

In addition, the seedlings were attacked significantly more by pine weevils than cuttings from the same family early in the growing season. The difference was small on the second occasion in the late growing season, when almost all plants had been attacked (Fig. 12a). However, seedlings were more damaged in terms of absolute debarked area both early and late in the growing season (Fig. 12b). Seedlings were also girdled significantly more frequently than cuttings (Fig. 12c). The differences in cultivation of cuttings and seedlings led to slight differences in their age and height (usually correlated with age), and seedlings with thicker stems (also usually correlated with age) are reportedly more resistant to pine weevil damage (Thorsen et al. 2001). Thus, the age and size differences between cuttings and seedlings contribute to their differences in resistance, but further studies are required to understand mechanisms underlying the variations.

5. Conclusion, Challenges & Future perspective

Exploiting potential defenses of the plants themselves is a strong candidate as a sustainable approach for protecting conifer seedlings against pine weevils. But plant defenses are complex and they are affected by numerous factors. Induced resistance provides plants with formidable and sophisticated defense "weapon" and use of the plant hormone MeJA allowed us to manipulate it to reduce pine weevil damage. However, applying MeJA to optimise protection is not as easy as tuning a console with different buttons. A major conclusion from the studies is that MeJA treatment can be applied a few months, rather than a few weeks, before planting, thus making it compatible with nursery practices. More specifically, if seedlings were sprayed three times with 10 mM solution at one-week intervals before winter storage, they could still be quite resistant to pine weevils when planted a few months later without another boost of MeJA treatment before planting, and the protection could be as good as a validated physical barrier protection. MeJA application is still one of the most practical and effective methods for triggering induced defense, although it has negative effects on seedlings' growth and potential side effects (e.g., increases in drought tolerance/susceptibility). Wound healing, as a plant tolerance trait, can also be impaired by MeJA treatment, if it is applied after the wounding, but we found that if MeJA was applied some time (>2 months) before the wounding it had no clear negative effect. A potential constraint for breeding weevil-resistant seedlings is that the heritabilities of examined resistance traits are low (according to our experiment with genetically improved seedlings from the breeding program) unless other resistance traits with higher heritabilities are found in further studies. We also found that cuttings are better planting materials than seedlings, at least in terms of reducing pine weevil damage.

However, both MeJA treatment and use of cuttings are still in a fundamental research phase and there are challenges for plant nurseries to adopt them. High cost is the major issue. MeJA is currently produced mainly for scientific research and the market demand is rather low. If it can be mass produced and the price reduced, MeJA-treated seedlings from nurseries will be more affordable for forest owners. MeJA can likely be sprayed by previous insecticide spraying systems or similar irrigation systems in plant nurseries. Thus, it might not be necessary to develop a customized system, or at least it may not be costly to modify current systems for automating MeJA treatment. However, cuttings are rather expensive as they require much more care and thus labor in the propagation process and cannot be produced on large scale with current techniques. Thus, although cuttings have great pine weevil resistance, they will not be commercially viable options unless there are major technical breakthroughs in their mass propagation.

To fully develop the idea of utilizing plant defenses as a reliable approach to reduce pine weevil damage, further studies are required to clarify some uncertainties. MeJA treatments (with details of timing, recurrence, dose etc.) that provide sufficient protection in controlled conditions have been developed. However, it is not known how MeJA application will affect seedlings' responses to abiotic factors (e.g., more frequent drought events due to climate change) and other biotic factors (e.g., aphids or other herbivores/omnivores) in the field. To improve understanding of how MeJA-treated seedlings cope with drought, studies of their physiological reactions and resistance to pine weevils of MeJA-treated seedlings under different water conditions are of interest. Interactions between MeJA-treated conifer seedlings and common non-coniferous plant species at planting sites also warrant attention, as the treatment might interfere with their interspecific competition. In addition, treatments involving different concentrations of MeJA in combination with ant baits could be worth testing, to assess their possible interactive effects on ants' behaviour and pine weevil damage. Such combinations could potentially confer strong protection by providing both MeJA-induced resistance and indirect protection from ant-aphid mutualism, thus enabling reductions in MeJA doses and their impairment of seedlings' growth. Other induction methods that involve wounding seedlings, with enough time to allow them to completely heal before exposure to pine weevils also warrant further tests, as such methods do not impair plants' growth if they become more resistant to pine weevils after complete recovery from the wound. Plant tolerance can go hand in hand with genetic resistance by screening elite families from available plant breeding materials that stably exhibit both high tolerance and resistance. Acquiring knowledge of how tolerance will be affected by field conditions and climatic changes is as important as elucidating other defenses. Further studies of the mechanisms underlying the differences between cuttings and seedlings are required. Development of methods that combine these plant defenses strategies by testing the resistance induction of elite families may also be needed to fully exploit plant defenses and obtain plants that are optimally prepared for future climate changes.

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Popular science summary

Plants have enormous value for humans, offering not only food but also many raw materials for production of many purposes in our daily life. However, plants are not only consumed by humans. Numerous other herbivorous foes (i.e., herbivores) also feed on them, including bacteria, fungi, insects, and other animals. It is not easy for plants to survive the diverse attacks they may encounter in any stages of their lives. As plants seem to be passive and immobile, this raises the question *How do they manage to protect themselves?* The answer is that a plant has defenses that work like human immune systems to some extent and can be categorized as constitutive or induced. Constitutive defense is always present and regarded as frontline protective features that any invader must overcome or penetrated, like the human skin that prevents most damaging agents from entering our bodies. Induced defense are activated when a plant perceives cues indicating potential attackers, or under subsequent attacks after it perceives initial cues. Methyl jasmonate (MeJA) is a signaling hormone that usually accumulates in damaged plants. Studies have shown that plants' responses to treatment with MeJA solution are similar to their responses to attacks by some herbivores. Thus, MeJA can be used as an activator to trigger plant induced defense.

Plant defenses against herbivores include resistance traits that enable them to prevent, stop or reduce damage and tolerance traits that enable them to withstand and recover from damage. Triggering induced defense with MeJA has conferred improvements in plant resistance traits and reduced damage by herbivores. Therefore, exploiting induced resistance is considered as a promising approach to protect plants from herbivores. Tolerance, in contrast, has been less studied since it is difficult to identify, and less straightforward than resistance, which can be evaluated by measuring pest infestations or damage severity. Among tolerance traits, the ability to heal wounds is important for young plants that suffer from bark damage. A potential complication is that both resistance and tolerance traits depend on the availability of plants' resources due to a presumed trade-off between resistance and tolerance to herbivores. Thus, if plants are treated with MeJA in efforts to enhance their resistance, it is essential to investigate whether the treatment also has negative effects on their wound healing or other tolerance traits.

Another important aspect to consider is that plants are constantly evolving and they must maintain sufficient resistance to herbivores to maintain their populations. Thus, screening resistant breeding families in current plant breeding programs and teasing out heritable resistance traits that can help future breeding efforts is highly valuable for utilization of plant constitutive defense. However, there are uncertainties regarding all types of defenses, and further research is essential to generate the knowledge we require to protect our plantations sustainably.

Swedish plantation forests are dominated by two conifer species (Norway spruce and Scots pine) and managed with clear-cutting regimes. These regimes are efficient, but clear-cuts also harbour pine weevils, which are considered the major threat to planted regeneration material. With market pressure for more sustainable wood products and ending the use of pesticides, exploitation of plant defenses is a strong candidate approach for reducing pine weevil damage to young coniferous seedlings (young plants grown from seed).

Previous studies have shown that applying MeJA solution a few weeks before seedlings are exposed to pine weevils can trigger induced resistance and reduce pine weevil damage, but it is not known if MeJA treatment can be applied a few months earlier without losing its efficiency to protect seedlings from pine weevil damage. The studies this thesis is based upon showed that seedlings can be well protected from weevils if MeJA treatment is applied a few months earlier at the end of their nursery cultivation, just before packing for winter storage.

Induced resistance can also be triggered when plants are damaged. Thus, I investigated if other methods involving inflicting damage can also trigger induced resistance that provide conifer seedlings with sufficient protection. The results showed that none of the other methods triggered strong resistance that greatly reduced weevil damage. Considering the possible trade-off between resistance and tolerance, I investigated effects of MeJA treatment on a tolerance trait (wound healing ability) in addition to its triggering of induced resistance. I found that it negatively affected healing rates of wounds inflicted before MeJA treatment, but not healing of wounds inflicted after it. Thus, since MeJA is normally applied to seedlings before they are exposed to pine weevils, its application should not impair the healing of wounds caused by pine weevil feeding later at planting sites.

In addition to induced resistance, constitutive resistance are also important elements of plants' armor. Therefore, I explored the possibility of utilizing constitutive resistance by examining the resistance of seedlings from different families from the Norway spruce breeding program in Sweden. I also examined the resistance of cuttings and seedlings belonging to the same family. The results revealed significant differences among families in terms of seedlings' resistance to pine weevil damage. However, we found that the examined resistance traits had low heritabilities, indicating that following generations of resistant families will not necessarily inherit high resistance. The results also show that cuttings, another type of material that can be used for regeneration, are more resistant to pine weevils than seedlings from the same family. Overall, the studies investigated several aspects of plant defenses. However, further studies are still required to fully understand some underlying mechanisms of several findings, as well as potential interactions between induced seedlings and both other herbivores and environmental factors.

Populärvetenskaplig sammanfattning

Växter har ett stort värde för mänskligheten, inte bara som föda utan även för att de ger råvaror till många olika slags produkter som vi använder i vårt dagliga liv. Människan är bara en av många organismer som helt eller delvis livnär sig på växter, allt från bakterier, svampar, insekter och andra djur så kallade herbivorer (växtätare). Alla stadier i växtens liv kan vara utsatta för angrepp från växtätare och varje attack kan vara dödlig för växten. Trots att växterna verkar passiva och är orörliga så har de flera möjligheter att skydda sig vid angrepp. Faktum är att växter har olika typer av försvar som i viss utsträckning kan liknas vid människans immunsystem och kan indelas i ett konstitutivt försvar och ett inducerat försvar. Det konstitutiva försvaret är ständigt närvarande och kan liknas vid en skyddande frontlinje som inkräktare måste övervinna eller ta sig igenom, ungefär som den mänskliga huden som kan hindrar skadliga ämnen från att ta sig in i kroppen. Växters inducerade försvar aktiveras när växten uppfattar tecken (signaler) som tyder på att den håller på att bli angripen och skadad eller med en viss fördröjning när en skada redan uppstått. Metyljasmonat (MeJA) är ett signalhormon som vanligen ansamlas i skadade växter. I studier har man visat att växter som behandlats med MeJA-lösning reagerar på liknande sätt som när de blir skadade eller angripna av vissa växtätare. Metyljasmonat kan därför användas för att aktivera växtens inducerade försvar.

Växternas försvar mot växtätare inkluderar resistensegenskaper som gör att de kan förebygga, stoppa eller minska skador och toleransegenskaper som gör att de kan motstå och återhämta sig från skador. Aktiveringen av växters försvar med MeJA har visat sig ge förbättrade resistensegenskaper och minskade skador av växtätare. Utnyttjandet av inducerade resistensegenskaper anses därför vara ett lovande sätt att skydda växter från växtätare. Tolerans har studerats i mindre omfattning än resistens eftersom det är svårare att identifiera och inte lika enkelt att skatta. Resistens skattas enkelt genom att man mäter skadegörares angreppsfrekvens och skadornas omfattning. Bland toleransegenskaper är förmågan att läka skador viktig för unga plantor som fått barkskador. Tolerans och resistensegenskaper är båda beroende av växtens resurstillgångar vilket gör att växten förväntas göra avvägningar mellan resistens mot och tolerans för växtätare. Därför är det viktigt att undersöka om MeJA-behandling för ökad resistens kan ha negativa effekter på läkning av skador och andra toleransegenskaper.

En annan viktig aspekt är att organismer ständigt utvecklas och växter som utvecklar tillräckligt bra resistensegenskaper mot växtätare överlever bättre än växter med sämre resistensegenskaper. Därför är screening av resistens och ärftlighet för resistensegenskaper i befintligt förädlingsmaterial av värde då det ger möjlighet att utnyttja dessa egenskaper i framtida förädling. Det finns fortfarande många osäkerheter kring växters försvar och ytterligare forskning är avgörande för att få den kunskap vi behöver för att skydda växter på ett hållbart sätt.

De svenska planterade skogarna domineras av två slags barrträd : gran och tall och skötseln bedrivs framförallt med hyggesbruk. Det är en effektiv skötselregim ur produktionssynpunkt men hyggen lockar och gynnar också snytbaggen som anses vara det största hotet mot planterad barrträdsföryngring på hygget. Med nuvarande marknadstryck för mer hållbara träprodukter och slutgiltig utfasning av bekämpningsmedel är möjligheten att utnyttja växternas eget försvar en stark kandidat för att ingå i en strategi för att minska snytbaggeskadorna på unga barrträdsplantor.

Tidigare studier har visat att MeJA–behandling av plantor några veckor innan de exponeras för snytbaggar kan aktivera plantans inducerade resistensegenskaper och minska snytbaggeskadorna. Däremot har det inte varit känt om MeJA-behandling några månader tidigare kommer att förlorar sin skyddseffekt när de sedan exponeras för snytbaggar. I en av studierna som denna avhandling bygger på visas att MeJA-behandling av plantor före vinterförvaringen, några månader före planteringen, är väl så skyddade som plantor som behandlades veckorna före planteringen följande år.

Den inducerade resistensegenskapen kan också aktiveras när växten skadas av annat än växtätare. Därför testade jag om några olika metoder att manuellt skada plantor också kan aktivera inducerade resistensegenskapen och ge barrträdsplantor ett tillräckligt skydd. Resultaten visade att ingen av de använda skademetoderna verkade aktivera ett tillräckligt starkt försvar för att markant minska snytbaggeskadorna. Med tanke på att växten troligen måste fördela sina resurser mellan resistens och tolerans undersökte jag också om MeJA-behandling påverkar en toleransegenskap, förmågan att läka skador. Jag fann att en skada som orsakats före MeJA-behandlingen läkte långsammare än skador som orsakats efter behandling med MeJA. Eftersom MeJA-behandling är tänkt att utföras innan plantorna planteras i fält är det inte troligt att det väsentligt skulle påverka läkningen av snytbaggeskador.

Förutom den inducerade resistensen (motståndskraften) är den ständigt närvarande konstitutiva resistensen en viktig del av växtens skydd. Därför utvärderade jag möjligheten att utnyttja konstitutiv resistens genom att undersöka resistensen hos plantor från olika familjer i det svenska förädlingsprogrammet för gran. Jag undersökte också resistensen hos sticklingar och fröplantor hos granar som tillhörde samma familj. Resultaten visade att det fanns signifikant skillnader mellan familjer när det gäller plantornas motståndskraft mot snytbaggar. Vi fann dock att de undersökta resistensegenskaperna hade låg ärftlighet vilket indikerar att kommande generationer av dessa familjer inte nödvändigtvis kommer att ärva föräldragenerationens resistensegenskaper. Resultaten visade också att sticklingar, en annan typ av föryngringsmaterial, är mer motståndskraftiga mot snytbaggeangrepp än fröplantor från samma familj. Sammantaget undersöktes flera aspekter av växtförsvar men ytterligare studier krävs för att fullt ut förstå de bakomliggande mekanismerna av vissa resultat, samt möjliga interaktioner mellan ”inducerade” plantor, andra växtätare och miljöfaktorer.

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Seasonal timing and recurrence of methyl jasmonate treatment influence pine weevil damage to Norway spruce seedlings

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Abstract

Defense can be induced in conifer seedlings to reduce pine weevil (*Hylobius abietis*) damage, by treatment with the plant hormone methyl jasmonate (MJ). Few studies have addressed important practical issues regarding the use of MJ such as treatment incidence and timing, seedling age and its compatibility with plant nursery practices. We examined if levels of pine weevil damage depend on seasonal timing and recurrence of MJ treatment, and if the observed effects depend on plant age. Norway spruce (*Picea abies*) seedlings from two age cohorts (1 year and 1.5 years old) received four MJ treatments: MJ application before winter storage in the previous year, after winter storage but before spring/summer planting, repeated MJ application (both before winter storage, and before planting) or no MJ application at all. Pine weevil damage was evaluated in a lab and field experiment. We found that the timing and recurrence of MJ treatment affected the amount of damage inflicted by pine weevils in different ways, but these effects were consistent among age cohorts. MJ application before winter storage provided the most effective protection, and this reduction in damage was comparable to that provided by a currently used physical protection method against pine weevil feeding. Our results indicated that MJ can be applied in line with nursery practices (before winter storage) and provides adequate protection for two growing seasons.

Keywords Conifer seedlings · Forest plant protection · *Hylobius abietis* · Induced defense · Nursery protocols

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Introduction

Insects are one of the major agents of damage to European forests (Matyjaszczyk et al. 2019). The problems caused by insects are usually more prominent in clear-cut forests as they are often regenerated through planting, and the seedlings are susceptible to insect herbivory during establishment. In these newly planted forests, the pine weevil (*Hylobius abietis*) is one of the most destructive insect pests because it consumes the stem cambium, often girdling seedlings (entire ring of bark removed), causing high levels of plant mortality (Hagner and Jonsson 1995; Långström and Day 2007). Protective measures against pine weevil damage are necessary to prevent up to 100% mortality and to date, insecticide treatment has been one of the most prevalent methods in Northern Europe (Eidmann et al. 1996; Nordlander et al. 2011; Örlander and Nilsson 1999; Von Sydow 1997). However, the use of insecticides in forest pest insect management is being phased out due to environmental and human health issues. Even though various silvicultural practices and physical protective methods against the pine weevil are available, their effects are variable depending on site conditions (Nordlander et al. 2011; Wallertz et al. 2018; López-Villamor et al. 2019); thus, complementary and sustainable methods of seedling protection are needed.

One potential strategy to reduce forest pest damage, which has received less attention, is to amplify the plant's own defenses. These defenses encompass resistance traits that enable plants to prevent, stop or reduce damage inflicted by insects, and tolerance traits that enable withstanding and recovering from damage. Defenses can be classified into two categories: (1) constitutive defenses which are present at all times, and (2) induced defenses which are activated immediately when damage occurs (inducible defenses), or activated under subsequent attacks after perceiving initial cues or stimuli relevant to a specific type of attack (priming of defense) (Mageroy et al. 2020; Wilkinson et al. 2019). Induced defenses, in particular, have been shown to be effective at reducing damage by aphids, spider mites, thrips and other insect pests, in crops such as grape, strawberry, tomato, soybean and lima bean (Choh et al. 2004; Omer et al. 2000; Thaler et al. 2001; Selig et al. 2016; Warabieda et al. 2005). These effects have been achieved by triggering defenses prior to insect attack, without causing physical damage, through the use of the plant hormone methyl jasmonate (MJ). MJ is the volatile counterpart of jasmonic acid (JA), which regulates plant responses to abiotic and biotic stresses, especially those involved in mediating insect induced resistance (Kahl et al. 2000; McConn et al. 1997; Miller et al. 2005). Application of MJ activates production of plant defensive compounds and expression of resistance-related genes, subsequently reducing insect herbivory (Yu et al. 2019). Thus, utilizing MJ-induced defenses has emerged as an effective method to increase plant protection against various crop pests (Stella de Freitas et al. 2019; Stenberg 2017).

In forest pest insect management, there has been less focus on the effects of exogenous MJ treatment on coniferous seedlings and the possibility of using it as a tool to boost forest regeneration. This is despite documented increases in the total amount of defensive compounds like terpenes, phenolic compounds and resin following MJ treatment in different coniferous species, including *Picea abies* (Heijari et al. 2005; Hudgins et al. 2004; Moreira et al. 2009; Zas et al. 2014). Changes in these chemical traits are known to be effective induced defense responses following stem damage or biotic attack (Franceschi et al. 2005; Hudgins et al. 2004). Furthermore, these MJ-induced changes not only occur locally but also systemically throughout the plant (Huber et al. 2005; Moreira et al. 2009). MJ treatment to coniferous seedlings has been shown to affect pine weevil feeding behaviour, resulting in an overall reduction in damage levels and the likelihood of girdling relative to untreated plants (Moreira et al. 2009; Fedderwitz et al. 2015). Moreover, these plant protective effects have been observed both in the lab and field. Field results from a previous study showed that MJ-mediated protection remained for two growing seasons after the initial treatment (Zas et al. 2014).

Besides the enhanced protective effects, it has been shown that MJ can negatively affect plant growth following application (Heijari et al. 2005; Gould et al. 2009). When defenses are triggered by MJ, resources could be allocated to synthesize defensive compounds and diverted away from growth. However, this growth reduction is not necessarily a hindrance for using MJ to protect conifer seedlings. In fact, a recent study shows that this short term growth reduction could be exploited by plant nurseries to stop growth when seedlings reach a certain height (Fedderwitz et al. 2019). In the long term, MJ treated seedlings can compensate the growth loss and this temporary reduction is compensated by lower mortality compared to untreated seedlings (Gould et al. 2008; Zas et al. 2014). Thus, MJ has the potential to emerge as a non-pesticide approach for conifer seedling plant protection.

Before MJ can be implemented as a practical protection tool, several aspects need to be further investigated in order to optimize its effects. First, the level of induced defense triggered by MJ is dose dependent but the required amounts to reach the desired defensive effect can vary among species and with different plant variables. For instance, higher concentrations of MJ can lead to stronger defensive responses and older plants can withstand higher concentrations (Zeneli et al. 2006; Heijari et al. 2008), while overdose can be phytotoxic or even lethal for younger plants (Gould et al. 2009). Second, the timing of induction is another crucial factor. Plant responses occur within minutes after JA accumulation (Koo et al. 2009), but it takes longer until induced defenses become effective at reducing herbivory (Karban 2011). So far, studies examining the efficacy of MJ against pine weevil damage have conducted the treatment a few days or weeks prior to insect exposure. However, if MJ treatment is to be implemented in nurseries, it should ideally be compatible with conifer seedling production practices. Briefly, these practices involve sowing of seeds in spring with plants being allowed to grow until late autumn; they are then packaged and stored in freezer rooms until the following spring/summer when they are planted. Treatment with MJ could, thus, potentially be conducted before winter storage or before planting, or even at both time points. Currently, we lack knowledge on whether differences in timing and recurrence of treatment, as well as seedling age, could affect the protective effects of MJ against pine weevil damage. Therefore, we conducted a lab and a field experiment using Norway spruce seedlings to answer the following questions:

(1) If MJ treatment is applied on one occasion, do seedlings receive similar amounts of pine weevil damage regardless of whether seedlings are treated before winter storage or before planting?
(2) If MJ treatment is applied recurrently over 2 years, both before winter storage (in autumn) and before planting (in spring/summer), does it result in less pine weevil damage compared to when MJ treatment is applied on only one occasion?
(3) Are the effects of recurrent application and timing of the MJ treatment also affected by the age of the seedlings, i.e. older seedlings that were sown earlier (1.5 years old) or younger seedlings sown later (1 year old)?

Materials and Methods

Plant material

All the Norway spruce seedlings used in the experiments were obtained from a commercial plant nursery (Bergvik Skog Plantor AB, Nässja) in Sweden. Half of the seedlings were from seeds sown in July 2015 (hereafter referred to as S2015 or the older cohort, Fig. 1) and overwintered outdoors in their first year. The other half of the seedlings were from seeds sown in April 2016 (hereafter referred to as S2016 or the younger cohort, Fig. 1). This plant material was originally part of a separate experiment that examined the growth of MJ-treated Norway spruce seedlings with that of untreated and long-night treated seedlings (Fedderwitz et al. 2019). Hence, we describe the initial MJ treatments related to the previous study in the paragraph below, but the follow-up treatments associated with the present study are described in the next section.

In July to August 2016, half of the seedling from the S2015 cohort were sprayed with 10 mM MJ (Sigma-Aldrich 95%, Ref. No. 392707) three times, and the interval between sprayings was 1 week. The other half of the seedlings (control group) in the S2015 cohort

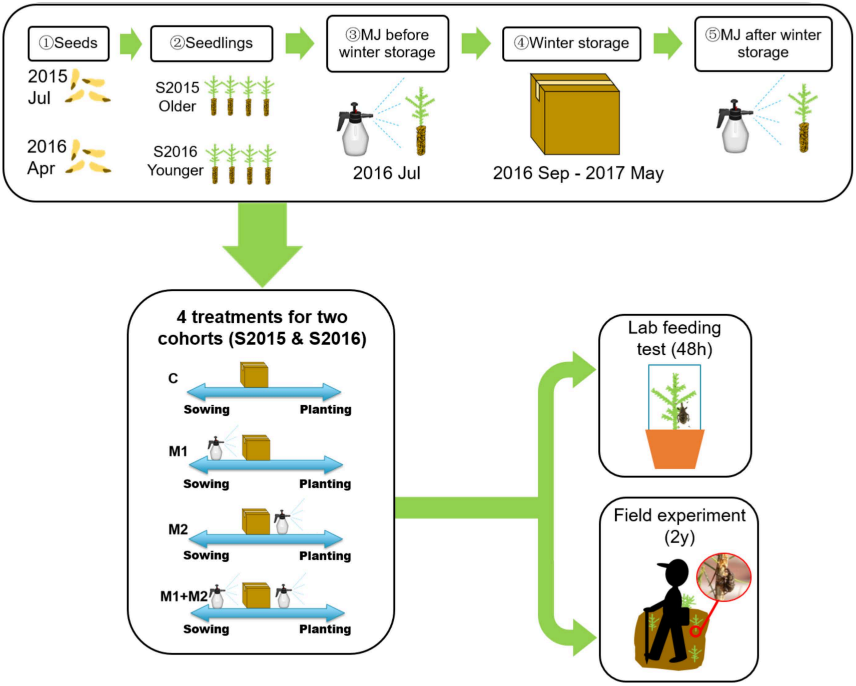

Fig. 1 Experimental design showing the timing of treatments relative to seed sowing and plant nursery operations. First, seeds of Norway spruce (*Picea abies*) were sown in July 2015 (S2015) or April 2016 (S2016) and allowed to grow until the autumn of 2017 (steps 1 and 2). Methyl jasmonate (MJ) was applied before or after winter storage (steps 3 to 5), or at both time points. Seedlings from each age cohort (S2015 and S2016) were subjected to four different treatments: MJ treatment before winter storage or before summer planting (M1 and M2, respectively), repeated MJ treatment both before storage and before planting (M1 + M2), and no MJ application at all (C). Plants were then assigned to a laboratory non-choice pine weevil (*Hylobius abietis*) feeding experiment or planted in a forest clear-cut and evaluated over 2 years

were sprayed with carrier solution (solution of 2.5% ethanol, v:v) with the same frequency. The seedlings in the S2016 cohort were treated in the same way and at the same time, as the S2015 cohort. All the treatments were performed outdoors and plants were kept growing outside until September 9, 2016 when they were subjected to a long night treatment (13 h darkness every day from 19:00 to 8:00) for 4.5 weeks. The long night treatment is a nursery practice used to prepare seedlings for winter storage and stop seedling growth at a desired height. After this treatment, all seedlings were packed in sealed boxes and stored in a freezer (−5 °C) over the winter. For the current study (Fig. 1), we used 200 seedlings from each age cohort (S2015 and S2016) and each treatment group (MJ-treated and control group).

Methyl jasmonate treatments

At the end of May 2017, seedlings described in the previous section were delivered from the nursery to our department (Swedish University of Agricultural Sciences, Uppsala, Sweden) and thawed at 5 °C. A week later, seedlings for the lab experiment were planted in plastic pots and seedlings for the field experiment were placed in seedling trays. These seedlings were then kept under greenhouse conditions for later use. The light period was 16 h (8 h dark) in the greenhouse, and day/night temperature was 20/16 C°. In order to test if the efficacy of MJ depends on whether treatments are conducted solely in 1 year or consecutively over 2 years, half of the previously MJ-treated seedlings from the S2015 cohort were sprayed with deionized water (treatment M1; MJ application before storage) and the other half with 10 mM MJ mixed with carrier solution (treatment M1 + M2; two MJ applications: before storage and before planting). For the remaining non-MJ-treated seedlings in the S2015 cohort, half of them were treated once with 10 mM MJ mixed with carrier solution (treatment M2; MJ application before planting), and the other half were sprayed once with deionized water (treatment C; control: no MJ applications at either time point). The same treatments were repeated on the S2016 seedling cohort (Fig. 1).

All treatments were performed only once on June 9, 2017 in a laboratory fumehood. MJ (Sigma-Aldrich 95%, Ref. No. 392707) was mixed with a carrier solution of 2.5% (v:v) ethanol to achieve the 10 mM concentration before spraying. The spray bottle was always pumped until the inner air pressure reached its limit of 2.5 bar, and shaken vigorously so that the MJ and carrier solution were well-mixed before spraying. The spray container was a Free-Syringe PC 1.5 litre plastic bottle (Jape Products AB, Hässleholm, Sweden). The nozzle was placed about 40 cm away from the plants and spraying was conducted so that the solution reached and covered the entire seedling in each pot. Each seedling was sprayed approximately for one second, then the pot was turned around 180 degrees and the spraying was repeated in the same way. Treatment of plants with deionized water was performed the same way as MJ treatment. The seedlings treated with MJ were dried at room temperature (inside a fumehood, light period 16 h) during 24 h, before they were moved back in the greenhouse together with other seedlings.

Lab experiment

Pine weevils

For the lab experiment, the pine weevils used in the feeding test were collected during their spring migration on May 27, 2017 at a sawmill (Balungstrands Sågverk AB) in

Enviken, Sweden. They were kept in darkness at a room temperature of 10 °C and fed with young Scots pine (*Pinus sylvestris*) stems and branches. One week before the feeding tests on June 16, 2017, pine weevils were placed at room temperature for acclimatization (light–dark cycle, 16 h–8 h) and supplied with water and Scots pine branches. Female pine weevils were individually placed in a Petri dish with a small Scots pine branch piece for 24 h. Those that fed on the branch during this period were selected and starved for 3 days before the feeding test.

Testing resistance against the pine weevil

In order to test how the timing and recurrence of MJ treatments on seedlings of two age cohorts affects pine weevil feeding under non-choice conditions, we conducted feeding tests in the lab (Fig. 1). Two weeks after the MJ treatment on June 22 and 24, 2017, 96 seedlings were exposed to pine weevils (12 seedlings for each treatment). Feeding tests were conducted in two rounds, one right after the other, due to limited space in the lab. Each pot containing one seedling was covered with a transparent plastic cylinder with a mesh opening at the top, and one female pine weevil was placed inside for 48 h. After the feeding test, absolute stem area debarked was calculated for each plant by using millimetre paper to measure the length and width of each scar, and adding up all the areas for each scar.

Seedling growth measurements

The height and diameter of seedlings were measured in the greenhouse to quantify and compare growth trajectories of MJ-treated and non-MJ treated plants. Five individuals from each treatment group were set aside for these growth measurements. The measurements began on June 8, 2017 and ended on September 10, 2017, with an interval of 6–11 days between measurements. A total of 10 measurements were taken.

Field experiment

To investigate how the timing and recurrence of MJ treatment affects pine weevil preference under field conditions, we set up an experiment in a 1-year-old clear-cut with sandy soil near Tierp, central Sweden (60°21′N, 17°26′E). The field was previously a commercial forest dominated by Scots pine and harvest was conducted in March 2016. A total of 330 seedlings were planted in 33 blocks on June 15, 2017. Each block was a rectangular patch (about 50 cm × 300 cm) that was previously scarified with an excavator before planting in 2017. Ten seedlings were planted in random order along the two long sides (5 seedlings per side) at the border between the humus and mineral soil. The eight treatment combinations (MJ × seedling cohort) were replicated once in each block, and an extra seedling treated with the commercial protection Conniflex® (physical barrier) from each cohort, was also included. Conniflex is a sand-glue mixture that covers the stem to physically protect seedlings from stem chewing by the pine weevils. It has been shown to efficiently reduce pine weevil damage and enhance seedling survival (Nordlander et al. 2009). Since such physical barriers are often used and have a well-established protective effect, we included it as a positive control in the field experiment to compare against the efficiency of MJ in reducing pine weevil damage.

Assessment of pine weevil damage

Pine weevil damage was assessed at two occasions, across two growing seasons (Fig. 1). The first measurements were done on September 21, 2017 to assess early pine weevil damage and seedling establishment. The second measurements were conducted the following year on June 28, 2018 to examine seedling survival over the winter and follow the development of pine weevil damage. During the assessments, we measured the following damage variables: (1) H_{damage} - the height from the ground (right above the root collar) to the upper side of the highest and last pine weevil feeding scar found on the stem; (2) P_{damage} - the estimated proportion of stem area damaged in relation to the total surface area up to the H_{damage} described in (1). Eight replicates of each treatment from 33 blocks were randomly chosen to calculate the mean diameter (D) of plants. Using the above mentioned measurements and the equation for calculating the circumference of a circle (estimated perimeter of the seedling stem), we estimated the debarked area (mm^2) for each plant as: $A_{debarked} = (\pi \cdot D) H_{damage} \cdot P_{damage}$. This method was used if the feeding scars were scattered and irregularly shaped. If the feeding scars were few and small, absolute stem area debarked by pine weevils was calculated by measuring the length and width of each scar with a ruler, and adding up the areas if more than one scar was found. In addition, the height of living seedlings, the frequency of pine weevil attack, as well as the number of dead seedlings and the possible cause of death were recorded. We categorized seedlings according to three possible causes of mortality: Drought – seedlings which appeared to have dried out but showed no evidence of pine weevil girdling; Girdled – seedlings which were girdled by pine weevil feeding; Other – seedlings which were dead due to other unknown cause (e.g., pulled out from the soil by birds or mammals in the area).

Statistical analysis

All analyses were conducted in R software version 3.5.2 (R Core team, 2018) using R studio 1.1.463 (RStudio team 2016), and all graphs were plotted using the *ggplot2* package (Wickham 2016). For the lab experiment, a linear model (*lm* command from the *nnet* package, Venables and Ripley 2002) was used to test the effect of MJ treatments, conducted at different times and occasions, on levels of pine weevil damage. This model included two fixed effects: treatment (4 levels: C, M1, M2, M1 + M2), age cohort (2 levels: S2015, S2016) and their interaction. Plant diameter before the feeding test was included as a continuous covariate, and the response variable explored was debarked area by pine weevils. Seedling growth (height increment) from greenhouse measurements was also tested with a linear model, including the same fixed effects as the pine weevil damage model. Initial height was added as a covariate in the growth model. For the field experiment, blocks where no pine weevil damage was observed on any of the plants were excluded since these blocks were considered to have no pine weevil pressure. A generalized linear mixed model (*glmer* command from *lme4* package, Bates et al. 2015) including the same fixed factors as for the lab experiment, was fitted for area debarked but it included block as a random factor. In addition to debarked area, attack rate (plant attacked or not) and mortality (alive or dead) in the field were also examined as response variables. A linear mixed model (*lmer* command from *lme4* package, Bates et al. 2015) was used to test the same fixed and random effects as the pine weevil field damage model, but for seedling growth (height increment). To test the significance of fixed effects and their interaction in all models, the *Anova* command from the *car* package (Fox and Weisberg 2011) was used. If significant main effects were found, Fisher's least significant difference (LSD) tests were conducted among treatment levels with the *emmeans* command from *emmeans* package (Lenth 2019). Due to the low number of damaged seedlings, which resulted in many zeros when analysing debarked area and attack rate, different data transformation and distributions were tested. Generalized mixed linear models that provided the best fit of the data were chosen. Even though models converged, no multiple comparisons among treatments followed for some models and we interpreted significance with caution.

Results

Lab experiment

There was a significant difference in area debarked by pine weevils among MJ treatments differing in timing and recurrence, but the effect of age and the interaction effect of age and MJ treatment were not significant (Table 1). Multiple comparisons showed that seedlings with treatment only before winter storage (M1) received the least damage and this was significantly lower than for control plants (66.5% reduction in damage; Fig. 2a, b). Consecutive MJ treatment (M1 + M2) also reduced damage (54.5% reduction; Fig. 2a, b), while MJ treatment right before planting (M2) had the least effect on pine weevil damage (a statistically non-significant 17.7% reduction; Fig. 2a, b) compared to the control treatment. This pattern was consistent between age cohorts (Fig. 2a, b).

Seedlings varied significantly in growth (height increment) among treatments and between age cohorts (Table S1). Overall, seedlings treated with MJ experienced a reduction in growth compared to control seedlings, and those from the S2015 cohort grew significantly more in height than those from the S2016 cohort (Fig. 3a and 3b). The height increment of the S2016 cohort was quite similar among MJ treatments (Fig. 3b), while there were significant differences among these treatments in the S2015 cohort (Fig. 3a).

Table 1 Results of Analysis of Variance (ANOVA) (df: degrees of freedom; F: F-value; P: *p* value) for linear models used to examine differences in stem area debarked (Debarked area, mm^2) by pine weevils (*Hylobius abietis*) in a lab feeding test. Models included methyl jasmonate treatments (M1, M2, M1 + M2 and C; see Fig. 1 for an explanation), Norway spruce (*Picea abies*) seedlings belonging to two age cohorts (Sown in 2015 or 2016), and their interaction. Seedling diameter (mm) was used as a continuous covariate in the model

Source of variance	Debarked area		
	df	F	P
Age cohort	1	1.393	0.242
Treatment	3	15.433	**< 0.0001**
Diameter	1	1.637	0.205
Treatment × Age cohort	3 × 1	1.253	0.297

Statistically signficant effects are shown in bold (P < 0.05)

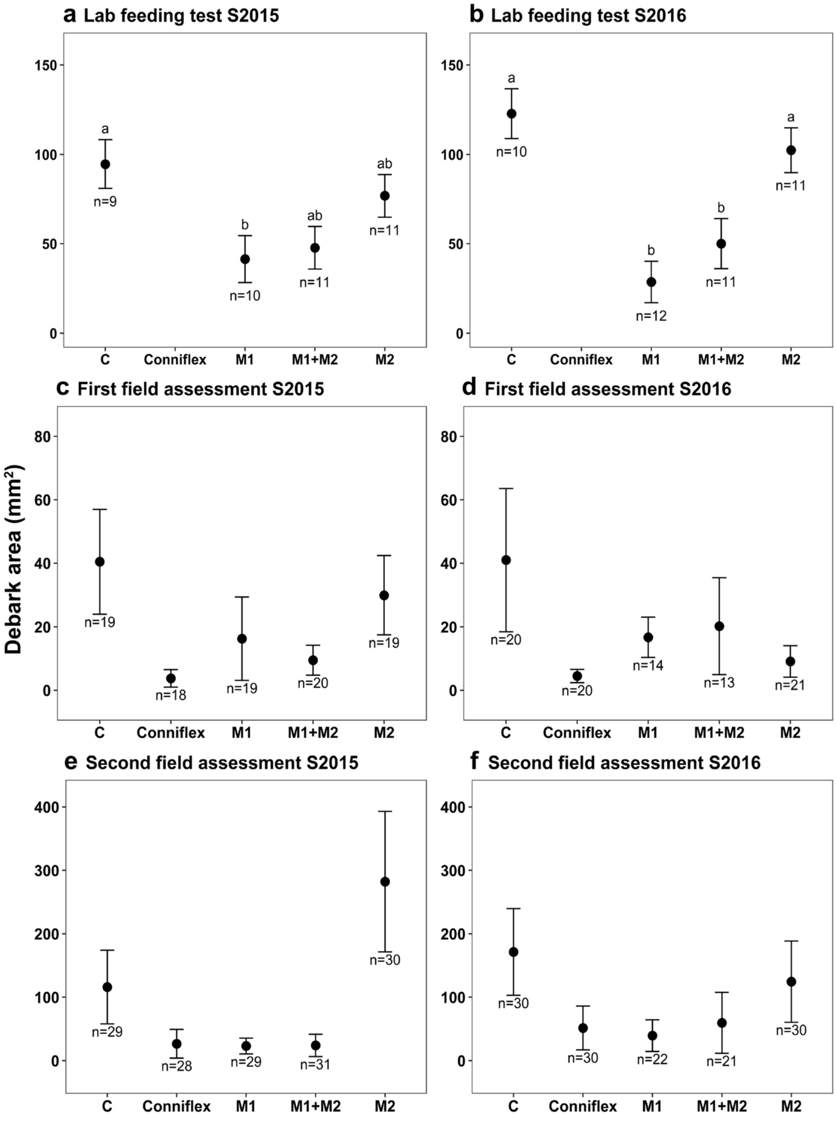

Fig. 2 Mean stem debarked area (mm^2) (± standard error) by pine weevils (*Hylobius abietis*) for Norway spruce (*Picea abies*) seedlings belonging to two age cohorts sown in 2015 (S2015; panels a, c and e) or in 2016 (S2016; panels b, d and f). For each cohort, results are presented per experimental treatment in the lab (panels a and b) and field (panels c and d, first assessment of damage in Sept. 2017; panels e and f, second assessment of damage in June 2018). Seedlings were treated with methyl jasmonate (MJ) before winter storage (M1), after (M2), or both before and after (M1 + M2); controls (C) received no MJ application at all (see Fig. 1). Seedlings with the physical barrier Conniflex were included as positive controls in the field experiment only. Different letters indicate significant differences among treatments at the 5% level (Tukey–Kramer tests), while means without letters are from models which converged but did not allow multiple comparisons (See Statistical analyses). Sample sizes (n) varied as some seedlings died during the experiment

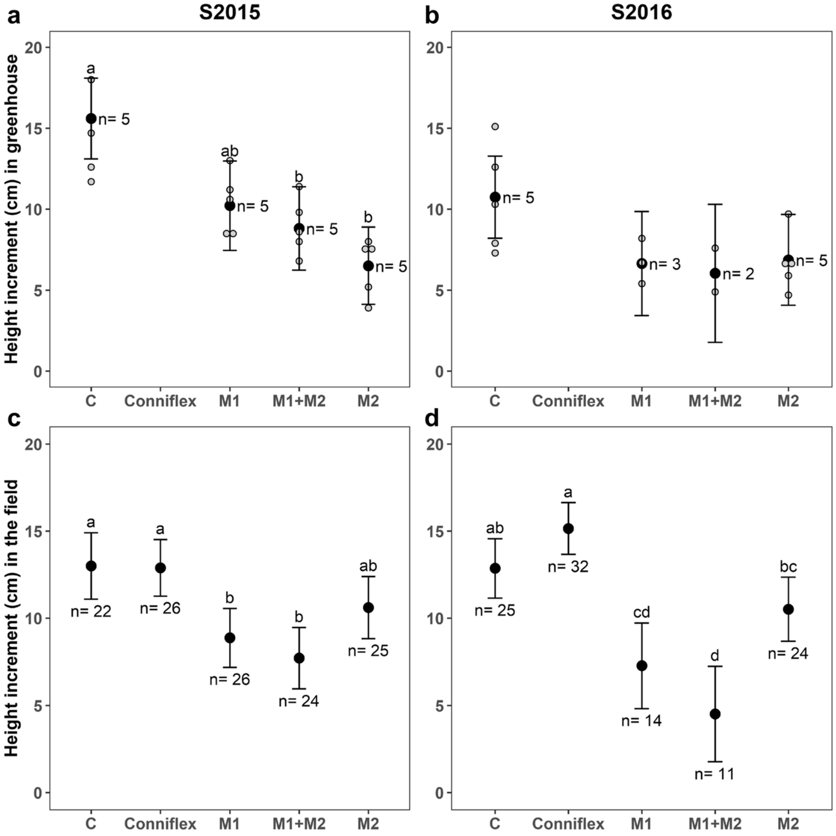

Fig. 3 Mean seedling height increment (final height subtracted with initial height, cm, ± 95% confidence intervals) of Norway spruce (*Picea abies*) seedlings belonging to two age cohorts sown in 2015 (S2015; panels a, and c) or in 2016 (S2016; panels b, and d). Plants were grown in a greenhouse for 95 days (panels a and b, actual data points in faint grey) or in the field between the time of planting (June 2017) until the second field assessment (June 2018) (panels c and d). Seedlings were treated with methyl jasmonate (MJ) before winter storage (M1), after (M2), or both before and after (M1 + M2); controls (C) received no MJ application at all (see Fig. 1). Seedlings with the physical barrier Conniflex were included as positive controls in the field experiment only. Different letters indicate significant differences among treatments that were significantly different at the 5% level (Tukey–Kramer tests). Significant letters are missing in panel b as some treatments had too few replicates for conducting such comparisons. Sample sizes (n) vary as some seedlings died during the measurement period

Field experiment

Similar to the lab experiment, we found that the timing and recurrence of MJ treatment significantly affected the amount of damage inflicted by pine weevils. By the first field assessment, which was 3 months after planting, pine weevils had damaged 44 seedlings (22.0%) in total, across 20 out of the 33 blocks. The attack rate was not significantly different among treatments and age cohorts, or their interaction (Table 2). With respect to pine weevil damage, we found significant differences in debarked area between the two age cohorts

Table 2 Results of analysis of deviance (df: degrees of freedom; Wald chi-squared statistic: χ^2 Wald Statistic; P: p-value) for generalized linear mixed models used to examine differences in seedling attack rate (plant attacked or not), plant mortality (alive or dead) and stem area debarked (Debarked area, mm^2) by pine weevils (*Hylobius abietis*) in a field experiment. The models included methyl jasmonate treatments (M1, M2, M1 + M2 and C; see Fig. 1 for an explanation), Norway spruce (*Picea abies*) seedlings belonging to two age cohorts (sown in 2015 or 2016), and their interaction. Two models were fitted, one for the 1st field assessment (year of treatment) and another one for the 2nd field assessment (year after treatment)

	Source of variance	Attack rate			Debarked area			Total mortality		
		df	$\chi^2_{\text{Wald Statistic}}$	P	df	$\chi^2_{\text{Wald Statistic}}$	P	df	$\chi^2_{\text{Wald Statistic}}$	P
1st field assessment (Sept. 2017)	Age cohort	1	0.24	0.624	1	20.79	**< 0.0001**	1	5.558	**0.018**
	Treatment	4	5.22	0.266	4	11.47	**0.022**	4	7.365	0.118
	Treatment × Age cohort	4 × 1	6.29	0.178	4 × 1	4.09	0.394	4 × 1	14.538	**0.006**
2nd field assessment (June 2018)	Age cohort	1	0.18	0.673	1	20.66	**<0.0001**	1	5.910	**0.015**
	Treatment	4	13.78	**0.008**	4	18.39	**0.001**	4	7.311	0.063
	Treatment × Age cohort	4 × 1	1.45	0.836	4 × 1	6.77	0.149	4 × 1	27.934	**<0.0001**

Statistically signficant effects are shown in bold ($P < 0.05$)

and among treatments (Table 2). Overall, MJ-treated seedlings received less damage than control seedlings (58.4% less damage; Fig. 2c, 2d). Seedlings with the physical barrier (Conniflex), which were included as a positive control, received the least damage (89.9% less than control; Fig. 2c, 2d). With regards to seedling age, the S2015 cohort as a whole received significantly more pine weevil damage than the S2016 cohort. Seedling mortality was mostly attributed to drought and girdling, while other causes were less common. The overall mortality of seedlings was significantly lower for the S2015 cohort (Table 2), while MJ treatment before storage (M1) and those treated consecutively (M1 + M2) for the S2016 cohort showed higher drought mortality compared to the other treatments (Fig. 4a, 4b).

By the second damage assessment, which was 1 year after planting, pine weevils had damaged 80 seedlings (26.7%) in total, across 30 out of 33 blocks. The attack rate was significantly different among treatments, but not between age cohorts or the interaction of treatment and age cohort (Table 2). Seedlings with the physical barrier (Conniflex) were less frequently attacked, while attack rate was similar among seedlings receiving MJ treatment (Table S2). With respect to pine weevil damage (area debarked), we found a similar pattern to that found in the lab experiment (Fig. 2a, 2b, 2e, 2f). The main effects of

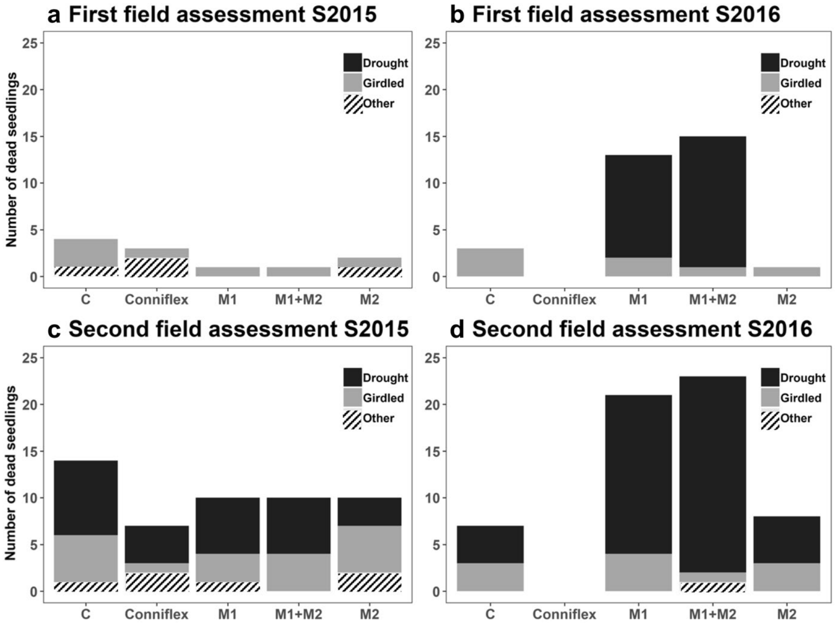

Fig. 4 Mortality (number of dead seedlings) of Norway spruce (*Picea abies*) seedlings belonging to two age cohorts (sown in 2015 or 2016) during two field assessments of pine weevil damage. A total of 33 seedlings for each treatment and age cohort (S2015: panels a and c; S2016: panels b and d) were planted in the field (panels a and b, first assessment of damage in Sept. 2017; panels c and d, second assessment of damage in June 2018). Seedlings were treated with methyl jasmonate (MJ) before winter storage (M1), after (M2), or both before and after (M1 + M2), controls (C) received no MJ application at all (see Fig. 1). Seedlings with the physical barrier Conniflex were included as positive controls. We categorized plants according to several possible causes of death: "Drought" included dead seedlings that appeared to have dried out but showed no signs of girdling; "Girdled" included dead seedlings for which an entire ring of barked had been removed by pine weevil feeding; "Other" included dead seedlings which died due other unknown reasons

treatment and age cohort on damage levels were significant (Table 2), and the overall pine weevil damage level for the S2015 cohort was significantly higher than that for the S2016 cohort. As in the lab experiment, seedlings with MJ treatment before winter storage (M1) received the least damage (78.6% less than control), and those with MJ treatment right before planting (M2) received the most damage (43.1% less than control) among all MJ treatments (Fig. 2e, 2f). We also found that the total mortality of seedlings almost doubled compared to what we observed during the first assessment, and the interaction of treatment and age was significant (Table 2). More girdled seedlings were found for the S2015 than the S2016 cohort, while higher mortality attributed to drought was observed for the S2016 cohort. This is mainly due to the high drought mortality of S2016 seedlings in the treatments M1 (MJ before winter storage treatment) and M1 + M2 (MJ treatment twice; before winter storage and before planting).

Seedling growth patterns in the field showed some similarity to those observed in the greenhouse. The height increment in the field was significantly affected by treatment, the interaction of treatment and age cohort, as well as initial height (Table S1). The height increment in the field showed similar patterns between the two age cohorts (Fig. 3c, 3d). For MJ treated plants, the height increment of the seedlings with MJ treatment only before planting (M2) eventually reached the same level as that of control seedlings, while the other two treatments (M1 + M2 and M2) still grew significantly less than control seedlings (Fig. 3c, 3d).

Discussion

Overall, our results show that MJ treatment reduces pine weevil damage and that the protective effects vary with the timing and recurrence of treatment, but not with the seedling age cohort. To our knowledge, this is the first study that has examined how an almost 1-year-long (~ 10 months) gap between MJ treatments can affect protection against pine weevil damage in Norway spruce seedlings. Our results show that a reduction in damage, comparable to that provided by currently used countermeasures such as physical barriers, can be achieved if MJ treatment is implemented at the right timing. Seedlings receiving MJ treatment before nursery winter storage the previous year, were least damaged by pine weevils both in lab and field. Recurrent treatment did not provide greater protection compared to treatments conducted at one occasion. We conclude that MJ treatment can be practically implemented in line with nursery practices and seedlings of different ages, below we discuss our findings in more detail.

Nearly all MJ treated seedlings in both lab and field experiments showed reduced pine weevil damage in terms of debarked area compared to control plants. This result is consistent with other studies that have used MJ to trigger induced defenses in seedlings of the same and other coniferous species (Moreira et al. 2009; Sampedro et al. 2010; Zas et al. 2014; Fedderwitz et al. 2015). The reduction in damage has been shown to be mediated, in part, by changes in feeding behaviour. For instance, pine weevils make fewer and smaller feeding scars on MJ treated Norway spruce seedlings compared to non-treated seedlings (Fedderwitz et al. 2015). Studies have also found that MJ-treated conifer seedlings have a lower risk of being girdled and this could be related to changes in the continuation (or not) of feeding by the pine weevils (Zas et al. 2014; Fedderwitz et al. 2015). Our results, thus, corroborate previous findings that MJ treatment can enhance seedling resistance against this insect pest.

However, the extent of damage reduction depends on the timing and recurrence of treatment. Our results indicated that MJ application before winter storage is the best timing for conducting treatment, as it offered the best protection to seedlings. A previous study testing MJ treatment at one and two occasions found that recurrent treatment provided better protection against pine weevils than single treatment (Zas et al. 2014). These results were in contrast to our study; however, they used a shorter time lag between their recurrent treatments (4 and 2 weeks before exposure to pine weevils). We found that consecutive MJ treatment across years was not necessary, as it did not result in a greater reduction of pine weevil damage than the MJ treatment conducted before storage. Similarly, another study did not find greater resistance to the pathogen *Diplodia pinea* when MJ treatment was repeated (Gould et al. 2009). Our results suggest that to enhance and maintain seedling resistance against pine weevil feeding, MJ application does not need to be recurrent or conducted right before exposure to this insect pest. Thus, our results may increase the potential of introducing MJ as plant protection tool in nurseries.

It is important to note that in our study, treatments with different timing and recurrences resulted in different doses of MJ, which could contribute to variation in its protective efficacy. The dose of MJ can be manipulated by either using the same concentration but different frequencies (or volumes) of spraying, or different concentrations but the same frequency (or volume) of spraying. Studies involving different MJ concentration but the same frequency of spraying, have shown that higher concentration triggers stronger induced chemical responses, with seedlings subsequently receiving less pine weevil damage (Moreira et al. 2009; Zas et al. 2014). Using the same concentration but repeated MJ treatment, also results in higher production of defensive chemical compounds and less pine weevil damage (Zas et al. 2014). In our study, treatments did not differ in concentration but resulted in different doses of MJ with respect to total volume of MJ (highest to lowest volume: M1 + M2 > M1 > M2). In line with previous studies, the treatment with the lowest dose (M2, 1 spraying) resulted in more pine weevil damage. But, in contrast to other studies, the treatment with highest MJ volume (M1 + M2) did not receive the least amount of pine weevil damage. Altogether, this suggests that the timing of application may be relatively more important than recurrence in mediating effective protection against the pine weevil.

Additionally, our results also indicate that differential short and long term effects of MJ on seedling resistance can occur based on the timing of treatment. We observed that seedlings treated with MJ only before planting (M2) received similar or slightly more damage compared to other treatments during the first growing season. Yet, these effects appear to have been short-lasting as these seedlings received much more damage during the second season. In another conifer species, Sitka spruce (*Picea sitchensis*), it has been shown that the emission of terpenoids reached a peak within a week after MJ treatment; defensive compounds like monoterpenoids, sesquiterpenoids, and diterpenoids accumulated in the outer stem tissue and reached their maximum about 1 month after MJ treatment (Miller et al. 2005). The same study and another study on Norway spruce found that terpene synthesis was still active 1 month after MJ treatment (Martin 2002; Miller et al. 2005). Furthermore, it also has been shown that MJ-induced resistance against insects or pathogens can be maintained for more than a year (Zas et al. 2014; Erbilgin et al. 2006; Zhao et al. 2010).

Following MJ treatment, increased plant resistance to pests can occur through two mechanisms: prolonged up-regulation of inducible defenses, and priming of defense responses (Wilkinson et al. 2019). Prolonged upregulation occurs when defenses remain elevated for a long period following MJ application, and provide resistance against subsequent attack. For instance, formation of anatomical defenses, such as resin ducts in Norway spruce and leaf trichomes in tomato, can remain at greater densities for weeks and years respectively, following MJ treatment (Boughton et al. 2005; Krokene 2015). On the other hand, defense priming can occur when defenses are sensitized following an initial stimulus, such as MJ. This sensitization allows inducible defenses to be rapidly and more strongly activated upon subsequent attack (Pastor et al. 2013; Wilkinson et al. 2019). For Norway spruce, a recent study showed that MJ treatment enhanced resistance against bark beetle colonization in mature trees, and these effects were mediated through defense priming (Mageroy et al. 2020). Our study does not allow us to discern among the mechanisms underlying greater seedling resistance after MJ application. However, prolonged upregulation of inducible defenses can be costly, especially compared to defense priming (Wilkinson et al. 2019). Given the observed reductions in seedling growth following MJ treatment (discussed below), this could be a more plausible mechanism mediating short- and long-term effects. However, a threshold level of MJ might be required to achieve longer term effects as plants receiving the lowest dose (M2) were less protected compared to other treatments during the 2 year (Fig. 2e,f).

In line with other studies, we observed the well-established negative effect of MJ treatment on growth (Heijari et al. 2005; Gould et al. 2009). These effects became evident shortly after treatment for those seedlings of which MJ was applied before planting (M1 + M2 and M2). The trade-off between growth and MJ-induced defense can also be seen in the longer term from the field growth results (Fig. 3c and 2e, 3d and 2f). How-ever, these differences are expected to even out with time as MJ-treated seedlings recover from this temporary growth loss (Zas et al. 2014). In addition to these growth reductions, we interestingly found differences in mortality among MJ treatments and their possible causes. Mortality caused by the pine weevil did not differ significantly among MJ treat-ments, while mortality due to drought seems to be affected by MJ and age cohort. MJ treat-ment may have alleviated drought-related mortality for the S2015 cohort, but it appears to have exacerbated it for the younger cohort (Fig. 4). This is an important factor to consider when evaluating MJ-mediated plant protection. Previous studies have documented vary-ing effects of MJ treatment on plant drought resistance and associated traits, such as root growth (Fedderwitz et al. 2019; Heijari et al. 2005; Moreira et al. 2012). Also, studies on cauliflower and soybean have shown that MJ application can alleviate drought stress (Wu et al. 2012; Mohamed and Latif 2017). However, its effects have been little studied in for-est regeneration as drought is considered to cause less mortality compared to biotic factors. Further studies will be required to tease apart the contribution of MJ treatment relative to other factors (e.g., age) in mediating drought susceptibility or tolerance.

Conclusion

We conclude that timing of MJ treatment is relatively more important than recurrence of treatment or seedling age for effective protection against pine weevil damage. Our results showed that MJ treatment can be conducted the year previous to planting and can con-fer protection comparable to standard physical barriers. Thus, the use of MJ is compatible with nursery practices. Despite the known initial growth reductions and any potential side effects (e.g. drought tolerance/susceptibility), the positive effects of MJ in reducing pine weevil damage should not be overlooked. MJ could be a complementary approach to other current or novel approaches to safeguard plant health in a sustainable way. Future studies should address how timing, recurrence and actual MJ dosage received by the plants mediate induced resistance and the duration of its efficacy.

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Author contributions AP, CB, FF and HB conceived and designed the experiment. YC conducted the experiment, carried out the statistical analysis and led the writing with input from AP, CB and HB. All authors contributed to subsequent revisions of the manuscript.

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Supplementary material

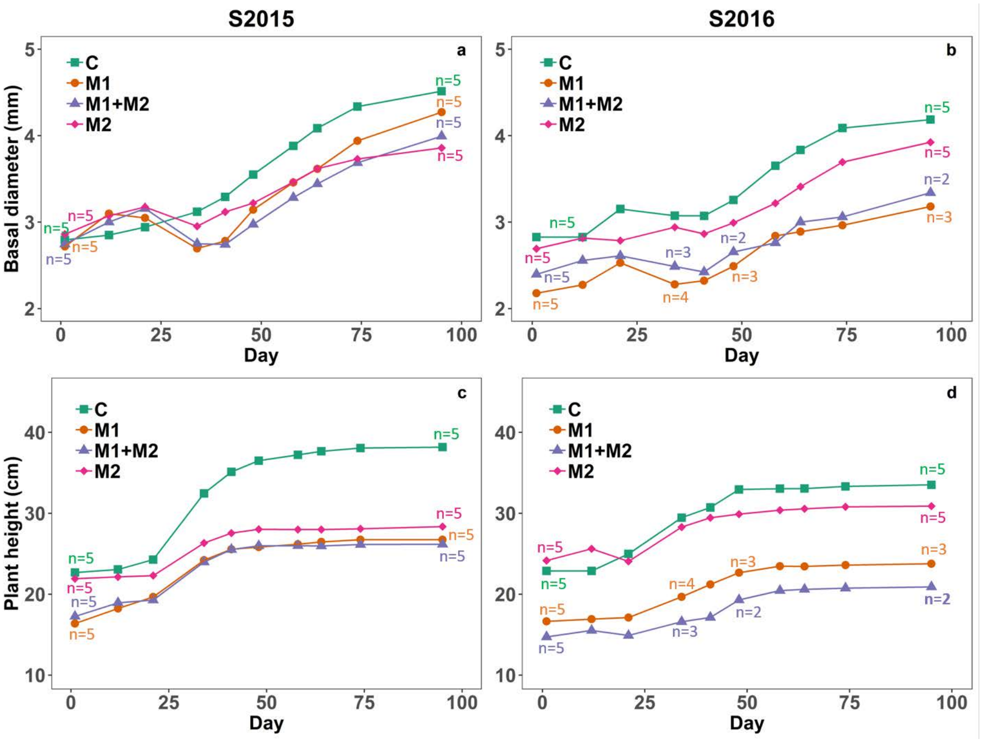

Fig. S1 Seedling basal diameter growth (mm, panels a and b) and height growth (cm, panels c and d) of Norway spruce (*Picea abies*) seedlings belonging to two age cohorts sown in 2015 (S2015; panels a and c) or 2016 (S2016; panels b and d). Seedlings were grown in a greenhouse for 95 days. The number of replicates (n) may decrease with time as some seedlings died before the end of the growth period. Seedlings were treated with methyl jasmonate (MJ) as follows: before winter storage (M1), after (M2), or both before and after (M1+M2); controls (C) received no MJ application at all (see Fig. 1). Seedlings treated with MJ in 2017 were sprayed one day before the first size measurement took place.

Table S1 Results of Analysis of Variance (ANOVA) (df: degrees of freedom; F: F-value; p: p-value) for models examining differences in height increment (final height subtracted with initial height, cm). The models included methyl jasmonate treatments (M1, M2, M1 + M2 and C; see Fig. 1 for an explanation) applied to Norway spruce (*Picea abies*) seedlings belonging to two age cohorts (sown in 2015 or 2016), and their interaction. Growth is shown for seedlings under greenhouse conditions for 95 days, and in the field from the time of planting (June 2017) until the second field assessment (June 2018). A linear model and a mixed generalized linear model were used to test the effect of age cohort and treatment on height increment in the greenhouse and field; initial height was included as a covariate. Significant effects (p<0.05) are in bold.

	Height increment in greenhouse			**Height increment in the field**		
Source of variance	**df**	**F**	**p**	**df**	**F**	**p**
Age cohort	1	9.650	**<0.0001**	1	0.06	0.940
Treatment	3	12.223	**0.005**	4	19.22	**<0.0001**
Initial height	1	0.036	0.851	1	2.55	**0.002**
Treatment × Age cohort	3 × 1	1.925	0.150	4 × 1	11.82	**0.036**

Table S2 Attack rate (number of attacked seedlings/total number of seedlings surveyed) by pine weevils (*Hylobius abietis*) of Norway spruce (*Picea abies*) seedlings belonging to two age cohorts (sown in 2015 or 2016). Attack rate is presented for each methyl jasmonate treatment (M1, M2, M1 + M2 and C; see Fig. 1 for an explanation) and for each field assessment. In the 1st field assessment (year of treatment), pine weevil damage was observed in 20 blocks and each treatment included 19-21 replicates. In the 2nd field assessment (year after treatment), pine weevil damage was observed in 30 blocks and each treatment × age combination included 30 replicates.

Age cohort	**Treatment**	**1st field assessment (Sept. 2017)**	**2nd field assessment (June 2018)**
S2015	C	6/20	11/30
	Conniflex	1/20	3/30
	M1	3/19	8/30
	M1+M2	4/20	9/30
	M2	9/20	12/30
	SUM	23/99	43/150
S2016	C	4/20	10/30
	Conniflex	3/20	4/30
	M1	7/20	10/30
	M1+M2	3/20	4/30
	M2	4/21	9/30
	SUM	21/101	37/150

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Comparing Exogenous Methods to Induce Plant-Resistance Against a Bark-Feeding Insect

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Exogenous application of the plant hormone methyl jasmonate (MeJA) can trigger induced plant defenses against herbivores, and has been shown to provide protection against insect herbivory in conifer seedlings. Other methods, such as mechanical damage to seedlings, can also induce plant defenses, yet few have been compared to MeJA and most studies lack subsequent herbivory feeding tests. We conducted two lab experiments to: (1) compare the efficacy of MeJA to mechanical damage treatments that could also induce seedling resistance, (2) examine if subsequent insect damage differs depending on the time since induction treatments occurred, and (3) assess if these induction methods affect plant growth. We compared Scots pine (*Pinus sylvestris*) seedlings sprayed with MeJA (10 or 15 mM) to seedlings subjected to four different mechanical bark damage treatments (two different bark wound sizes, needle-piercing damage, root damage) and previous pine weevil (*Hylobius abietis*) damage as a reference treatment. The seedlings were exposed to pine weevils 12 or 32 days after treatments (early and late exposure, hereafter), and resistance was measured as the amount of damage received by plants. At early exposure, seedlings treated with needle-piercing damage received significantly more subsequent pine weevil feeding damage than those treated with MeJA. Seedlings treated with MeJA and needle-piercing damage received 84% less and 250% more pine weevil feeding, respectively, relative to control seedlings. The other treatments did not differ statistically from control or MeJA in terms of subsequent pine weevil damage. For the late exposure group, plants in all induction treatments tended to receive less pine weevil feeding (yet this was not statistically significant) compared to control seedlings. On the other hand, MeJA significantly slowed down seedling growth relative to control and all other induction treatments. Overall, the mechanical damage treatments appeared to have no or variable effects on seedling resistance. One of the treatments, needle-piercing damage, actually increased pine weevil feeding at early exposure. These results therefore suggest that mechanical damage shows little potential as a plant protection measure to reduce feeding by a bark-chewing insect.

Keywords: simulated herbivory, root damage, methyl jasmonate, forest regeneration, true insect herbivory, wounding

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INTRODUCTION

Induced plant defenses can be triggered experimentally by exogenous application of methyl jasmonate (MeJA), a hormone naturally present in plants. MeJA is a methyl ester of jasmonic acid (JA), which is involved in one of three signaling pathways mediating stress responses in plants. These pathways are (1) the octadecanoid pathway, which relies on JA, (2) the shikimate pathway which mainly involves salicylic acid (SA), and (3) the ethylene pathway, which relies on ethylene molecules (Dicke and van Poecke, 2002; Kant et al., 2015). The octadecanoid pathway is most important for defense responses following insect damage (McConn et al., 1997; Kahl et al., 2000). In particular, MeJA has been shown to be involved in several plant processes such as root growth, damage signaling, and promoting plant defenses against chewing herbivores and necrotrophic pathogens (Creelman and Mullet, 1995; Thaler et al., 2012). Given its role in defense induction, exogenous MeJA application is increasingly being proposed as a plant protection strategy against various insect pests and pathogens (Moreira et al., 2012b; Zas et al., 2014; Yu et al., 2019). Inducing defenses with MeJA prior to exposure to pests has been shown to reduce levels of damage, negatively affect herbivores and increase the likelihood of plant survival. These effects have been found to occur not only in crops such as rice and soybean (Chen et al., 2018; Senthil-Nathan, 2019), but also in conifer seedlings (Zas et al., 2014; Jiang et al., 2016). Thus, it has great potential to become a practical tool within pest management and a sustainable alternative to insecticides in conifer plant protection.

Before MeJA can be promoted as a practical plant protection measure, it is necessary to consider how the use of MeJA compares to other methods of plant defense induction. Other methods to trigger plant induced defenses include previous insect feeding and mechanical damage (Mattiacci et al., 1994); but little is known on whether these responses are comparable to those induced by MeJA (Moreira et al., 2012b). Simulated herbivory, mainly by mechanical wounding and true insect herbivory, has been shown to cause defense-related responses in several plants, e.g., *Nicotiana sylvestris*, *Pinus resinosa*, and *Arabidopsis thaliana* (Baldwin, 1988; Mattiacci et al., 1994; Lombardero et al., 2006; Herde et al., 2013), and could potentially be used as a method of induction. Moreira et al. (2012b) showed that exogenous application of MeJA, mechanical stem wounding and real herbivory by the pine weevil *Hylobius abietis*, resulted in chemical defensive responses that were quantitatively similar in Maritime pine (*Pinus pinaster*) seedlings. Likewise, insect herbivory caused chemical and anatomical changes related to increased defense in Sitka spruce (*Picea sitchensis*) (Miller et al., 2005). In some plants, these defensive responses do not only happen in the damaged area, but also in undamaged parts (Wu and Baldwin, 2009). A few other studies have investigated the role of root damage on aboveground induced defenses. For example, in a study with oilseed rape (*Brassica napus*), belowground insect herbivory or mechanical damage to roots increased the proportion of indole glucosinolates in the leaves (Griffiths et al., 1994). Indole glucosinolates are defensive compounds that accumulate after damage in e.g., *A. thaliana* and other *Brassicacea* (Agerbirk et al., 2008). In general, less is known about the defense induction effects following belowground simulated herbivory (Erb et al., 2012).

Depending on the type and strength of the damage stimulus, plants can also be primed, so that once attack happens defense responses can occur more quickly and stronger (Wilkinson et al., 2019). Thus, it has been suggested that mechanical damage and previous insect herbivory can also serve as methods of defense induction to increase protection against insect pests. Regardless of the defense induction method used, most studies have focused on the extent of defensive chemical responses following induction (Miller et al., 2005; Moreira et al., 2012a), but very few have actually examined subsequent herbivory to corroborate that the induction method indeed provides efficient protection. To determine whether a method is suitable for plant protection against insect pests, a herbivore damage test following induction treatment is necessary.

Aside from its protective effects, induction methods that enhance plant resistance (such as MeJA) can result in a negative effect on growth. This trade-off can occur because plants have limited resources to be allocated among defense, growth, development and reproduction. Thus, when a plant invests more resources in defense, it is expected that less resources are available for other purposes (Herms and Mattson, 1992). Some studies have shown that application of MeJA/JA results in fewer fruits and seeds in tomato plants, and growth reductions in young conifers (Redman et al., 2001; Gould et al., 2009; Sampedro et al., 2011). In the case of tomatoes, however, fruits from JA-treated plants were larger than those from control plants (Redman et al., 2001). Thus, even if MeJA can result in a trade-off, a loss in growth or reproduction could be compensated by other benefits such as larger fruits or increased survival in the case of conifer seedlings (Redman et al., 2001; Zas et al., 2014). The effects of other induction methods on growth are less known, thus it would be of interest to investigate how such effects compare to those of MeJA.

Here, we examined and compared the efficacy of MeJA to other plant-resistance inducing methods, i.e., various kinds of mechanical damage and true insect herbivory, in providing plant protection against a bark-chewing insect. As a model system, we used the pine weevil-conifer seedling system as different studies have shown that application of MeJA enhances resistance of conifer seedlings against this herbivore (Zas et al., 2014; Fedderwitz et al., 2016; López-Goldar et al., 2020). Moreover, a study in *Pinus pinaster* examined chemical responses following mechanical stem damage, true insect herbivory and MeJA treatment, and the results showed that these induction methods all increased chemical responses to an equivalent magnitude (Moreira et al., 2012a). It would be interesting to test whether those observed changes in defensive chemistry eventually result in less insect feeding. The pine weevil, *H. abietis* (Coleoptera: Curculionidae), is an important pest of planted conifer seedlings at regeneration sites where forest stands are clear-cut. Adult pine weevils are attracted to these sites, because they use conifer stumps as breeding substrate. If seedlings are planted during the first 3 years after clear-cutting, the parental generation and their adult offspring will feed on the stem bark of these seedlings. The feeding can cause seedling deformations, reduced growth, and high seedling mortality (Leather et al., 1999). Given increasing restrictions on the use of insecticides due to environmental and human health issues (Lalík et al., 2020), there is timely incentive to explore methods of plant protection based on plants' intrinsic defenses and how they compare to each other.

Another factor that could be essential, yet rarely addressed in other studies, is the time interval from induction stimulus to exposure of plants to the insect pest. Various time intervals between MeJA treatment and exposure to the insect have been used in the pine weevil-conifer seedling system, with less pine weevil damage being observed a week, 1 month or even longer after MeJA treatment (Heijari et al., 2005; Sampedro et al., 2010; Fedderwitz et al., 2016; Chen et al., 2020). Several studies examining defensive chemical changes in conifer seedlings showed increased concentration of terpenes or resin 2 weeks, 4 weeks, or up to 1 month after MeJA treatment (Martin et al., 2002; Miller et al., 2005; Zas et al., 2014). Thus, time is also an important factor to consider when examining induced resistance responses after using different induction methods. For example, in a study with Loblolly pine (*Pinus taeda*), decreased resin flow was observed 1 day after wounding treatment but resin levels were higher than normal 30 days after mechanical treatment (Knebel et al., 2008). Thus, examining resistance at various time periods after induction stimulus could provide more comprehensive insight into how and when to apply these stimuli to achieve the greatest effect on plant resistance.

The purpose of the study was to investigate and compare how MeJA and other potential mechanical defense induction methods affect subsequent damage to conifer seedlings by the pine weevil. Resistance to pine weevil damage was used as a measure of the extent of induced resistance, with plants that were less damaged being considered to have experienced a greater induction following treatment. Additionally, we investigated if these effects depend on the time between induction stimulus (i.e., damage treatment) and exposure to weevils, and how the different treatments affect the growth of Scots pine seedlings. We chose two time intervals (12 and 32 days after stimulus) based on a pilot study and our previous studies on MeJA (e.g., Fedderwitz et al., 2016; Chen et al., 2020). Mechanical bark damage treatments (rectangular scars of different sizes inflicted on the stem, stem needle-piercing bark damage, and root damage) were chosen based on the types of damage that seedlings may encounter naturally, but exclude any chemical or microbial stimuli from the insect feeding. True insect herbivory was also included as a reference treatment. We intended to answer the following questions:

1. How does bark mechanical damage and previous herbivory treatments influence the levels of pine weevil damage to seedlings, relative to treatment with MeJA?
2. Do the effects of these treatments on pine weevil damage to seedlings differ depending on the time since induction occurred? More specifically, 12 and 32 days after treatment?
3. How is seedling growth affected following these non-MeJA treatments relative to when MeJA is used?

MATERIALS AND METHODS

Insect Material

To examine the induced resistance of seedlings following different treatments, we conducted two experiments where we subsequently exposed treated and control seedlings to pine weevils. The experiments were conducted in 2017 and 2020. The pine weevils used in these experiments were collected on May 27, 2017 and May 31, 2020, respectively, at the same sawmill (Balungstrands Sågverk AB, Enviken, Sweden) during their yearly migration. Before experiments, the weevils were kept in wooden rearing boxes in constant darkness at a room temperature of 10°C. Stems and branches from young Scots pine trees, and water tubes were placed inside each box; food and water were replaced once every month.

One week before the experiment, the pine weevils were brought for acclimatization from the cool dark room to the lab, where feeding tests were conducted (light-dark cycle 16 L/8 D, room temperature). The weevils were placed in plastic buckets with ventilated lids, and supplied with water and Scots pine branches. Female pine weevils were selected and placed individually in a Petri dish with a small Scots pine branch piece for 24 h. Those that fed on the branch during this period were selected and placed all together in a bucket, supplied only with water, in order to starve for 48 h before each feeding test.

Experiment 1: Plant Induction Treatments and Subsequent Feeding Tests at One Time Point

In order to test differences in resistance against pine weevils using different potential defense induction methods, six treatments (and undamaged controls) were applied to plants in order to trigger the induced defense of Scots pine seedlings. Since our regular nursery did not have enough seedlings, two provenances of Scots pine seedlings, known as Hade (plant height: 6–8 cm, Stora Enso Plantor AB, Sör Amsberg, Sweden) and Gotthardsberg (plant height: 7.8–13.5 cm, Stora Enso Plantor AB, Sjögränd, Sweden), were obtained from two nurseries instead. On July 17, 2017, seedlings were planted in round plastic pots (diameter: 14 cm) with commercial standard gardening soil (S-Jord, Hasselfors garden, Sweden) and kept in a greenhouse (light-dark cycle 16L/8D, temperature 20/16°C) for 1 month until the different treatments were applied. These two provenances were sown approximately at the same time of the year, and plants were 1-year-old when they were used in the experiment. However, Gotthardsberg has its origin further south in Sweden relative to Hade, and was larger in size when they were delivered to the lab.

After inflicting different potential induction treatments, area debarked by pine weevils in a feeding test was used as an inverse measure of induced resistance (seedlings receiving less damage were those considered to exhibit higher induced resistance). The following treatments were inflicted on plants 12 days before exposing them to pine weevils:

(1) Control (C): These seedlings (*n* = 34 for each provenance) received no damage at all.
(2) Methyl jasmonate treatment (MeJA): Seedlings (*n* = 34 for each provenance) were sprayed with MeJA. The concentration of MeJA (10 mM) was created by mixing MeJA (Sigma-Aldrich 95%, Ref. No. 392707) with a carrier solution of 2.5% (v:v) ethanol. Spraying was conducted once with a plastic bottle equipped with a spraying nozzle (Free-Syringe PC 1.5 liter, Jape Products AB, Hässleholm, Sweden), in a laboratory fume hood. The spraying bottle was pumped to reach the inner air pressure limit (2.5 bar) and shaken vigorously to mix the MeJA and carrier solution before each spraying occasion. Seedlings were placed in a row and the spraying nozzle was aimed horizontally at about 40 cm from the seedlings. The spraying bottle was moved manually along the seedling row, and the pots were turned 180° to spray the other side of the plants. Each seedling got approximately one second of spraying on each side, and all aboveground parts were moistened with the solution.
(3) Previous weevil feeding (WF): Seedlings (*n* = 34 for each provenance) were wounded by pine weevil feeding. One pine weevil was allowed to feed restrictively using a small custom-made cage with a transparent plastic tube (diameter: 10 mm; 25 mm long; and the top of the tube was sealed with holed plastic foil). A small opening was carved with a scalpel on each cage, to allow the pine weevil to feed on the area of the stem where the cage was attached. The cage and the pine weevil were removed when the insect had fed on about 50% of the circumference, with a vertical length of about 0.5 cm. The average scar area inflicted by the pine weevil (± standard error) was 32.2 ± 3.1 mm^2.
(4) Piercing-needle damage to the stem bark (P): Seedlings (*n* = 34 for each provenance) were needle-pierced with a handmade tool consisting of a row of five insect pins (No. 00, diameter 0.3 mm). The five pins were fixed on a 1 cm long straight line, with a 2∼4 mm gap between pins on an eraser. With the tool, five vertical holes could be created simultaneously in the stem bark. The depth of each hole reached the xylem of the stem. Fifty holes were created by ten repeated piercings right below where the lowest needles grow, and these were evenly spread out around the stem circumference. The piercing damage area was ∼14 mm^2 (area of each hole ($0.3^2 \times 3.14$) × 50 holes). This treatment imitates sap-sucking insect damage.
(5) Root bark damage (RD): Seedlings (*n* = 34 for each provenance) were wounded with a scalpel on the root bark. A rectangular scar was created on the main root bark right below the soil surface. The width of the scar was about 50% of the main root circumference, and the length was 0.5 cm. All phloem tissue was removed and the xylem was exposed within the scar. This treatment imitates damage by root-bark feeding insects such as *Hylastes* sp. beetles.
(6) Small stem window (WinS): A rectangular scar was inflicted on the stem of each individual seedling (*n* = 17 for each provenance) with a scalpel, and was located about 1 cm above the soil or right below where the lowest needles grow. The width of the scar was about 50% of the stem circumference and the vertical length was 0.5 cm. All phloem tissue was removed, and the xylem was exposed within the scar. The average scar area (± standard error) inflicted was 16.9 ± 4.2 mm^2. This treatment imitates pine weevil damage.
(7) Large stem window (WinL): A rectangular scar was inflicted on the stem of each individual seedling (*n* = 17 for each provenance) as described for the WinS treatment above, but the vertical length of the scar was 1 cm. The average scar area (± standard error) inflicted was 23.3 ± 4.2 mm^2. We originally intended to include only one treatment with one stem scar/window, but we considered that the wound may be too small to trigger induced resistance, and thus inflicted a larger scar on half of the seedlings (thus *n* = 17 for each stem window treatment per provenance).

Twelve days after the treatments, 288 seedlings in total were exposed to pine weevils in feeding tests. Each treatment included 48 seedlings (24 for each provenance), except treatment WinL and WinS which each included 24 seedlings (12 for each provenance). The remaining seedlings in each group were monitored for their height and diameter growth without exposure to pine weevils (see description below). Each seedling was exposed to one female pine weevil for 48 h. Potted seedlings and the corresponding pine weevil were enclosed by a plastic transparent cylinder with mesh net at the top to allow air flow, but prevent insects from escaping. After the feeding test, the number of feeding scars was recorded for each seedling, and the length and width of each feeding scar were measured using millimeter paper. Areas of all scars were added together to obtain the total stem area debarked per seedling. The number of girdled (when an entire ring of stem bark around the circumference is removed) seedlings were recorded as well. Due to limited lab space, the plant treatments and corresponding feeding tests were replicated in time and thus conducted in four consecutive rounds (two rounds per week). Each round consisted of 72 seedlings. Pine weevil individuals were not reused after each test.

A total of 120 seedlings were used to compare plant growth among treatments. For each treatment, 20 seedlings (10 for each provenance) were kept in a greenhouse for growth measurements [except treatment WinL and WinS which each included 10 seedlings (5 for each provenance)]. The settings in the greenhouse were 16 h light/8 h dark, and day/night temperature was 20/16°C. The aboveground height and basal diameter of seedlings was measured once a week for three consecutive weeks from August 15 to September 4, 2017. The first measurements of height and diameter were conducted 1 day before the different treatments were applied. The average height (± standard error) and diameter (± standard error) of the provenance Hade (height: 14.82 ± 0.24 cm; diameter: 2.18 ± 0.04 mm) was significantly lower than that of Gotthardsberg (height: 21.92 ± 0.47 cm; diameter: 2.46 ± 0.04 mm).

Experiment 2: Plant Induction Treatments and Subsequent Feeding Tests at Two Time Points

To further investigate if Scots pine resistance to weevils differed depending on the time since induction treatments were applied, we conducted a follow-up experiment. In this experiment, we examined five induction methods (in addition to non-damaged controls) and evaluated their effect on seedling resistance to pine weevil damage at two time points after treatment, 12 and 32 days. We included needle-piercing (P) of the stem bark and stem window damage (treatments P and WinS in experiment 1, respectively; average scar area Win S ± standard error: 17.8 ± 3.3 mm^2), previous pine weevil feeding (WF in experiment 1; average scar area ± standard error: 29.0 ± 3.7 mm^2), MeJA treatment (two levels: 10 mM and 15 mM) and an undamaged control group (C). The greenhouse settings and experimental set-up were the same as for experiment 1, except that only seedlings of the Hade provenance (average height: 13.21 ± 0.13 cm, diameter: 2.36 ± 0.02 mm; Stora Enso Plantor AB, Sör Amsberg, Sweden) were used. On July 16, 2020 (roughly a month earlier in the season relative to when experiment 1 was conducted), damage treatments were inflicted after seedlings had grown for 28 days in the greenhouse (planted on June 18, 2020). Seedlings were kept in this same greenhouse until it was time to expose them to pine weevils and evaluate induced resistance. The pine weevil exposure feeding tests were conducted 12 days after damage treatments (July 29, 2020, referred to as early exposure hereafter) for 5 of the treatments (8 seedlings × 5 treatments: C, 10 mM MeJA, 15 mM MeJA, WinS and Piercing), and 32 days after damage treatments (August 17, 2020, referred to as late exposure hereafter) for all six treatments (12 seedlings × 6 treatments: C, WF, 10 mM MeJA, 15 mM MeJA, WinS, and Piercing). Due to logistical challenges of restricting the amount of previous pine weevil damage (WF treatment) on seedlings and the limited number of seedlings available, we included this treatment only at late exposure. Each seedling was exposed to a starved female pine weevil for 48 h in the lab (light-dark cycle 16L/8D, room temperature), and damage inflicted was measured as described in experiment 1. Seedling height and basal diameter were measured once a week since they were planted. The room temperature during the feeding test was not recorded. However, data from the Swedish Meteorological and Hydrological Institute showed a different average air temperature for Uppsala, Sweden of 16.2 and 21.2°C, respectively, for feeding tests that happened in the early and late exposure groups.

Statistical Analyses

All statistical analyses were conducted in R software version 3.6.3 (R Core team, 2020) using R studio 1.2.5042 (RStudio Team, 2020), and graphs were generated using the *ggplot2* package (Wickham, 2016). Model fit was checked by visualizing residuals vs. predicted values using the *plot* command in R, and we found that models fitted well.

Experiment 1: Plant Induction Treatments and Subsequent Feeding Tests at One Time Point

We examined the effect of all treatments on area debarked by pine weevils, by fitting a linear mixed model (*lmer* command from *lme4* package, Bates et al., 2015). The model included treatment (C, MeJA, P, RD, WF, WinL, and WinS), plant provenance (Gotthardsberg, Hade), and their interaction, as fixed explanatory variables, round (n = 4, due to limited laboratory space the experiment was replicated in time with a total of four consecutive rounds) as a random variable, and seedling height (measured before pine weevil exposure) as a continuous covariate. A generalized linear mixed-effects model (*glmer* command from *lme4* package) was used to analyze the effect of treatment on number of scars (family = Poisson) and girdling (family = binomial) including the same explanatory variables as for area debarked. To analyze the effect of treatment on seedling height and diameter increment, a linear model (*lm* command from the base R *stats* package, R Core team, 2020) was used. Explanatory variables included treatment, provenance, the interaction of treatment and provenance, and seedling initial height (from the beginning of the growth observation period) as a covariate. After model fitting, significance of main effects and interactions was tested with analysis of deviance using the ANOVA command from the *car* package (Fox and Weisberg, 2019). When main effects were significant ($P < 0.05$), treatment means were compared using a Dunnett's test from the *contrast* command in the *emmeans* package (Lenth, 2020) and using treatment C and MeJA as reference levels.

Experiment 2: Plant Defense Induction Treatments and Subsequent Feeding Tests at Two Time Points

We examined the effects of treatment and the timing of exposure to pine weevils on seedling resistance by fitting several models. A general least square model (*gls* command from the *nlme* package, Pinheiro et al., 2020), which allows for heterogeneous error variance, was fitted with area debarked and the number of feeding scars as response variables. The explanatory variables in the models were treatment (C, MeJA 10 mM and 15 mM, P, WinS), timing of exposure (12 or 32 days) and their interaction as fixed explanatory variables, and plant height as a continuous covariate. The variance function *varIdent()* in the *weights* = argument was used to specify heterogeneous error variance for the two fixed variables.

As the number of treatments was different for the two time points (early and late exposure), we also examined the effect of treatment on seedling resistance separately for each time point. To examine differences in pine weevil area debarked and number of feeding scars at early exposure, we fitted a general least square model (*gls* command from the *nlme* package, Pinheiro et al., 2020) for each variable separately. The model included treatment (C, MeJA 10 mM and 15 mM, WinS, and P) and seedling height as explanatory fixed variables, and the variance function *varIdent()* in the *weights* = argument was used to specify heterogeneous error variance for treatments. After model fitting, treatment estimated means were calculated by using *emmeans* command in the *emmeans* package (Lenth, 2020).

To examine differences in area debarked by pine weevils at late exposure, a linear model (*lm* command from the default *stats* package, R Core team, 2020) was fitted with area debarked being square-root transformed. The model included treatment (C, MeJA 10 mM and 15 mM, P, WF, WinS) and seedling height (measured before pine weevil exposure) as explanatory variables. A negative binomial generalized linear model (*glm.nb* command from the *MASS* package, Venables and Ripley, 2002) was used when analyzing the effect of treatment on number of scars, and this included the same explanatory variables as for area debarked.

The effect of treatment on seedling height increment and basal diameter increment (increment = last measurement–the first measurement for each individual) were analyzed with a linear model (*lm* command from the default *stats* package, R Core team, 2020). In this model, the explanatory variables were treatment (C, MeJA 10 mM and 15 mM, P, WF, WinS) and plant initial height as a covariate.

After model fitting, significance of main effects and interactions were tested with analysis of deviance using the ANOVA command from the *car* package (Fox and Weisberg, 2019). When main effects were significant ($P < 0.05$), differences among treatment levels were examined using *emmeans* command in the *emmeans* package (Lenth, 2020). If main effects were not significant, estimated means were still obtained using the *emmeans* command and used for plotting figures.

RESULTS

Experiment 1: Plant Induction Treatments and Subsequent Feeding Tests at One Time Point

Area debarked by pine weevils did not differ among Scots pine seedlings exposed to different induction treatments (**Table 1** and **Figure 1A**), and these effects were consistent for the two seedling provenances examined (non-significant treatment × provenance interaction, **Table 1** and **Supplementary Figures 1A,B**). Likewise, the number of pine weevil feeding scars was not significantly affected by induction treatments (**Table 1** and **Figure 1B**). However, a significant interaction between treatment and provenance with respect to the number of feeding scars was found, with Hade receiving overall fewer scars (**Supplementary Figures 1C,D**). Moreover, Hade showed a significantly higher girdling rate than Gotthardsberg (21% vs. 14% of seedlings were girdled, respectively) in the feeding test (**Table 1** and **Supplementary Figure 2**). Seedlings in the piercing treatment were 22% more debarked in area than control seedlings, and significantly more debarked than seedlings with MeJA treatment (Dunnett's test, df = 248, t-ratio = 2.68, $P = 0.040$). All seedlings receiving previous pine weevil feeding (WF), and mechanical stem windows (WinL and WinS) were fed upon by pine weevils in the feeding tests, while a few seedlings from other treatments remained undamaged (**Supplementary Figure 2**). Moreover, previous pine weevil feeding (WF) and small stem window (WinS) resulted in similar levels of debarked area and number of scars compared to seedlings in the MeJA treatment (**Figures 1A,B**). In addition, seedlings in the MeJA, WF, and WinS treatments experienced reductions in area debarked of 18, 12, and 22%, respectively, compared to controls. Among the three treatments for which seedlings experienced slightly less debarked area compared to controls, seedlings with previous pine weevil feeding (WF) were more girdled than those in the MeJA and WinS treatments (girdling rate 37, 13, and 13% respectively) (**Supplementary Figure 2**).

In contrast to results from feeding tests, the growth of Scots pine seedlings varied significantly among induction treatments (**Table 2**). Multiple comparisons indicated that MeJA treated seedlings had a significantly lower height growth than seedlings in all other treatments (**Figures 2A,C**). The non-significant interaction of provenance and treatment indicated that height growth patterns were similar for the two provenances across treatments (**Supplementary Figures 3A,B** and **4**). Diameter growth was also significantly affected by the different defense induction treatments (**Table 2**), and it differed for the two provenances (**Supplementary Figures 3C,D**). Overall, seedlings treated with MeJA, and those that received root damage (RD), previous weevil feeding (WF), and small stem window (WinS)

TABLE 1 | Results of analysis of deviance (df: degrees of freedom; χ^2: Chi-square value; LR χ^2: likelihood ratio Chi-square value; P: P value) from several models examining the effect of treatment on subsequent pine weevil damage in experiment 1.

	Debarked area			Number of feeding scars			Girdling rate		
Source of variance	**df**	**χ^2**	***P***	**df**	**χ^2**	***P***	**df**	**LR χ^2**	***P***
Treatment	6	11.32	0.08	6	4.75	0.58	6	6.40	0.38
Provenance	1	6.38	**0.01**	1	26.28	**<0.01**	1	5.30	**0.02**
Height	1	2.23	0.15	1	0.41	0.52	1	0.10	0.75
Treatment × Provenance	6 × 1	8.23	0.22	6 × 1	20.04	**<0.01**	6 × 1	5.92	0.43

More specifically, these models examined the effect of different plant defense induction treatments [Large stem window damage (WinL), small stem window damage (WinS), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), 10 mM MeJA (MeJA), and undamaged seedlings as controls (C)] on levels of damage (area debarked, mm^2), number of feeding scars, and girdling rate by pine weevils (H. abietis) in Scots pine (P. sylvestris) seedlings for experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment).

The models included the fixed variables: treatment, provenance (Hade, Gotthardsberg), their interaction, and seedling height (cm, measured a day before feeding test) as a continuous covariate.

Significant effects ($P < 0.05$) are in bold.

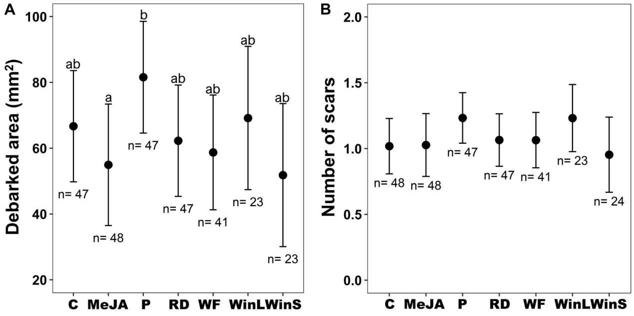

FIGURE 1 | Pine weevil damage to Scots pine (*P. sylvestris*) seedlings in experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment) after receiving different induction treatments [Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)]. **(A)** Estimated mean debarked area (±95% confidence intervals), and **(B)** Estimated mean number of feeding scars (±95% confidence intervals) for each treatment. Sample sizes (*n*) used in the statistical analyses are also shown. Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates.

did not differ in diameter growth. Yet, seedlings receiving needle-piercing damage (P) or a large stem window (WinL) grew significantly more in diameter than MeJA treated seedlings (**Figures 2B,D**).

Experiment 2: Plant Induction Treatments and Subsequent Feeding Tests at Two Time Points

Pine weevil damage to Scots pine seedlings differed among treatments, and between the two time points at which seedlings were subsequently exposed to pine weevils. Overall, seedlings were significantly more damaged at late exposure, than at early exposure (**Table 3**). At early exposure, seedlings in the needle-piercing treatment (P) received significantly more feeding damage (both in terms of debarked area and feeding scars) than seedlings in the MeJA treatment (15 mM), and it was the only group receiving 250% more damage by pine weevils relative to control seedlings (**Table 3** and **Figures 3A,B**). Seedlings in the MeJA treatments experienced a non-statistically significant reduction in area debarked (46% less for 10 mM, and 84% less for 15 mM) compared to control seedlings; while, seedlings in the WinS treatment received similar damage to controls (**Figure 3A** and **Supplementary Figure 5A**). Although not statistically significant, seedlings in the piercing and WinS treatments received 110 and 87% more feeding scars, respectively, than controls; while MeJA treated (10 mM MeJA and 15 mM MeJA) seedlings received 38 and 85% less scars, respectively, than controls (**Figure 3B** and **Supplementary Figure 5B**). In addition, many plants were not damaged at all at the early exposure, especially for seedlings that were treated with 15 mM MeJA, which had only one seedling damaged by pine weevil, while seedlings with needle-piercing had only one seedling undamaged. More than half of the seedlings treated with 10 mM and control seedlings were undamaged (**Supplementary Figure 6A**).

At late exposure, area debarked and number of feeding scars did not differ significantly among treatments (**Table 3** and **Figures 3A,B**). Seedlings in the control group, nonetheless, tended to receive the most pine weevil damage in terms of debarked area. Seedlings in the 10 mM MeJA, 15 mM MeJA, P, WF, and WinS treatments had 27, 32, 12, 17, and 23% less damage, respectively, than control seedlings (**Figure 3A** and **Supplementary Figure 5A**). The number of feeding scars was quite similar among treatments, and only seedlings with the previous pine weevil feeding (WF) and 15 mM MeJA treatments showed a 24 and 4% reduction, respectively, compared to controls. On the other hand, seedlings in the 10 mM MeJA, P, and WinS treatments received 15, 14, and 15% more scars than controls (**Figure 3B** and **Supplementary Figure 5B**). Across all treatment, the number of undamaged seedlings was lower, compared to those in the early exposure group (**Supplementary Figure 6**).

Similar to experiment 1, we found that different induction treatments significantly affected plant height and diameter growth (**Table 2**). Only seedlings treated with 15 mM MeJA grew significantly less (42% lower) in height than control seedlings. Seedlings treated with 10 mM MeJA and 15 mM MeJA grew significantly less (37 and 49%, respectively) in height than seedlings induced by previous pine weevil feeding (WF) (**Figure 4A** and **Supplementary Figure 7A**). For diameter growth, only seedlings treated with 15 mM MeJA grew significantly less (60%) than control seedlings. Seedlings receiving needle-piercing (P), previous pine weevil feeding (WF), and small window damage (WinS) treatments grew slightly more in diameter (21, 10, and 15% more, respectively) than control seedlings. Seedlings in these three treatments grew significantly more than seedlings treated with MeJA (10 and 15 mM) (**Figure 4B** and **Supplementary Figure 7B**). We also noted that wounds created by the different induction treatments had healed completely, or healed to at least half the original damaged area, by the time of late exposure (**Supplementary Figures 8A–C**).

TABLE 2 | Results of analysis of variance (ANOVA) (df: degrees of freedom; *F*: *F*-value; *P*: *P*-value) from several linear models examining the effect of treatments on plant growth in experiments 1 and 2.

		Height increment			**Diameter increment**		
	Source of variance	**df**	***F***	***P***	**df**	***F***	***P***
Experiment 1	Provenance	1	18.86	**<0.01**	1	0.28	0.61
	Treatment	6	15.36	**<0.01**	6	3.02	**<0.01**
	Initial height	1	5.46	**0.02**	1	14.33	**<0.01**
	Treatment × Provenance	6	1.72	0.10	6	3.28	**<0.01**
	Residuals	102			101		
Experiment 2	Treatment	5	4.63	**<0.01**	5	9.18	**<0.01**
	Initial height	1	0.22	0.64			
	Initial diameter				1	0.036	0.85
	Residuals	63			65		

More specifically, these models examined the effect of different plant defense induction treatments [Large stem window damage (WinL), small stem window damage (WinS), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), 10 mM MeJA (MeJA), and undamaged seedlings as controls (C)] for experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment), and [Small stem window damage (WinS), needle-piercing damage to the stem bark (P), previous weevil feeding damage (WF), 10 mM MeJA, 15 mM MeJA, and undamaged seedlings as controls (C)] for experiment 2 (Insect feeding tests were conducted at two time points, 12 and 32 post-treatment) on growth (height increment, cm, and diameter increment, mm) in Scots pine (P. sylvestris) seedlings.

The models included as explanatory variables: treatment, provenance (only included in experiment 1, Hade and Gotthardsberg), their interaction, and initial seedling height (cm) or initial seedling diameter (mm) (both were measured on the day of planting) as a continuous covariate.

Significant effects ($P < 0.05$) are in bold.

DISCUSSION

Our study showed that simulated bark damage as treatments for potential plant defense induction can affect levels of pine weevil damage to Scots pine seedlings. However, these effects varied depending on the type of damage inflicted and when plants were exposed to the insects after induction stimulus occurred. We found that none of the mechanical induction methods increased seedling resistance to a greater extent than MeJA, and that a shorter time between the induction stimulus and exposure to pine weevils resulted in lower damage levels. One type of stem damage, needle-piercing, even increased subsequent feeding by pine weevils to very high levels relative to all other treatments. In terms of growth, only MeJA negatively affected seedling height growth and diameter relative to the control group, in line with previous studies. All in all, our results indicate that the previous damage treatments evaluated in this study do not provide enhanced seedling resistance to bark-feeding insect damage. We discuss our findings below.

Even though studies on other conifer plants have shown that both mechanical wounding and insect herbivory can trigger induced defensive responses (Miller et al., 2005; Moreira et al., 2012a), our bark damage treatments did not result in significantly greater seedling resistance. One explanation could be that even if defensive chemistry is enhanced or altered, these changes are not enough to sufficiently deter pine weevil feeding. Previous studies have not exposed mechanically damaged plants to subsequent insect feeding, and have assumed that increased defensive chemistry responses will result in less feeding (i.e., greater resistance). Our results show that this assumption may not always be true. Exposure to the pest after induction stimulus is essential if these methods are being evaluated for use within plant protection. Our study directly examined the extent of protection provided by previous mechanical damage, and we find that it is not sufficient against damage by a bark-chewing insect. Another factor that could also be important is the extent of damage or tissue loss. A recent study on tobacco (*Nicotiana tabacum*) plants showed that the amount of leaf tissue loss is important for the level of defense induction (Lin and Felton, 2020). The authors found higher levels of trypsin protease inhibitors (which result in anti-nutritive effects and reduced insect herbivore growth) in plants subjected to whole leaf removal relative to partial leaf damage (Lin and Felton, 2020). On the other hand, a study with 1-year old Scots pine seedlings found that moderate and severe mechanical stem damage resulted in similar negative effects on plant morphology and physiology (Bansal et al., 2013). Seedlings received either one (moderate damage) or two (severe damage) window-like stem bark scars (inflicted with a scalpel), and each scar was about 10 mm in length and covering 1/3 of the stem circumference (Bansal et al., 2013). These scars are similar and even slightly greater in total area to our WinS and WinL treatments, for which we inflicted scars of 5 or 10 mm in length, respectively, across 1/2 of the stem circumference. The authors found that both treatments significantly reduced photosynthesis, needle mass and needle area relative to undamaged controls (Bansal et al., 2013). In our study, seedlings in the WinS treatment received similar pine weevil damage to those in the MeJA treatment, and even received less damage (albeit non-statistically significant) than those with larger window wounds (experiment 1, WinS and WinL, **Figure 1A**). Our results and those of Bansal et al. (2013) suggest that greater tissue loss or damage may instead be detrimental for the plants, and would not necessarily result in greater enhanced resistance to subsequent insect feeding. However, evaluation of a broader range of stem damage levels may be needed to conclusively elucidate if the extent of tissue loss plays a role.

In addition to stem bark damage, root herbivory has also been shown to trigger subsequent defensive responses in aboveground plant tissues, e.g., in cotton *Gossypium herbaceum* (Bezemer et al., 2004) and tobacco *N. tabacum* (Kaplan et al., 2008b). After belowground damage occurs, it has been observed that a reduction in herbivore growth rate, body size and food consumption of aboveground herbivores can occur (Bezemer et al., 2003; Soler et al., 2005; Van Dam et al., 2005). Thus, root herbivory has the potential to decrease overall plant damage levels of aboveground herbivores. However, there are also cases where it has not resulted in increased resistance aboveground. The magnitude of defensive responses in aboveground tissue may not be large or effective enough to decrease herbivore damage, as in the case of cotton *G. herbaceum* (Bezemer et al., 2004). Moreover, some aboveground herbivores may even benefit from belowground herbivory and inflict more damage

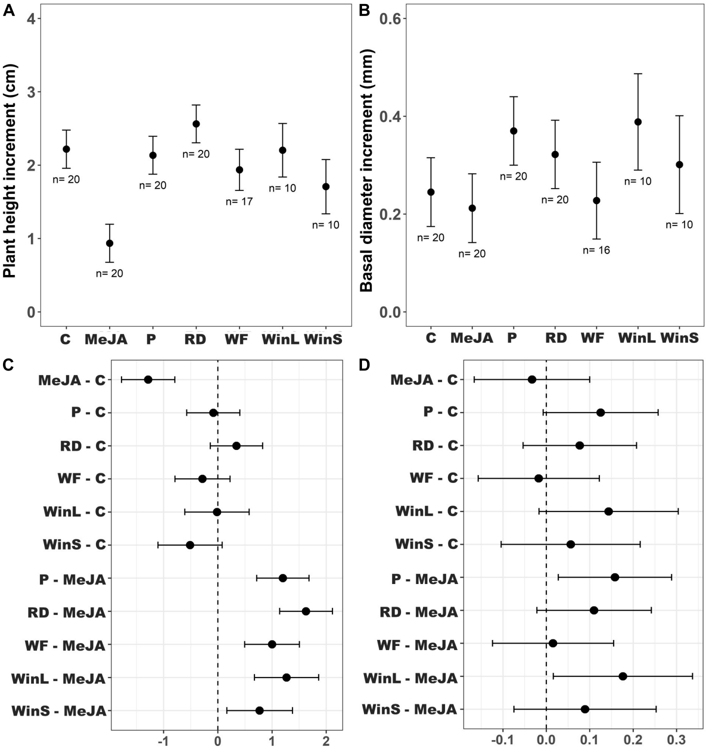

FIGURE 2 | Growth increment of Scots pine (*P. sylvestris*) seedlings and treatment mean comparisons using Dunnett's test among different plant defense induction treatments [Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)] in experiment 1. Insect feeding tests were conducted at one time point, 12 days post-treatment. The growth of seedlings was followed for 21 days post-treatment. Sample sizes (*n*) used in the statistical analyses are also shown. **(A)** Estimated mean plant height increment (cm ± 95% confidence intervals), **(B)** estimated mean basal diameter increment (mm ± 95% confidence intervals), **(C)** differences between treatments in estimated mean height increments (cm ± 95% confidence intervals), and **(D)** differences between treatments in estimated mean diameter increments (mm ± 95% confidence intervals). If an interval does not include zero, the difference between estimated means is considered to be statistically significant.

(Soler et al., 2007; Kaplan et al., 2008a). In our study, we found that root-damaged seedlings tended to grow more (15% more in seedling height increment and 33% more in basal diameter increment) and received 7% less weevil damage (debarked area) compared to control seedlings, but this difference was not statistically significant. This result suggests that the extent of root tissue loss may not have been large enough to trigger aboveground defensive responses that affected the pine weevils.

In contrast to all other induction treatments, seedlings in the needle-piercing treatment received much greater damage levels (as extreme as 250% more damage) than controls. Pine weevils are known to be attracted to the odors or compounds emitted by recently damaged seedlings (Nordlander, 1991). Given the multiple wounds that the needle piercing treatment inflicted on the stem, it could be possible that it stimulated their feeding. Even though two other treatments also inflicted large wounds (WinS and WinL), increased damage levels to the extent of those receiving piercing damage, were not observed for seedlings in these treatments. This indicates that patterns of damage are also relevant and can differentially influence pine weevil feeding behavior. However, we noted in experiment 2 that needle-piercing wounds had healed by the late exposure and at this time point, seedlings received somewhat less pine weevil damage than controls. This suggests that the cues emitted by freshly damaged seedlings could stimulate feeding, but decrease with time.

Although none of the previous damage treatments enhanced seedling resistance to a greater extent than MeJA, treatment with MeJA was also not as significantly effective as reported in previous studies. Only seedlings treated with a higher MeJA concentration (15 mM) were significantly less damaged

TABLE 3 | Results of analysis of deviance (df: degrees of freedom; χ^2: Chi-square value; LR χ^2: likelihood ratio Chi-square value; *P*: *P*-value) from several models examining the effect of treatment on subsequent pine weevil damage in experiment 2.

	Source of variance	**Debarked area**			**Number of feeding scars**		
		df	**F/χ^2**	***P***	**df**	**LR χ^2**	***P***
Both time points	Time point	1	82.36	**<0.01**	1	88.83	**<0.01**
	Treatment	4	13.05	**0.01**	4	22.07	**<0.01**
	Height	1	0.36	0.55	1	1.13	0.29
	Time point × Treatment	4	2.88	0.72	4	1.24	0.87
12 days after induction treatment	Treatment	4	12.53	**0.01**	4	18.85	**<0.01**
	Height	1	0.24	0.62		0.23	0.63
32 days after induction treatment	Treatment	5	0.29	0.92	5	6.67	0.25
	Height	1	1.41	0.24	1	8.06	**<0.01**

More specifically, these models examined the effect of different plant defense induction treatments and time point of exposure to pine weevils since induction, on area debarked (mm^2) and number of scars in experiment 2 (Insect feeding tests were conducted at two time points, 12 and 32 days post-treatment).

Models included treatments [Small stem window damage (WinS), needle-piercing damage to the stem bark (P), previous weevil feeding damage (WF), 10 mM MeJA, 15 mM MeJA and undamaged seedlings as controls (C); previous weevil feeding damage (WF) was not included at 12 days after induction], time point (12 or 32 days after induction), their interaction and plant height as a continuous covariate.

Significant effects (P < 0.05) are in bold.

compared to seedlings in the piercing-needle treatment after early exposure. It could be that the dose (the net amount and frequency of MeJA treatment) we used could partly explain our results. The effect of MeJA treatment on pine weevil damage has been shown to be dose dependent (Moreira et al., 2009; Zas et al., 2014). In one of our previous experiments, a higher dose of MeJA (three consecutive sprayings of 10 mM MeJA) resulted in greater Norway spruce resistance to pine weevil damage relative to plants receiving a lower dose (one spraying of 10 mM MeJA) (Chen et al., 2020). The low dose of MeJA in our previous study on Norway spruce was the same as the low dose used in this study on Scots pine seedlings, and the amount of debarked area received by these two conifer species were similar in both studies. Other studies have also used higher doses and concentrations, which have resulted in greater resistance to pine weevil damage. For example, MeJA concentrations of 100, 40, and 22 mM were used on Maritime pine (*P. pinaster*) (Moreira et al., 2009; Moreira et al., 2012a,b), 50 mM MeJA on Norway spruce (Fedderwitz et al., 2016), and 25 mM MeJA on Maritime pine, Monterrey pine (*Pinus radiata*), Scots pine and Norway spruce (Zas et al., 2014). Therefore, it appears that the MeJA dose in this study was not enough to significantly reduce pine weevil damage. Moreover, it seems that induced resistance can be better achieved by several sprayings of MeJA at lower concentrations instead of one application with a higher concentration. Concentrations higher than 10 mM can be detrimental to seedlings, and result in treatment-related damage (e.g., loss of needles, needle browning in Norway spruce; Fedderwitz et al., 2019), and we indeed observed some needle-browning in seedlings treated with 15 mM MeJA (**Supplementary Figure 8D**). Our results are thus, an important contribution to development of methods for optimum use MeJA as a seedling protection tool. Finding the MeJA treatment concentration and frequency that provides effective resistance, minimizes phytotoxicity and is compatible with nursery needs

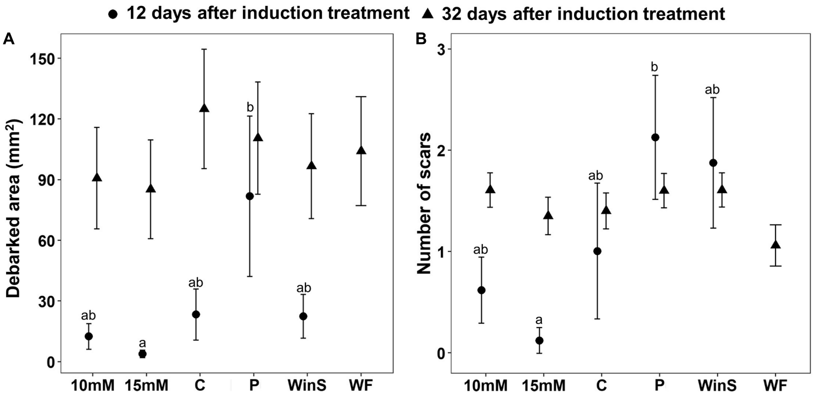

FIGURE 3 | Pine weevil damage to Scots pine (*P. sylvestris*) seedlings after receiving different induction treatments [10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF, not included in the weevil exposure test conducted 12 days post-treatment)] in experiment 2. Plants that were exposed to pine weevils 12 days after induction treatments are referred to as early exposure, and those that were exposed 32 days after, are referred to as late exposure in the main text. **(A)** Estimated mean debarked area (mm^2 ± standard error), and **(B)** Estimated mean number of feeding scars (±standard error). Sample size (n) is equal to 8 for all other treatments, except that the Control group (C) is equal to 7 for the early exposure, and $n = 12$ for all treatments for the late exposure. Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates.

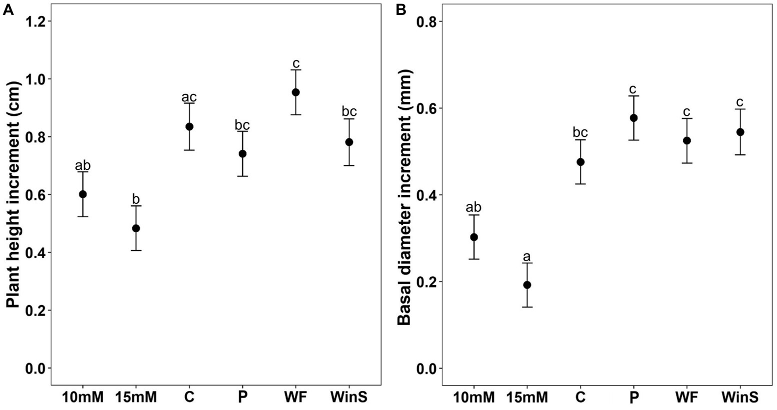

FIGURE 4 | Growth increment of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments [10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF)] in experiment 2 ($n = 12$ per treatment, insect feeding tests were conducted at two time points, 12 and 32 days post-treatment). The growth of seedlings was followed for 32 days post-treatment. **(A)** Estimated mean height increment (cm ± standard error), and **(B)** Estimated mean diameter growth (mm ± standard error). Different letters above means indicate statistically significant differences ($P < 0.05$) between mean estimates.

and practices is essential for MeJA implementation (Fedderwitz et al., 2019, Chen et al., 2020).

Timing since induction stimulus is also an essential factor in development of plant protection strategies aimed at increasing seedling resistance prior to pest exposure. We found that the effect of previous damage and MeJA on triggering seedling resistance to pine weevils differed depending on the time since treatment. Overall, we found that plants exposed early to pine weevils (12 days after treatment) received less damage relative to those in the late exposure group (32 days after treatment). These results could indicate that treatment effects were short-lasting and tended to lose their efficacy with time. As discussed in previous paragraphs, an explanation could be that the extent of tissue loss/damage could play a role and that MeJA doses used were not enough to induce effective resistance. We observed that seedlings in the 10 mM and 15 mM MeJA groups, were often not eaten by weevils at all (**Supplementary Figure 6**) or received considerably less pine weevil feeding damage at 12 days relative to 32 days after MeJA application. Seedlings in other induction treatments also showed a similar tendency, with less damage at 12 days relative to 32 days but not as pronounced as for those in the MeJA group. Thus, if a peak in induced resistance occurs, this peak is likely closer to 12 days rather than 32 days after treatment.

Another potential cause for the different damage levels at these two time points could be that pine weevil feeding behavior differed. The average air temperature in Uppsala, Sweden at the time when the late exposure occurred (average air temperature: 21.2°C) was 5°C higher than during the early exposure (average air temperature: 16.2°C), according to data from Swedish Meteorological and Hydrological Institute (station 97510 Uppsala Aut, 59°50′50″N, 17°37′55″E, SMHI, 2020). The weevils were acclimatized in the lab for a week before the feeding test, thus, weevils in the late exposure group might have been affected by the warmer room temperature compared to those in the early exposure group. Pine weevils have been shown to consume almost four times more bark of Scots pine twigs at 20°C compared to 15°C (Leather et al., 1994). The behavior of pine weevils may thus, have been affected by a higher room temperature and resulted in increased feeding at the late exposure time point. All in all, from a plant protection perspective, our results on the timing of induction suggest that it may be better if the treatment stimulus occurs closer rather than further from pest exposure. However, additional studies where temperature is controlled for, and different levels of tissue loss and other time points since treatment are included, would help to tease apart their effect on seedling resistance.

We were also interested in examining any potential cost of induction treatments with respect to plant growth. As documented in other studies on MeJA-induced plant defense (Heijari et al., 2005; Vivas et al., 2012), we observed a significant growth reduction in seedlings receiving MeJA treatment compared to the control seedlings. Such a trade-off between growth and defense has indeed been found for seedlings of several coniferous species, e.g., Maritime pine (Moreira et al., 2012b), Monterrey pine (Gould et al., 2008), and Norway spruce (Chen et al., 2020). Also, in line with other studies, we found that growth was even more reduced for plants receiving the higher concentration of MeJA (15 mM). This suggests that resources were diverted away from growth and presumably invested in defense, yet it only resulted in a slight reduction in area debarked relative to seedlings in the control group.

Some of the non-MeJA treatments also exhibited different relationships between growth and resistance. For example, seedlings in the piercing treatment (P) and large window (WinL) treatments received relatively higher subsequent pine weevil damage compared to all other treatments in experiment 1. Yet, there was a slight reduction in height growth and even a tendency to grow more in diameter compared to the control group. This is in line with another study that Scots pine seedlings with stem bark damage had significantly more radial growth compared to undamaged controls (Bansal et al., 2013). Moreover, seedlings with root damage showed a tendency to grow more in height, compared to control seedlings. A study on field corn *Zea mays* showed that plant dry weight was greater for plants damaged by the western corn rootworm (*Diabrotica virgifera*) relative to those not experiencing any root damage (Godfrey et al., 1993). It is also possible that the non-MeJA treatments do not induce but instead "prime" seedling defenses, which is much less costly compared to fully inducing defenses (Wilkinson et al., 2019). We observed, for example, a slight reduction in debarked area for seedlings in other non-MeJA induction methods after the late exposure, but no growth reduction compared to controls. This is in contrast to plants receiving MeJA treatments, especially at the high concentration, which exhibited distinct growth reductions, and only a reduction of 20–30% in area debarked compared to control seedlings. However, we are not able to discern from our study which of these mechanisms was involved.

CONCLUSION

Our study showed that bark damage induction treatments and a low dose of MeJA did not effectively increase the resistance of Scots pine seedlings. Induction methods that include needle-piercing stem wounding can even be detrimental for seedlings, as we found that this type of damage resulted in even more damage by pine weevils relative to all other treatments. Apart from MeJA treatments, none of the damage treatments had negative effects on seedling growth in terms of height and diameter. All in all, our results suggest that mechanical damage may not be sufficient to trigger induced resistance responses that provide adequate seedling protection. Thus, these methods of induction would not be suitable for larger scale implementation to protect conifer seedlings. Instead, improving the use of MeJA and finding optimal concentrations that enhance resistance but minimize negative effects, remains as a promising alternative. Nonetheless, further studies varying the degree of tissue loss as well as the time period between induction treatment and insect exposure, would be of interest. In addition, studies that examine the levels of chemical defense in seedlings following the treatments and subsequent exposure to insect feeding in both lab and the field, are needed to enhance our knowledge on the mechanisms of induced defense in conifer seedlings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

AUTHOR CONTRIBUTIONS

AP, CB, and HB conceived and designed the experiment. AB carried out the pilot experiment as part of her master's thesis. AP, CB, HB, and YC re-evaluated and improved the experimental design. YC conducted the experiment, carried out the statistical analyses, and wrote the draft of the manuscript with input from AP, CB, and HB. All authors contributed to subsequent revisions of the manuscript and agreed to publish the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.695867/full#supplementary-material

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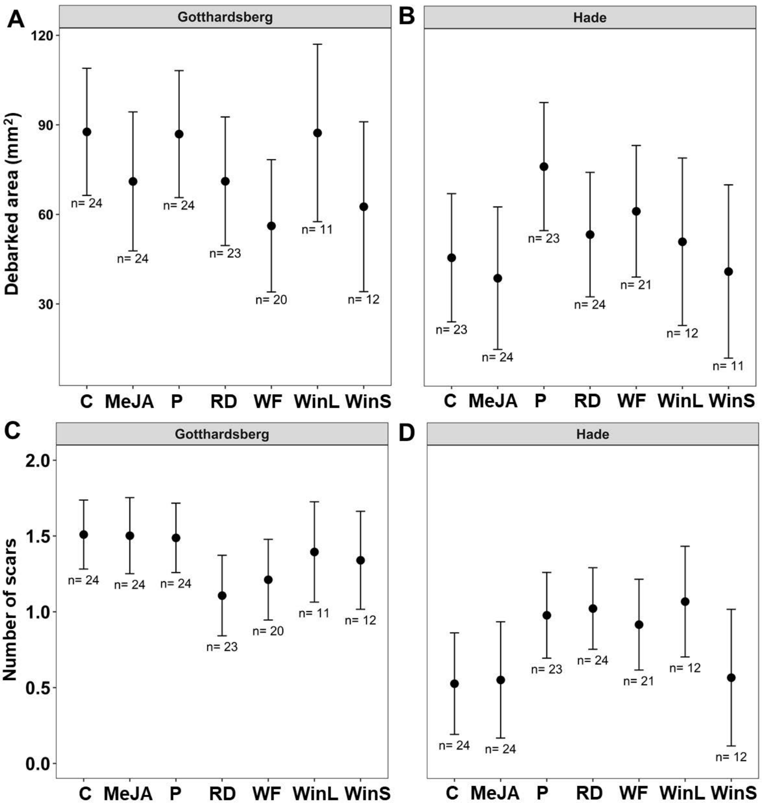
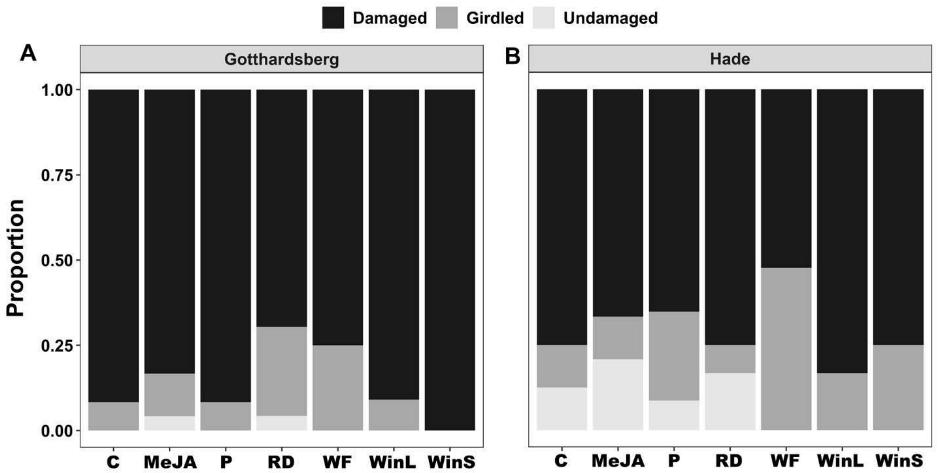
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

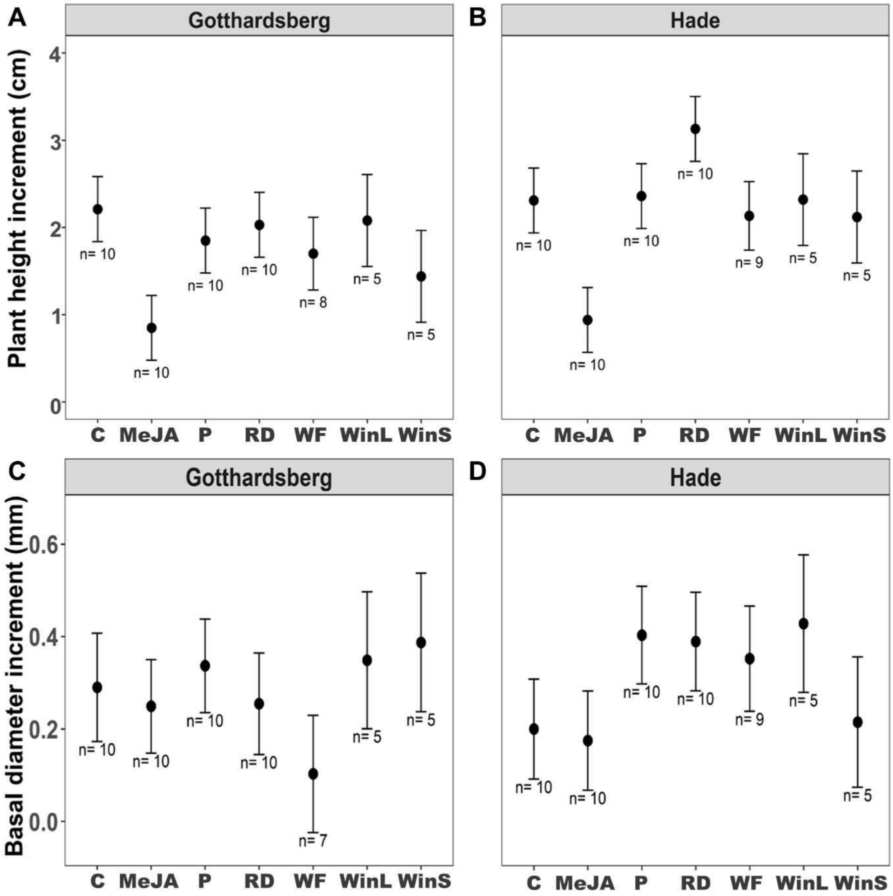
Supplementary Figures



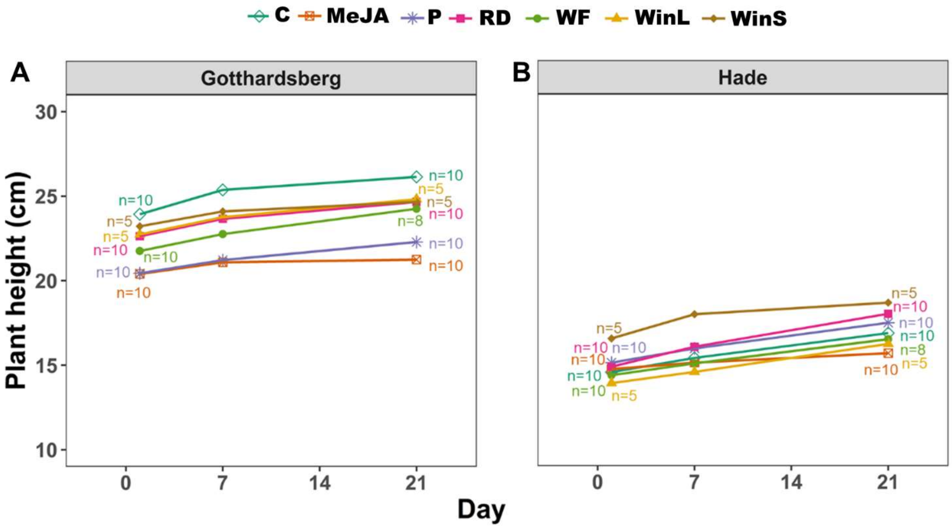
Supplementary Figure 1. Estimated mean debarked area (mm^2 ± 95% confidence intervals) and number of feeding scars (± standard error) for two provenances of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments (Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)) in experiment 1. Insect feeding tests were conducted at one time point, 12 days post-treatment. Panels **(A)** and **(C)** show the provenance Gotthardsberg, debarked area and number of scars; panels **(B)** and **(D)** show provenance Hade, debarked area and number of scars. Sample sizes (n) used in the statistical analysis are also shown.



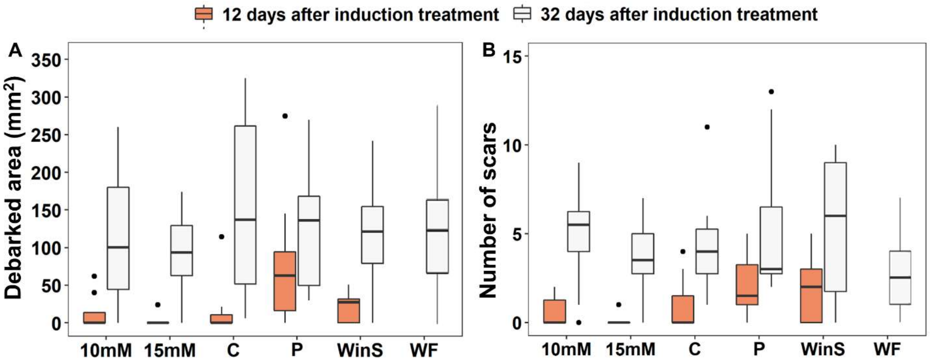
Supplementary Figure 2. Proportion of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments (Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)) that were undamaged, damaged and girdled for two provenances during experiment 1 (insect feeding tests were conducted at one time point, 12 days post-treatment). **(A)** Provenance Gotthardsberg and **(B)** Hade. "Damaged" refers to seedlings that received pine weevil damage but were not girdled during the feeding test; "Girdled" refers to seedlings for which an entire ring of stem bark around the circumference was removed by the pine weevil; "Undamaged" refers to seedlings that did not receive any pine weevil damage during the feeding test.



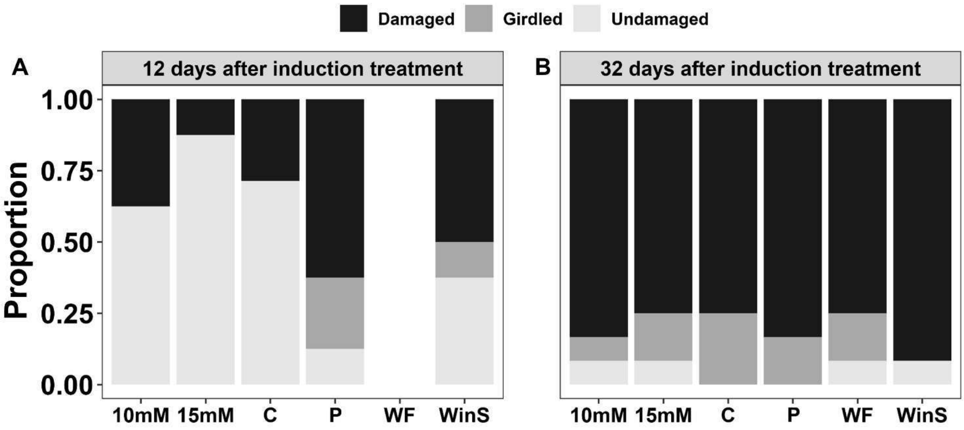
Supplementary Figure 3. Estimated mean height increment (cm ± 95% confidence intervals) and diameter increment (mm ± 95% confidence intervals) for two provenances of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments (Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)) in experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment). Panels **(A)** and **(C)** show provenance Gotthardsberg, plant height and diameter; panels **(B)** and **(D)** show provenance Hade, plant height and diameter. The growth of seedlings was followed for 21 days post-treatment. Sample sizes (n) used in the statistical analyses are also shown.



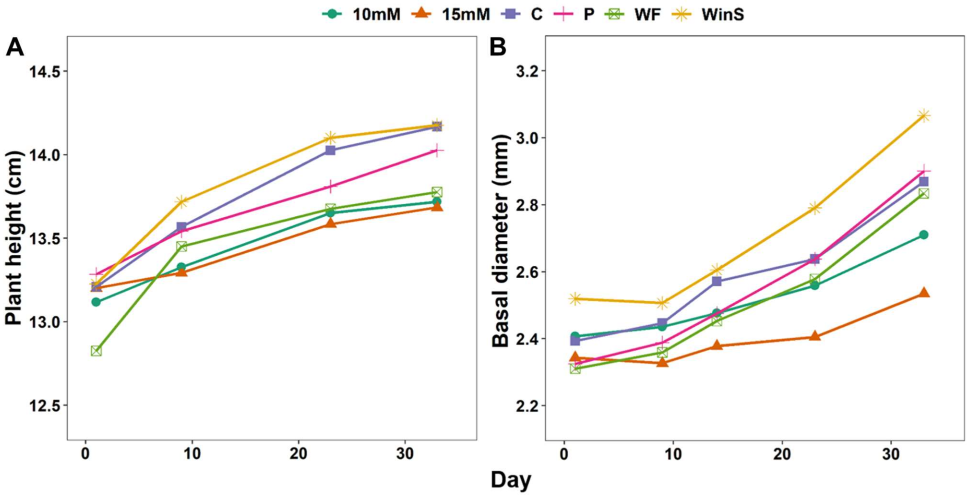
Supplementary Figure 4. Height of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments (Undamaged seedlings as controls (C), 10 mM MeJA (MeJA), needle-piercing damage to the stem bark (P), root bark damage (RD), previous weevil feeding damage (WF), large stem window damage (WinL), and small stem window damage (WinS)) in experiment 1 (Insect feeding tests were conducted at one time point, 12 days post-treatment). **(A)** Provenance Gotthardsberg and **(B)** Hade. Weekly measurements were conducted over 21 days, but data for day 14 is missing.



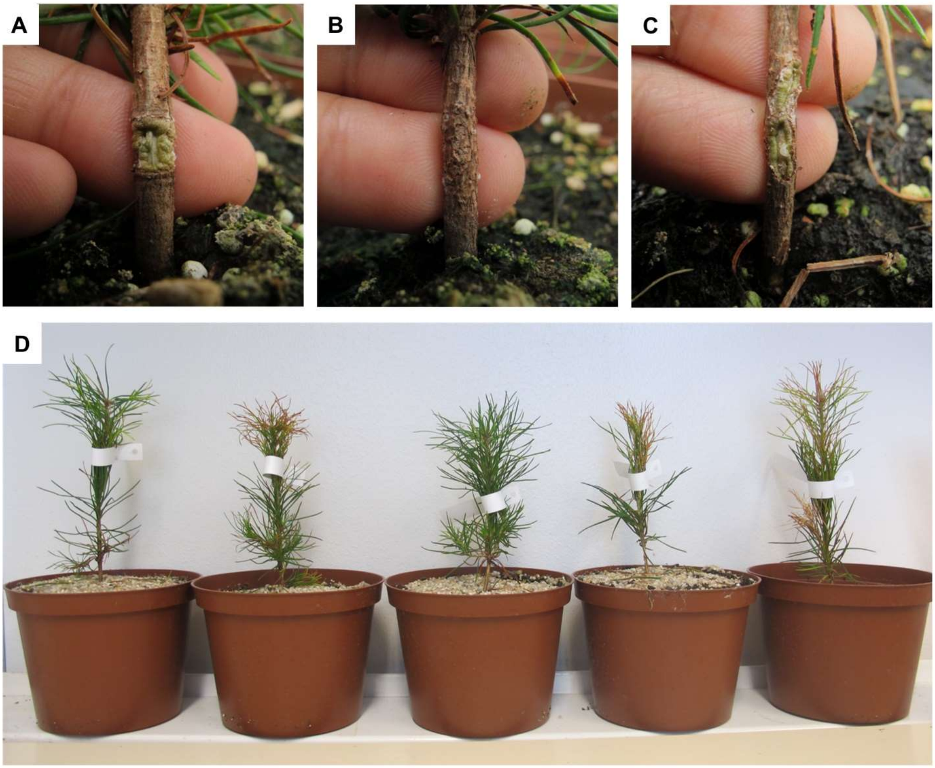
Supplementary Figure 5. Area debarked by pine weevils and number of scars on the stem of Scots pine seedlings in experiment 2 (Insect feeding tests were conducted at two time points, 12 and 32 days post-treatment). Plants that were exposed to pine weevils 12 days after induction treatments (10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF) (not included in the weevil exposure test conducted 12 days post-treatment)) are referred to as early exposure, and those that were exposed 32 days after, are referred to as late exposure in the main text. **(A)** Boxplot for debarked area (mm^2) (raw data), and **(B)** Boxplot for number of feeding scars (raw data). Ends of lines represent the sample minimums and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the solid dots represent potential outliers.



Supplementary Figure 6. Proportion of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments (10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF) (not included in the weevil exposure test conducted 12 days post-treatment) that were undamaged, damaged and girdled during experiment 2 (Insect feeding tests were conducted at two time points, 12 and 32 days post-treatment). In panel **(A)** 12 days (n = 8 for all treatments except that n = 7 for the control (C) group) and **(B)** 32 days after treatments were inflicted. Plants that were exposed to pine weevils 12 days after induction treatments are referred to as early exposure, and those that were exposed 32 days after, are referred to as late exposure in the main text. "Damaged" refers to seedlings that received pine weevil damage but were not girdled during the feeding test; "Girdled" refers to seedlings for which an entire ring of stem bark around the circumference was removed by the pine weevil; "Undamaged" refers to seedlings that did not receive any pine weevil damage during the feeding test.



Supplementary Figure 7. Seedling height and basal diameter of Scots pine (*P. sylvestris*) seedlings receiving different plant defense induction treatments (10 mM MeJA, 15 mM MeJA, undamaged seedlings as controls (C), needle-piercing damage to the stem bark (P), small stem window damage (WinS), and previous weevil feeding damage (WF)) in experiment 2 (n = 12 per treatment, insect feeding tests were conducted at two time points, 12 and 32 days post-treatment). **(A)** Plant height (cm) and **(B)** plant diameter (mm).Weekly measurements were conducted over 33 days, but height data for day 14 is missing.



Supplementary Figure 8. Pictures of seedlings from experiment 2 (Insect feeding tests were conducted at two time points, 12 and 32 days post-treatment) receiving small stem window damage (WinS), needle-piercing damage to the stem bark (P), previous pine weevil damage (WF) and 15 mM MeJA, 32 days after defense induction treatments occurred, which is referred to as the late exposure group in the main text. **(A)** WinS; **(B)** Piercing; **(C)** WF; and **(D)** 5 seedlings from the 15mM MeJA group showing that a few of them exhibited needle-browning at the top and on side branches.

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After pesticides have been banned from use in Swedish plant nurseries, efficient alternatives are needed to control pine weevil damage on conifer seedlings. This thesis investigated potential non-pesticide methods that are based on plant defenses. Among the investigated methods, we have a special interest in comprehending plant induced resistance by using the plant hormone methyl jasmonate (MeJA). The results increased our knowledge about plant defenses and highlighted the compatibility of tested methods.

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