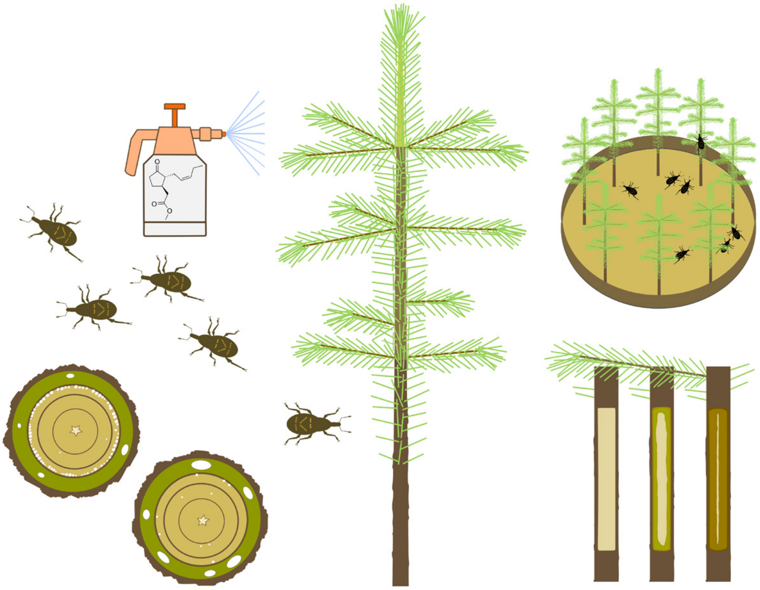
Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2024:40
Faculty of Forest Sciences

Enhancing spruce defence against an insect pest

Effects of somatic embryogenesis and methyl jasmonate treatment

Kristina Berggren Nieto



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SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Uppsala 2024

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Cover: Spruce embling surrounded by the four projects of the thesis; SE+MeJA on pine weevil resistance, pine weevil preference arena, stem resin ducts, and stem wound healing. (Illustration: Kristina Berggren Nieto, 2024)

ISSN 1652-6880
ISBN (print version) 978-91-8046-344-7
ISBN (electronic version) 978-91-8046-345-4
https://doi.org/10.54612/a.70h5b2nmsb

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Print: SLU Grafisk service, Uppsala 2024

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Abstract

Enhancing plant resistance against pests has the potential to become a sustainable alternative within forestry. Exogenous application of the plant defence signalling hormone methyl jasmonate (MeJA) as well as propagation through somatic embryogenesis (SE) has been shown to increase Norway spruce (*Picea abies*) plant's resistance to the major forest regeneration pest the pine weevil *Hylobius abietis*. The aim of this project was to investigate why SE plants display higher resistance. We explored the effects of SE alone and in combination with MeJA on Norway spruce plants' resistance and tolerance, pine weevil behaviour, as well as the underlying mechanisms to their enhanced resistance. Our results suggest that SE and MeJA together have the potential to synergistically enhance plant resistance against weevil damage, and reduce mortality in the field for at least three growing seasons. MeJA treatment and, to a lesser extent SE alone, can influence pine weevil feeding preferences over time based on plant palatability. Moreover, emblings displayed a higher density of phloem constitutive resin ducts, which may contribute to explain their increased resistance. MeJA-treated emblings formed both fewer and smaller traumatic resin ducts than treated seedlings, implying traumatic ducts do not play a large role in the greater resistance seen for MeJA-treated emblings. Finally, SE and MeJA displayed opposing effects on the onset of wound healing and healing rate; MeJA accelerated onset but decreased overall healing rate, and vice versa for emblings. In conclusion, SE plants appear to be differentially palatable to the pine weevil, and exhibit defensive responses different from seedlings, both in how they resist and recover from damage. These findings increase the understanding of plant stress responses, defence traits and pest behaviour, and offer a sustainable approach to plant protection, encouraging the use of our results in practical applications.

Keywords: emblings, forestry, *Hylobius abietis*, induced defence, Norway spruce, *Picea abies*, pine weevil, plant, resistance, plant tolerance, regeneration pest

Förbättrat försvar hos gran mot en insektsskadegörare: Effekter av somatisk embryogenes och metyljasmonatbehandling

Sammanfattning

Att öka växters motståndskraft mot skadegörare har potential att bli ett hållbart alternativ inom skogsbruket. Exogen applicering av signalhormonet metyljasmonat (MeJA) likväl som förökning genom somatisk embryogenes (SE) har visat sig öka granplantors (*Picea abies*) motståndskraft mot skogsföryngringsskadegöraren snytbaggen (*Hylobius abietis*). Syftet med detta projekt var att undersöka varför SE-plantor uppvisar högre motståndskraft. Vi undersökte effekterna av SE enskilt och i kombination med MeJA på granplantors resistens och tolerans, snytbaggens beteende, samt bakomliggande mekanismer till deras ökade resistens. Våra resultat tyder på att SE och MeJA tillsammans har potential att synergistiskt öka plantornas motståndskraft mot snytbaggeskador, samt minska dödligheten i fält under minst tre växtsäsonger. Vidare kan MeJA-behandling, och i mindre utsträckning endast SE, påverka snytbaggens födopreferenser över tid baserat på växternas smaklighet. Dessutom uppvisade SE-plantorna en högre densitet av kådkanaler i floemet, vilket kan bidra till att förklara deras ökade motståndskraft. MeJA-behandlade SE-plantor bildade både färre och mindre traumatiska kådkanaler jämfört med fröplantor, vilket antyder att dessa inte spelar någon större roll för den ökade motståndskraft som ses hos MeJA-behandlade SE-plantor. Slutligen hade SE och MeJA motsatta effekter på sårläkningens start och hastighet; MeJA påskyndade läkningsstarten men minskade totala hastigheten och vice versa hos SE-plantor. Sammanfattningsvis verkar SE-plantor vara olika smakliga för snytbaggen och uppvisa försvarsreaktioner som skiljer sig från fröplantor, både när det gäller motstånd och återhämtning från skador. Våra resultat ökar förståelsen för växters stressreaktioner och motståndskraft, samt skadegörares beteende, och erbjuder ett hållbart tillvägagångssätt för växtskydd, vilket uppmuntrar användning av våra resultat i praktiska tillämpningar.

Keywords: föryngringsskadedjur, *Hylobius abietis*, inducerat försvar, *Picea abies*, rödgran, SE-plantor, skogsbruk, snytbaggar, växtresistens, växttolerans

Preface

I must admit, having studied conservation biology, I did not know much about trees, plant defence responses, insect behaviour, or forestry practices when I started my PhD. I was rather one of those who thought working with wild mammals would be the coolest thing to do. Although, insects I have always thought of as very captivating animals as well. However, thanks to my previous research projects, I had begun to develop a fascination for plant physiology and their interactions with insects, as well as sustainable protection methods. Then, when I saw this project, it really caught my interest. Now, after working with plants these last years, I have come to the elevating realization of how evolved, complex and amazing plants are and I do not think I ever want to stop learning about them. Therefore, this thesis is my tribute to the astonishing plants. Consisting of a mixture of scientific findings and my own reflections, I hope you will enjoy reading it as much as I have enjoyed writing it.

Dedication

To my family and friends, for being so awesome. Especially to my parents; for giving me life and the possibility to do whatever I want with it.

To Carlos, for inspiring me and for pushing me to go ahead. Without you, I probably never would have started this journey.

To nature, for being so spectacular.

”What is commonly called a pest is nature’s way of bringing back into balance an imbalance that man has created” Alan Chadwick

”We should never believe anything we have not dared to doubt”
Queen Kristina

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. Berggren, K., Nordkvist, M., Björkman, C., Bylund, H., Klapwijk, M. J., and Puentes, A. (2023). Synergistic effects of methyl jasmonate treatment and propagation method on Norway spruce resistance against a bark-feeding insect. *Frontiers in Plant Sciences*, 14:1165156. https://doi.org/10.3389/fpls.2023.1165156

II. Berggren, K., Tudoran, A., Chen, Y., Bylund, H., Björkman, C., Tikkinen, M., Egertsdotter, U., and Puentes, A. Effects of propagation method and methyl jasmonate treatment in Norway spruce plants: can pine weevils tell the difference? (manuscript)

III. Berggren, K., Tudoran, A., Björkman, C., Bylund, H., Farrar, S. L., Tikkinen, M., Muscarella, R., and Puentes, A. Enhanced resistance to insect damage in somatic embryogenesis propagated plants: The role of traumatic resin ducts (manuscript)

IV. Berggren, K., Tudoran, A., Chen, Y., Tikkinen, M., Bylund, H., Björkman, C., Egertsdotter, U., and Puentes, A. Opposing effects of propagation method and methyl jasmonate treatment on stem bark wound healing in Norway spruce plants. (submitted manuscript)

The contribution of Kristina Berggren Nieto to the papers included in this thesis was as follows:

I. Conceived and designed the experiments together with AP, CB, HB and MK. Conducted the experiments together with AP and collected the data. Wrote the manuscript together with AP and with input from the other co-authors.

II. Conceived and designed the experiment together with AP and AT. Compiled the data and conducted the statistical analyses. Wrote the manuscript with input from the co-authors.

III. Conceived and designed the experiment together with AP. Conducted the experiments and collected the data together with AT. Compiled the data and conducted the statistical analyses. Wrote the manuscript with input from co-authors.

IV. Conceived and designed the experiment together with AP. Compiled the data and conducted the statistical analyses. Wrote the manuscript together with AP and with input from the other co-authors.

Abbreviations

ABA	Abscisic acid
DAMPs	Damage-associated molecular patterns
DDT	Dichlorodiphenyltrichloroethane
ET	Ethylene
HAMPs	Herbivore-associated molecular patterns
HIPVs	Herbivore induced plant volatiles
IPM	Integrated pest management
JA	Jasmonic acid
MeJA	Methyl jasmonate
PEMs	Pro-embryogenic masses
PGRs	Plant growth regulators
SA	Salicylic acid
SE	Somatic embryogenesis
VOCs	Volatile organic compounds

1. Introduction

1.1 Plant protection

Why do we need plant protection? For hundreds of millions of years, plants and insects have coexisted, and as a result of continuous herbivore attacks, plants have evolved a highly sophisticated defence system (Bruce 2015; Acevedo et al. 2015; Chaudhary et al. 2018). Why then do they need our help to protect themselves? The answer is simple. Humans have relied on plants as a source of food and material since the beginning of their time, as have herbivores. When humans learned how to cultivate plants, not only did we create a readily available food source for us, but also for herbivores.

As the human population grew, in recent centuries, there was a shift towards large-scale monocultures and improved plant growth, quality of the material, and yield (Tilman et al. 2001; Scherr and McNeely 2008). However, this resulted in an increased plant vulnerability towards biotic disturbances, such as insect infestation. With intensified cultivation and reduced plant resilience (Bradshaw et al. 2004), herbivore competition and damage also increased, and the pest problem became a pressing concern.

1.1.1 What defines a pest insect?

Going back to the quote on the early pages of this thesis "What is commonly called a pest is nature's way of bringing back into balance an imbalance that man has created" (Alan Chadwick). The way we cultivate plants has disrupted the natural balance between plants and insects, creating ideal conditions for some insect populations to thrive. A classic example of how a non-pest can become a major pest due to human intervention is the Colorado potato beetle (*Leptinotarsa decemlineata*). Native to the Rocky Mountains of North America, this beetle fed on wild Solanum species without causing significant damage. However, the introduction of potato in the mid-1800s created an abundant food source for the Colorado potato beetle, turning it into a major pest (see e.g. Headings 2004; Weber 2003). With this in mind, an insect might have a minimal ecological impact but become a pest if it significantly reduces crop yields. The concept of a "pest" is ultimately defined by its impact on human endeavors, and thus, a herbivore is labelled as a pest when it is considered to reduce the value of a plant to humans (Ayres and Lombardero 2000; Aukema et al. 2011).

1.1.2 The introduction of synthetic pesticides

A defining moment in the history of plant protection came in the mid-20th century, with the introduction of modern synthetic chemical pesticides (U.S. Environmental Protection Agency, 2024). This innovation revolutionised pest control. Never had it been so easy to eradicate pests from cultivated plants. However, this convenience came at a cost. The detrimental consequences of these pesticides on natural ecosystems and the society unfolded gradually over the following decades (Carson 2002). Dichlorodiphenyltrichloroethane, more commonly known as DDT, serves as a cautionary example. DDT was effectively and extensively used to fight, for example, malaria and typhus mosquitos as well as agricultural pests. However, it was found to be very persistent in nature and to accumulate in fatty tissues, creating a cascading effect throughout the food chain. One of the more notable consequences of DDT was that it caused eggshell thinning in birds (Kolaja and Hinton 1977; Carson 2002), leading to population decline of especially predatory birds (see e.g. Grier 1982; U.S. Environmental Protection Agency, 2024). For example, the white-tailed sea eagle (*Haliaeetus albicilla*) almost got extinct in Sweden (Helander et al. 2008). After the ban of DDT in the 1970s, these populations have slowly recovered. Additionally, DDT has been found to have carcinogenic properties, and due to its persistency in nature, it is still found in living organisms.

1.1.3 Current use of pesticides

Although the use of DDT is an extreme example, it is just one out of many examples of how chemical pesticides can affect natural ecosystems and human health. Yet, despite their drawbacks, global pesticide use has increased over the last decades alongside a growing population (Figure 1). With over 2 million tons of pesticides applied annually (Sharma et al. 2019), the need to reduce our reliance on these chemicals is critical. Creating an urgent need to develop more sustainable, innovative plant protection methods.

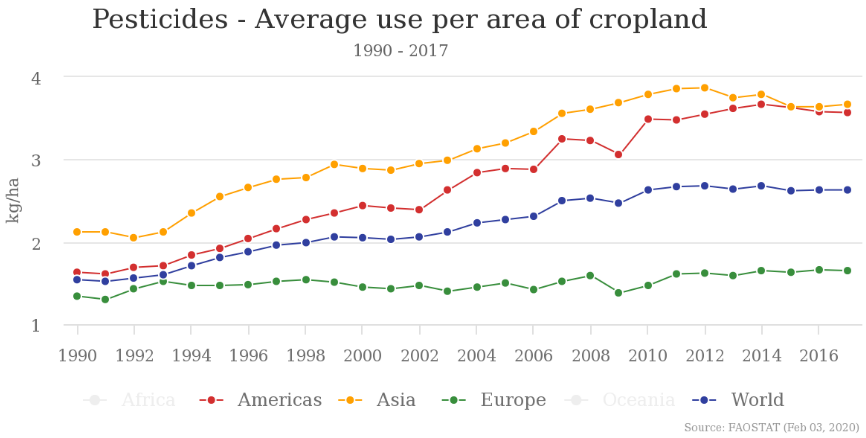

Figure 1. Global use of pesticides. Highlighted in this figure is the average use of pesticides per area of cropland in the Americas, Asia, Europe, and the World average. Figure: FAOSTAT (Feb 03, 2020)

1.1.4 The multifaceted need for novel plant protection

The demand for novel plant protection methods does not only encompass the need to reduce the use of chemical pesticides. Pests can also develop resistance to existing control methods, reducing their efficacy over time. A well-known example is the Colorado potato beetle mentioned above, which has developed resistance to a multitude of insecticides, including DDT (Alyokhin et al. 2008). Moreover, climate change and other global environmental shifts may alter plant-herbivore interactions in several ways. Changes in global transport patterns can introduce new invasive species that become pests in previously unaffected areas. A warmer climate might lead to longer, more widespread outbreaks of existing pests. Rising temperatures can allow pests to expand their range into previously unsuitable areas, such as the emerald ash borer (*Agrilus planipennis*), an invasive beetle from Asia destructive to ash trees (*Fraxinus* spp.). Milder winters, along with other potential changes associated with climate change, may be a contributing factor to its spread in North America (Liang and Fei 2014; Cuddington et al. 2018). Additionally, warmer seasons can facilitate more breeding cycles and faster reproduction and metabolism in herbivores, leading to larger populations with increased feeding activity (more and faster feeding). Warmer climates may also increase stress and damage to cultivated plants, e.g. from drought or storms, making them more susceptible and vulnerable to herbivore attacks. Thus, not only do we need to develop new methods, we also need to diversify these strategies and include integrated pest management, i.e. the combination of different methods. However, to make these new methods as effective as possible, we need to understand the complex ecological and physiological details of plant-insect interactions.

1.2 A co-evolutionary arms race: plants vs. insects

Plants are sessile organisms, meaning, they cannot run away from herbivore attack. Despite this, plants can effectively defend themselves against herbivore attacks through the evolution of a diverse array of defence strategies, all without a nervous system (Chaudhary et al. 2018). These strategies aim to prevent or reduce stress and damage, and ensure survival (Walling 2000; Nishida 2014; Basu et al. 2018). To counteract, insects have adapted to host plant defences in various ways. For example, by avoiding certain plants or plant tissues, suppressing plant defences (e.g. reducing toxicity via excretion or detoxification) (Kant et al. 2015), or counteracting digestive enzyme inhibitors released by the plant (Mainguet et al. 2000; Schoonhoven et al. 2005; Zhang et al. 2023). Some insects even exploit plant defence chemicals as cues to locate suitable food sources or oviposition sites (Fraenkel 1959; Hopkins et al. 2009; Yang et al. 2021). This constant arms race between plants and insects determines the outcome of an encounter; the insect overcoming or withstanding plant defences, leaving, or facing mortality.

1.2.1 The different aspects of plant defence

Plant defences can be broadly categorized as resistance and tolerance (Figure 2) (Stout 2013). Resistance is the ability of a plant to fight back a herbivore attack and directly reduce damage or stop the attack altogether (Nuñez-Farfán et al. 2007; Mitchell et al. 2016). A resistant plant may possess physical barriers like thorns or trichomes (hair-like structures) that deter herbivores, or chemical substances like toxins or repellants that make them unpalatable or poisonous (War et al. 2012). These traits can decrease herbivore performance by affecting their growth, development, or preference for the plant as a food source or oviposition site (Karban and Myers 1989).

In contrast to resistance, tolerance does not affect insect biology or behaviour (Smith 2005). Instead, tolerance focuses on the plant's ability to withstand herbivore damage and minimize its negative impact (Nuñez-Farfán et al. 2007; Mitchell et al. 2016). A tolerant plant may compensate by increasing their growth rate, photosynthetic capacity, or resource allocation, to recover from damage (Strauss and Agrawal 1999; Chaudhary et al. 2018).

Plants can deploy two main types of defences: constitutive and induced. Constitutive defences are pre-formed and always present in the plant, while induced defences are only activated upon specific stimuli, such as herbivore attack or environmental stresses (Gatehouse 2002; War et al. 2012). This strategy allows for a more efficient use of resources, with activation of selected responses upon specific threats (Fagerström et al. 1987; Clark and Harvell 1992), as well as a more diverse defence system (Karban et al. 1997). Induced defences can also work indirect, e.g. through the production and release of volatile organic compounds (VOCs) that attract natural enemies of the herbivore, such as predatory insects (Gatehouse 2002; Chaudhary et al. 2018). By understanding the diverse defence mechanisms in plants, we can develop new plant protection strategies that mimic or enhance these natural mechanisms.

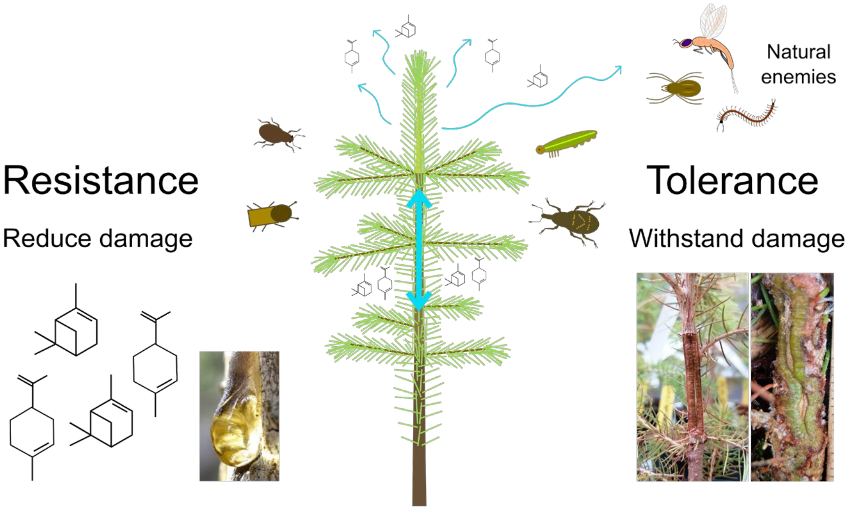

Figure 2. Overview of plant defences: Resistance is how the plant fight back an attack, e.g. through production of toxic secondary metabolites (volatile and non-volatile). Tolerance is how the plant reduce the negative impact from an attack, e.g. through wound healing. Volatiles attract natural enemies. Illustration: Kristina Berggren Nieto

1.3 Novel plant protection using plant's own defence

Utilization of plant's own defence system to improve plant resistance against pests has received increased attention lately (Stenberg et al. 2015; Mitchell et al. 2016; Lalík et al. 2020; Dreischhoff et al. 2020; Hernández-Suárez and Beitia 2021). Herbivore attack triggers plant stress responses, which can be both local (at the site of attack) and systemic (in the whole plant) (Savatin et al. 2014; Erb and Reymond 2019). These responses are initiated by elicitors, e.g. plant-derived damage-associated molecular patterns (DAMPs) released from wounded tissue, or herbivore-associated molecular patterns (HAMPs) found in insect saliva, cuticle, pheromones or feces (Acevedo 2015; Erb and Reymond 2019). Signalling hormones known as chemical elicitors, such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA), further mediate these plant stress responses (Savatin et al. 2014). Chemical elicitors can be exploited to manipulate plant's defence system for enhanced resistance against herbivores, e.g. through direct activation of defences (Walters et al. 2014; Bruce et al. 2017; Siah et al. 2018; Yassin et al. 2021), or by priming the plants. Priming functions like a vaccine and prior exposure to a stressor prepares the plant for future attacks, which can result in a faster and stronger upregulation of defences following, for example, a second herbivore attack (Conrath et al. 2006; Wilkinson et al. 2019). This approach offers a promising strategy for sustainable pest management by utilizing the plant's own defence mechanisms against herbivory.

1.3.1 Enhancing plant defence with methyl jasmonate

There is a growing interest in the potential use of methyl jasmonate (MeJA), the volatile ester derivative of JA, to enhance plant resistance (e.g., Walters et al. 2014; Bruce et al. 2017). MeJA is a naturally occurring stress-signalling plant hormone; a chemical elicitor of plant defence mechanisms (Yu et al. 2019). Studies have shown that exogenous MeJA application can activate defence responses and subsequently reduce insect feeding (Figure 3) and damage in various crops like soybean, rice, strawberry, and Andean lupin (Chen et al. 2018; Senthil-Nathan 2019; Mouden et al. 2021; Erazo-García et al. 2021). Similarly, MeJA treatment has been shown to effectively enhance conifer resistance to insect pests such as the pine weevil (*Hylobius abietis*) (e.g. Zas et al. 2014; Puentes et al. 2021), the spruce bark beetles (*Ips typographus*) (Mageroy et al. 2020), and the Japanese pine sawyer (*Monochamus alternatus*) (Chen R et al. 2020), as well as fungal pathogens like *Pythium ultimum* and blue-stain fungi (Kozlowski et al. 1999; Krokene et al. 2008). MeJA treatment triggers tree defences and the increased resistance often involves formation of traumatic resin ducts and production of defensive compounds like terpenes and phenolics (Krokene et al. 2008; López-Villamor et al. 2021). It has also been shown that MeJA can function as a priming agent (described above) in Norway spruce (Mageroy et al. 2020).

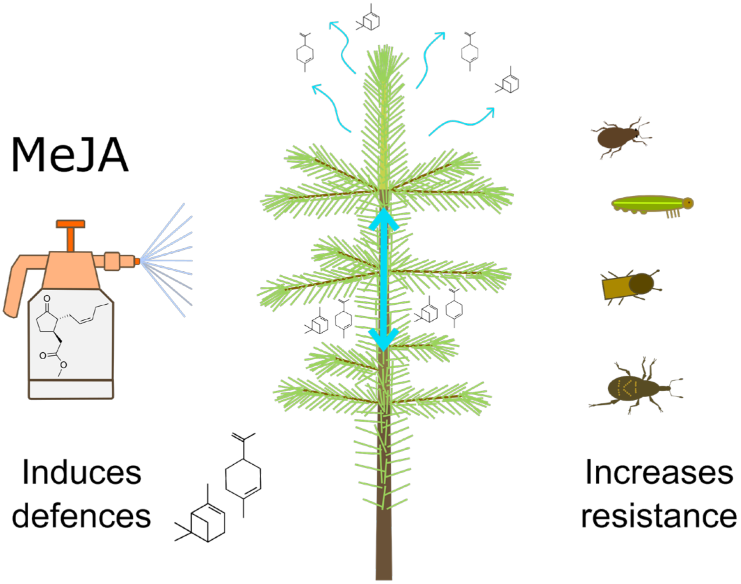

Figure 3. Induction of plant defences through MeJA application, which subsequently increases plant resistance to herbivorous insects. Illustration: Kristina Berggren Nieto

1.4 Enhancing plant defence with SE

A recent study has shown that a propagation method can influence plant resistance to pests. In a field trial, Puentes et al. (2018) found that Norway spruce plants propagated via somatic embryogenesis (SE) suffered about 30% less feeding damage and 10% lower attack from the pine weevil (*Hylobius abietis*) compared to traditionally propagated seedlings. This finding revealed a previously unknown plant protection advantage associated with SE propagation.

1.4.1 The SE process

SE is a vegetative propagation technique that was first described in carrot (*Daucus carota*) in 1958 (Steward et al. 1958; Reinert 1959). It has been used since the late 20th century in agriculture and forestry to generate various economically important crops (e.g. wine grapes, cacao trees, bananas) (Duarte-Aké and De-la-Peña 2016; Etienne et al. 2016; López et al. 2022; Lelu-Walter et al. 2013) and tree species (e.g. spruce, larch, firs) (Jain et al. 1995; Lelu-Walter et al. 2013). Norway spruce (*Picea abies*) was the first conifer species to be regenerated via SE, in 1985 (Hakman et al. 1985;

Chalupa 1985). For the SE process, somatic cells or tissue from a mother plant are induced in a controlled laboratory environment (*in vitro*) to form somatic embryos (Mo et al. 1995; Klimaszewska et al. 2016; Egertsdotter 2019). Induction takes place via the use of specific plant growth regulators (PGRs), such as auxins and cytokinins (von Aderkas et al. 2015; Méndez-Hernández et al. 2019). Stimulated by the PGRs, these embryos then initiate a multiplication process (proliferation, or cloning process) to form pro-embryogenic masses (PEMs), which are clusters of early-stage embryos (Figure 4). To stop the multiplication process and stimulate embryo maturation, the initial PGRs are replaced with another PGR; abscisic acid. Given the right conditions, the mature embryos will germinate and grow into plantlets (Figure 4), which can then be transplanted to a growing medium and cultivated in nurseries as regular plants (Egertsdotter 2019).

1.4.2 Can early stress prime SE plants defence?

Considering the conditions during SE propagation, the process itself may be the cause of the increased pine weevil resistance observed in emblings. As the SE process involves exposure to high levels of PGRs, several of which are known to be involved in plant stress responses (Méndez-Hernández et al. 2019; Müller 2021), it may trigger stress mechanisms in plants during their embryonic stages (Jiménez 2001; von Aderkas et al. 2015; Méndez-Hernández et al. 2019). Additionally, embryos may in some cases be exposed to other stressors such as extreme pH and heat shock during the SE process (Winkelmann 2016; Méndez-Hernández et al. 2019). This exposure to stressors during the propagation has been shown to increase the levels of secondary metabolites, important for plant defence, in SE-propagated plants compared to those produced from seeds or growing wild (Lamhamedi et al. 2000; Fulzele and Satdive 2003; Domínguez et al. 2010). Exposure to stress early in life may influence plant defence responses later in their life through priming (Conrath et al. 2006; Wilkinson et al. 2019). This opens up exciting new avenues for exploring how we can utilize plant stress responses for sustainable pest management strategies.

Figure 4. Different stages of the SE propagation process. Cell proliferation form pro-embryogenic masses (PEMs) (colony development) consisting of clusters of early-stage embryos in different stages of development. These embryos mature and start growing into small plantlets, which eventually turn into emblings. Photos: Kristina Berggren Nieto, except the bottom left photo of a magnified PEM by Josefine Lind Björs

1.5 Novel forest plant protection

Forests are a vital resource for many wood-related products. Due to the economic consequences of insect damage to trees, past research has yielded a diverse toolbox for managing insect pests, including insecticides, silvicultural techniques, and biological control methods. However, the potential of using insect-resistant trees as pest control has long been recognized as an ideal strategy (Hanover 1975) since it offers a promising solution for controlling forest insects. Both SE and MeJA have the potential to be utilized within plant protection as resistance-enhancing methods.

1.6 Conifers

Conifers, an ancient group of woody plants, include some of the planet's longest-living plants, with lifespans stretching over thousands of years. They are a group of cone-bearing seed plants that encompass approximately 630 species distributed across the globe and have been integral components of terrestrial ecosystems for millions of years, shaping landscapes and influencing biodiversity. Unlike deciduous trees, conifers retain their foliage throughout the year, a trait known as evergreen. This adaptation enables conifers to thrive in diverse environmental conditions, ranging from boreal forests to arid mountain slopes (Farjon 2017). Conifers play crucial roles in ecosystem functioning and biodiversity conservation. Their dense foliage provides habitat and food sources for a myriad of organisms, including insects, birds, and mammals (Figure 5). Moreover, conifer forests contribute to carbon sequestration, mitigating the impacts of climate change by storing vast amounts of carbon in their biomass and soil (Taiz and Zeiger 2014). Within this group, the Pinaceae family is the most abundant and widespread, especially in the Northern Hemisphere. Two Pinaceae members, Norway spruce and Scots pine (*Pinus sylvestris*) dominate Europe's boreal and subalpine forests, and are thus the most economically valuable tree species in this area (Buras and Menzel 2019). Like all plants, conifers face constant attacks from herbivores and pathogens. The most devastating threats worldwide come from infestations by tree-killing bark beetles accompanied by their symbiotic fungal partners. However, the remarkable success of conifers can be attributed in part to their complex defence mechanisms, evolved over millennia to deter invasion.

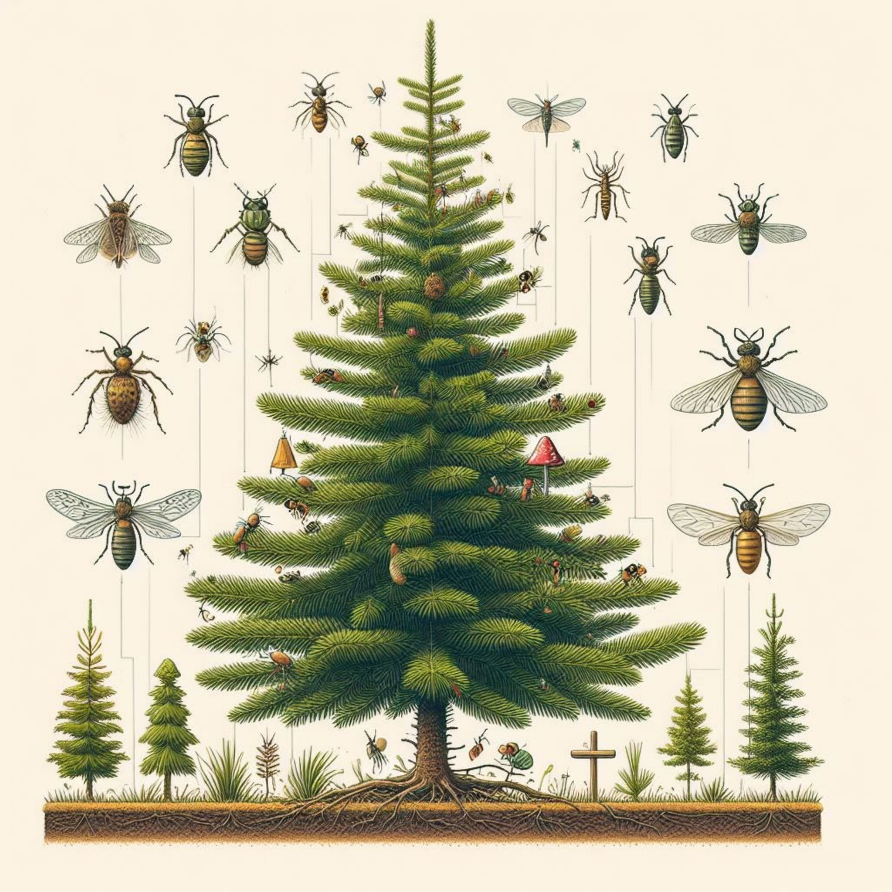

Figure 5. The myriad of organisms connected to a conifer. Illustration: Carlos Palo Nieto

1.6.1 Conifer defence – resin characteristics

The main defence system in conifers is the resin system (Trapp and Croteau 2001), a unique system consisting of both a chemical and physical defence (Ferrenberg et al. 2014; López-Villamor et al. 2021). The resin system involves the production of oleoresin (resin hereafter), which is a sticky mass produced and stored in resin blisters, cells or ducts (Berryman 1972; Lewinsohn et al. 1991). Ducts are found in all or some of the main parts of the tree (Celedon and Bohlmann 2019), and resin is toxic to herbivores due to the involvement of mainly terpenoids (e.g. Trapp and Croteau 2001; Martin et al. 2002; Celedon and Bohlmann 2019). In Norway spruce, constitutive resin ducts occur both in the cortex/phloem and in the secondary xylem (Figure 6) (Bannan 1936; Wu and Hu 1997; Franceschi et al. 2005). Upon damage, the resin-filled ducts break, releasing a flow of sticky resin that covers the wounded area and flush out potential invaders (Nagy et al. 2000). Damage induces the formation of traumatic resin ducts in the outer margin of the xylem (Figure 5), as well as the production of secondary resin, which is stored in these ducts (Nagy et al. 2000; Martin et al. 2002; Franceschi et al. 2005). Additionally, MeJA treatment has been shown to induce the formation of traumatic resin ducts (Hudgins et al. 2003; Vázquez-González 2019). A positive correlation has been reported between increased size or abundance of resin ducts and resistance to insects (Baier et al. 2002; Kane and Kolb 2010; Moreira et al. 2012). Thus, the increased resistance observed in SE propagated and in MeJA-treated Norway spruce, may be caused by an increase in resin ducts in the stem tissues, suggesting that an examination of resin duct distribution and density can provide valuable knowledge about the underlying mechanisms of the increased insect resistance.

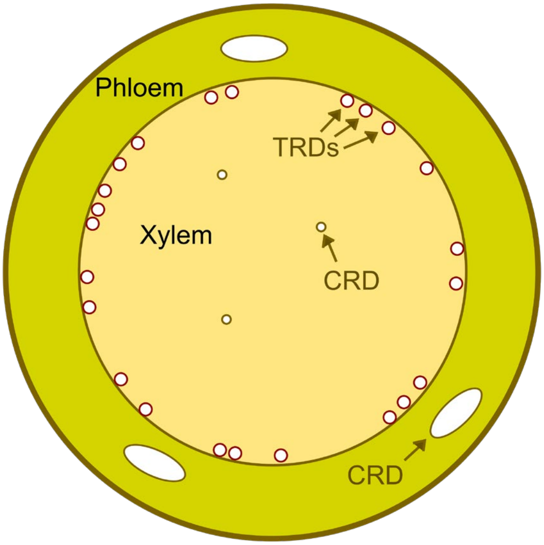

Figure 6. The figure illustrates a transversal stem section of a Norway spruce plant showing the positioning of the phloem, xylem and resin ducts. CRDs = constitutive resin ducts (always present in the plant); TRDs = traumatic resin ducts (formed after induction of defences). Illustration: Kristina Berggren Nieto

1.7 SE in future forestry

Somatic embryogenesis (SE) has the potential to reform conifer propagation in forestry. This technique allows for mass-production of certain genotypes – essentially clones – from selected “plus” trees with desirable traits. Compared to traditional breeding methods using seeds or cuttings, SE offers a rapid means to produce plants, which accelerates the testing of these improved genotypes (Klimaszewska et al. 2016; Egertsdotter et al. 2019). Although SE has been applied for decades, the inherent resistance to the pine weevil in Norway spruce produced via SE was not discovered until recently (Puentes et al. 2018). This suggests a largely unexplored potential for SE to enhance intrinsic pest resistance in conifers. Notably, there is a growing interest in SE for commercial conifer production in the Nordic European countries (e.g., Lelu-Walter et al. 2013; Egertsdotter et al. 2019; Rosvall et al. 2019a; Rosvall et al. 2019b). Therefore, it is of high time to delve deeper into this “SE effect” on resistance. Understanding the underlying mechanisms and how they influence pest behaviour is essential for maximizing the benefits of SE in developing a new generation of pest-resistant conifers, as well as evaluating the potential of SE to be used as a novel plant protection tool.

2. Aims/Objectives

The aim of this project was to investigate why Norway spruce emblings, propagated through somatic embryogenesis (SE), display a higher resistance to pine weevil damage than regular nursery seedlings, as documented recently. Although SE has been used as a propagation method for Norway spruce for more than 30 years, no previous study had explored the possibility of an inherent pest resistance in SE propagated plants. Focus has been on consequences and underlying mechanisms of the observed SE-effect, such as insect behavioural and plant physiological responses, as well as on factors that could moderate this effect. As the pine weevil is a major regeneration pest on planted conifer seedlings, these research findings may contribute to developing plants with increased resistance to feeding damage as well as reduced negative effects of damage, and eventually be used to improve forest regeneration.

The four research objectives of the project were:

1) Examine potential effects on pine weevil resistance when combining SE with the defence-inducing hormone MeJA (**Paper I**).

2) Examine the effect of SE on pine weevil behaviour (**Paper II**).

3) Examine the underlying mechanisms of the SE-effect (**Paper III**).

4) Examine the wound healing ability of SE propagated plants (**Paper IV**).

3. Materials and Methods

3.1 The pine weevil – conifer regeneration system

The pine weevil (*Hylobius abietis* L.) is one of the most important regeneration pests in Europe and Asia, posing a significant threat to forest regeneration efforts (Långström and Day 2004; Nilsson et al. 2010). Adult weevils are strongly attracted to the odours emitted by the stumps of freshly cut conifer trees to find breeding substrate (Figure 7) (Nordlander 1991; Långström 1982), and can travel far distances in search of new breeding areas (Solbreck 1980). They lay their eggs near or inside the root bark of stumps (Nordlander 1991; Nordlander et al. 1997), with new generations emerging within 1-2 years (Figure 8A-B) (Bejer-Petersen et al. 1962; Nordenhem 1989; Wainhouse et al. 2014). This continued weevil presence in clear-cuts, which can be up to three years depending on the geographical location, allows both the parental and new generations to feed on the stems of planted conifer seedlings (Figure 7) throughout their growing season (spring till autumn in Nordic countries) (Wallertz et al. 2014). The parental generation stays while the new generation eventually leaves to search for new oviposition sites (Nordenhem 1989). Their extensive feeding often girdles the seedlings (removal of an entire ring of bark phloem from the stem circumference, exposing the xylem) (Figure 8C), leading to high mortality rates and the need for replanting in heavily infested areas (Nordlander 1991; Lalík et al. 2020; Leather et al. 1999; Mattsson 2016). Without proper seedling protection, weevils can cause substantial seedling damage and economic losses (von Sydow, 1997; Örlander and Nilsson 1999; Petersson and Örlander 2003).

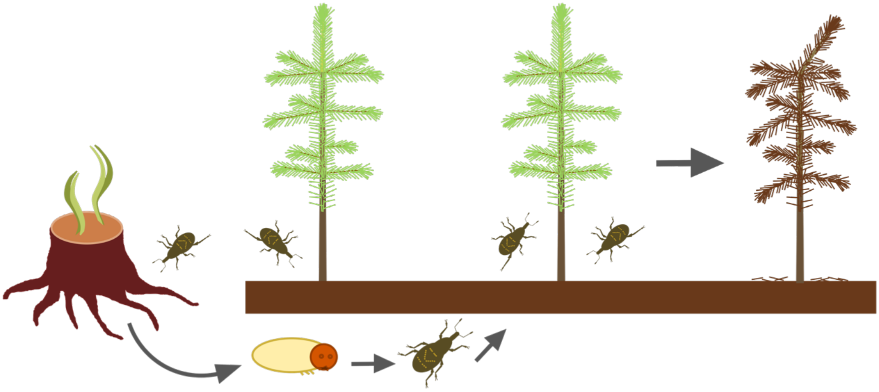

Figure 7. Pine weevil regeneration: Pine weevils are attracted by volatiles from fresh conifer stumps. They lay their eggs in the roots of the stumps and feed on conifer seedlings. Larval development takes place in the stump roots. Adult weevils emerge from the ground and feed on conifer seedlings together with the parental generation, causing seedling damage and mortality. Illustration: Kristina Berggren Nieto

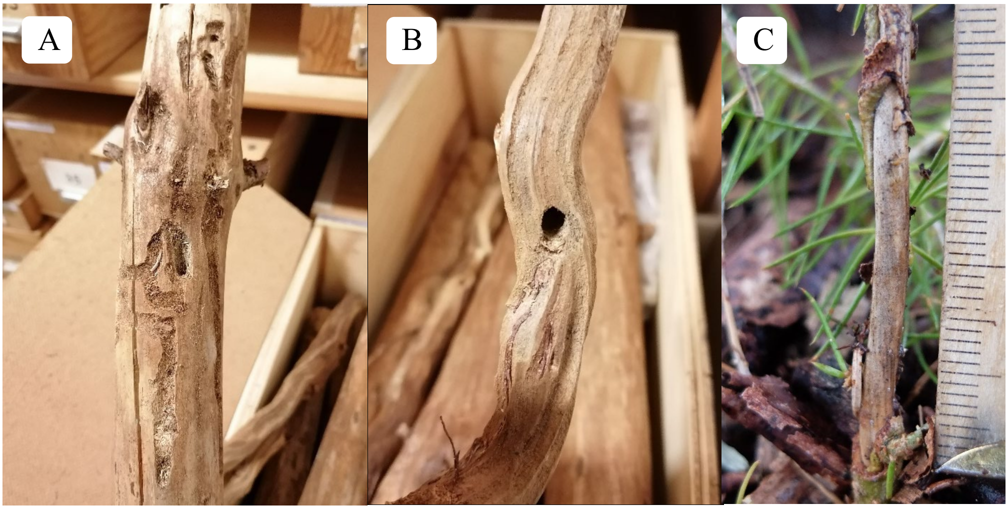

Figure 8. Picture A and B shows pine weevil larval galleries and exit holes in roots. Picture C shows a spruce seedling stem girdled by pine weevils. Photos: Kristina Berggren Nieto

3.2 Plant material

Plant material consisted of 1-3 years old Norway spruce (*Picea abies* (L.) H. Karst) somatic plants (emblings) and regular nursery seedlings. Emblings were obtained from the Forestry Research Institute of Sweden (Skogforsk)

(**Paper I**) originating from 19 full-sib families (from trees belonging to the clonal archive used in breeding trials of Norway spruce in Sweden), and the Natural Resources Institute Finland (Luke), originating from five full-sib families (of progeny tested plus trees) (**Paper II**, **III**, and **IV**). Embling proliferation and maturation were either carried out on Petri plates (Skogforsk/Umeå) (**Paper I**) or in temporary immersion system (TIS) bioreactors (**Paper II**, **III**, and **IV**). For a detailed description of the methods, see relevant paper. Seedlings were obtained from two Swedish commercial plant nurseries (**Paper I**) and from Luke Suonenjoki research nursery (**Paper II**, **III**, and **IV**). Seedlings from Sweden (**Paper I**) consisted of containerized and bare-root seedlings, while seedlings from Finland included only containerized seedlings (**Paper II**, **III**, and **IV**). Upon arrival to the Swedish University of Agricultural Sciences (SLU), Uppsala campus, all plants for the laboratory or greenhouse experiments were repotted in 1L (ø 13 cm) or 2L (ø 16.5 cm) plastic pots and kept in a greenhouse (16h/8h light/dark and ~18/15 °C day/night). Plants for the field experiment were kept in plug trays (ø 6.5 cm per plug) in the greenhouse for 3.5 weeks until being relocated and planted in the field.

3.3 Experimental set-up

3.3.1 Methyl jasmonate treatment

In all experiments, half of the plants from each plant type were treated once (**Paper I**, **II** and **IV**) or twice (**Paper III**) with 10 Mm methyl jasmonate (MeJA). This concentration has been previously shown to be effective in similar-sized conifer seedlings (Chen et al. 2021). Induction times varied and can be found in each paper. Preparation and application in summary: MeJA (95%, Sigma-Aldrich, ref. 392707) was first dissolved in ethanol to create a concentrated solution. Deionized water was then added to achieve a final ethanol concentration of 2.5% (v:v). The mixture was shaken vigorously until a uniform, milky emulsion formed. The solution was transferred to a hand-sprayer bottle and pressurized (2.5 bar). Spraying procedure: Plants were sprayed outdoors, positioned in adjacent rows (Figure 9). The spray nozzle was held approximately 30 cm from the plants, ensuring all aboveground parts (except new-year shoots) received the solution for roughly one second per plant. Untreated plants underwent the same spraying process using deionized water instead of the MeJA solution.

MeJA-treated plants were kept in a separate greenhouse to prevent contamination of the control group.

Figure 9. Exogenous application of methyl jasmonate (MeJA) in an outdoor setting. Seedlings were placed in rows and sprayed with MeJA from two sides. Spraying occurred about 30 cm from the plants and for approximately one second per plant. Photos: Kristina Berggren Nieto

3.4 Pine weevil resistance experiment: field

3.4.1 Study site and block design

The field experimental site in **Paper I** was a non-scarified clear-cut (harvested autumn 2018) (Figure 10) close to Tierp in central Sweden (60°21’N, 17°26’E). Planting occurred on 18-19 June 2019 in nine blocks, each containing 72 plants (except one with 80 plants). Within each block, plants were positioned one meter apart in a rolling manner of the treatment combinations, across nine columns with eight positions (ten columns in the larger block). The positioning ensured that no plants of the same treatment were adjacent horizontally or vertically. The four treatment combinations included: 1) MeJA-treated embling, 2) non-treated embling, 3) MeJA-treated containerized or bare-root seedling, and 4) non-treated containerized or bare-root seedling, with each treatment replicated twice per column. Additionally, a reference block (72 plants) comprising only untreated containerized seedlings was included near the experimental blocks to estimate pine weevil pressure in the clear-cut without the influence of treatments.

Figure 10. Field site (left) and lab cylinder (right). Photos: Kristina Berggren Nieto

3.4.2 Data collection in the field

The field experiment (**Paper I**) included a total of 328 emblings, 228 containerized, and 100 bare-root seedlings, of which half (164 emblings, 114 containerized, and 50 bare-root seedlings) were treated with MeJA. The field experiment lasted three-years, from June 2019 to September 2021. Three variables related to plant resistance were recorded: whether the plant had been attacked or not by pine weevils (yes or no), damage caused by pine weevil feeding on the stem (area debarked), and mortality (alive or dead). Inventories were carried out late in the growing season of each year: September 2, 2019 (11 weeks after planting; all three variables), September 15, 2020 (attack and mortality), and September 29, 2021 (attack and mortality). To estimate the total debarked area per plant we measured the following variables: (1) height debarked - height from the ground (just above the root collar) to the top of the uppermost pine weevil feeding scar on the stem, and (2) percentage debarked - the proportion of the stem area damaged (%) in relation to the total surface area up to the debarked height described in (1). The debarked area (cm^2) for each plant was calculated from these measurements as: Total area debarked = stem circumference ($\pi \cdot d$) × (height debarked × percentage debarked).

3.5 Laboratory and greenhouse experiments

All laboratory and greenhouse experiments were carried out at the Swedish University of Agricultural Sciences in Uppsala, Sweden.

3.5.1 Insect material

Pine weevils used in the laboratory experiments were collected during their spring migration in May of 2019 (**Paper I**) and 2022 (**Paper II**) at a sawmill (Balungstrands Sågverk AB) in Enviken, Sweden. To maintain them, weevils were kept in a dark room at 10 °C with access to water and young Scots pine stems and branches as food source. For acclimatization one (**Paper I**) or two (**Paper II**) weeks before an experimental start, weevils were transferred to room temperature (~20 °C, 16h light/8h dark) and provided with Scots pine branches and water. In order to starve the weevils, food was removed three to four days prior to the start of an experimental round. Due to pine weevils being the most active during the dark hours in the lab (Fedderwitz et al. 2014), the dark and light hours were reversed during the two-week acclimatization period in **Paper II** (meaning the lab was kept dark in the day and with the lights on in the night). This was done in order to observe them during our waking hours.

3.5.2 Pine weevil resistance experiment: lab (no choice)

The laboratory experiment (**Paper I**) included a total of 324 emblings, 300 containerized, and 24 bare-root seedlings, of which half (162 emblings, 150 containerized, and 12 bare-root seedlings) were treated with MeJA. The experiment was replicated nine times following each other (called rounds) during July-August 2019. Each round consisted of a new set of 72 plants, and lasted for three or four days. It was a no choice test and each plant was obligatorily exposed to one starved pine weevil enclosed in a transparent plastic cylinder with the potted plant (Figure 10). The experiment was conducted under room temperature conditions (~25 °C) with natural light. Plants were placed closely together in rows on tables and the same order of rolling treatment was used within the block as in the field (treatment 1: MeJA-treated embling; 2: non-treated embling; 3: MeJA-treated containerized or bare-root seedling, 4: non-treated containerized or bare-root seedling). In each round, the order of treatments in the columns/positions was changed. At the end of each round, we recorded whether the plant had been attacked or not (yes or no) by the pine weevil, as well as damage caused by pine weevil feeding on the stem (area debarked). The debarked area was calculated for each plant by measuring each feeding scar using graded millimetre templates, and adding all areas together (cm^2).

3.5.3 Pine weevil preference (arena) experiment

The pine weevil preference laboratory experiment (**Paper II**) included a total of 160 plants; 40 per treatment of which half were treated with MeJA. We used two multi-choice arenas (1 m in diameter) constructed by an acrylic bottom plate and 38 cm high walls of cardboard with a thin nylon net as a "roof". Potted plants were placed in openings in the bottom plate, in total 16 positions in the arena circumference (Figure 11). To ensure that only the above-ground parts were accessible to pine weevils, the soil was covered with silver tape. To prevent volatile emission bias from damaged/undamaged plants, each plant's stem bark was lightly scraped with a scalpel near the base during the setup. The experiment was replicated over time in five rounds (July-August 2022) and a new set of plants and pine weevils were used each round. Treatments (non-treated embling, MeJA-treated embling, non-treated seedling, and MeJA-treated seedling) were arranged in a block design with blocks 1 to 4 occupying positions 1-4, 5-8, 9-12, and 13-16, respectively (Figure 11). Plants were randomized within each block and round, ensuring no treatments were neighbouring each other. At the start of a round, a total of 32 pine weevils (16 males and 16 females) that had been starved for 72 hours, were released in the center of the arena (at 9 am the first day). All rounds were conducted at room temperature (~20 °C; 8/16 hours dark/light). Data were collected twice a day during the dark hours (see Insect material section above) (at 11 am and 4 pm), i.e. 2, 7, 26, and 31 hours after the pine weevils were released into the arena. Observations included the number of pine weevils present on each plant at the time point of observation. After 48 hours, pine weevils and plants were removed from the arena. Pine weevil attack (yes or no) and the amount of debarked area (mm^2) on each plant were recorded. The sum of the number of pine weevils observed on the plants at each time point was used to calculate the total number of pine weevils per treatment.

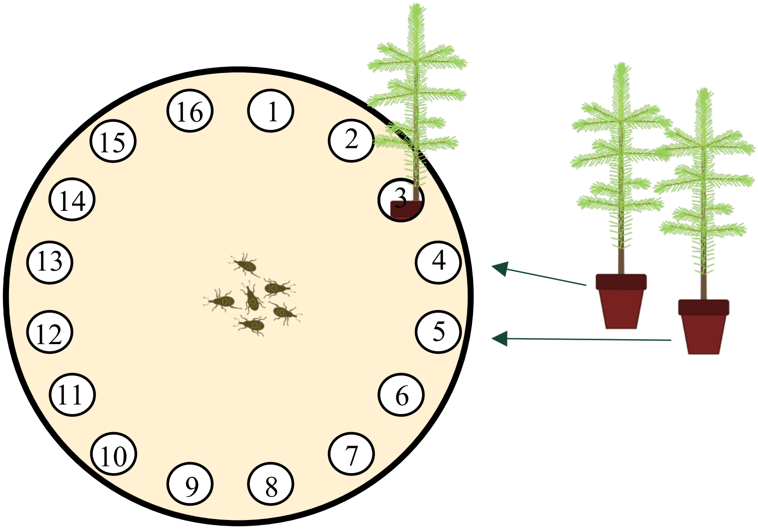

Figure 11. Arena set-up showing the 16 plant positions. Pine weevils were released in the center after all plants were positioned. Illustration: Kristina Berggren Nieto

3.5.4 Resin duct experiment

The resin duct experiment (**Paper III**) included a total of 59 emblings and 59 seedlings, of which ~half (30 emblings and 29 seedlings) were treated with MeJA. About 2 months after the initial MeJA treatment (August 31, 2021) all plants were sampled by cutting off an approximately 2 cm long part of the lower stem and immediately submerged it in formalin acetic acid (FAA) solution (85% ethanol, 5% formalin (37% aqueous solution), 5% acetic acid, and 5% glycerol) (Figure 12) (following Moreira et al. 2015). After 48 hours, the FAA was replaced with 70% ethanol (v/v) to store the stems until sectioning and staining. Using a sliding microtome (Figure 13 left), stems were transversely sectioned (~40 µm thick) in August-November, 2022, and sections were stained in a 1:1 ratio mix of Safranin and Astra Blue for 10 minutes (Figure 13 right). To remove excess stain, sections were immerged first in distilled water and then in ethanol baths of different concentrations (50%, 70%, and 100%) (Figure 13 right), and mounted on microscope slides. Finally, the sections were dried in an oven (~60 °C) for approximately three days. Using a slide scanner, images of the sections were obtained, and the images were analysed using QuPath image analysis software (Bankhead et al. 2017). We counted all axial resin ducts in the phloem and xylem (constitutive (CRDs) and traumatic (TRDs) resin ducts separately), and measured their size (lumen area) and the area of the phloem and xylem (excluding the pith) (mm^2). We then calculated the mean size of CRDs (mm^2), density (number of CRDs per mm^2 tissue), and conductive area (percentage tissue area occupied by CRD area) following Vázquez-González et al. (2019). Finally, we calculated the number and mean size (mm^2) of CRDs and TRDs in the xylem separately, to compare constitutive and induced defences.

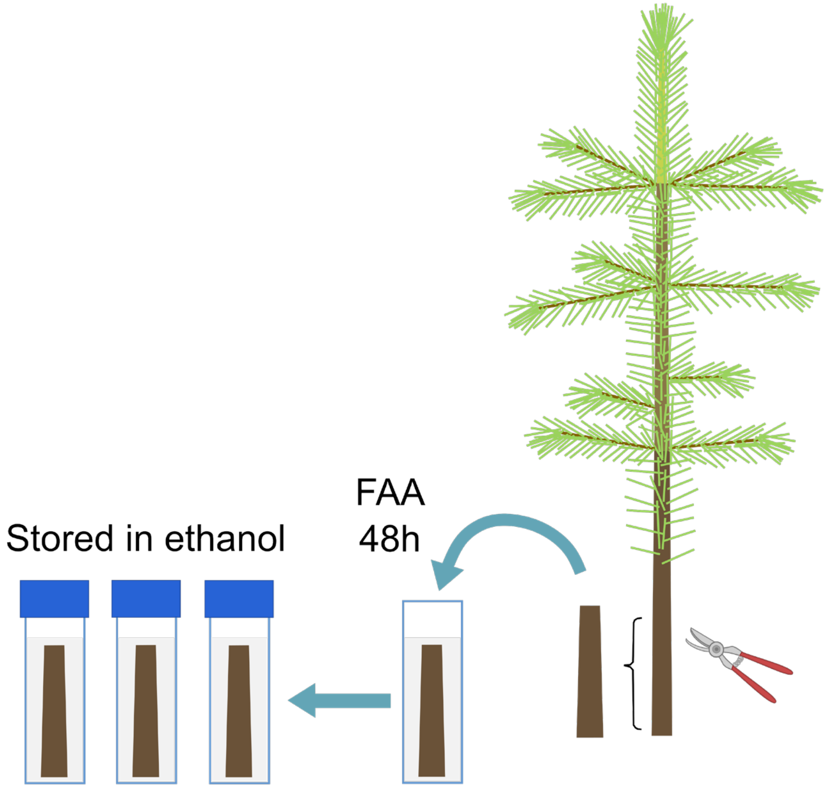

Figure 12. Stem sampling and fixing in FAA prior to stem sectioning. Illustration: Kristina Berggren Nieto

Figure 13. Stem sectioning in a sliding microtome (left) and staining + cleaning of sections in different ethanol baths (right). Photos: Kristina Berggren Nieto

3.5.5 Wound healing experiment

The wound healing greenhouse experiment (**Paper IV**) included a total of 76 emblings and 80 seedlings, of which ~half (39 emblings and 40 seedlings) were treated with MeJA. Wounding was performed over two consecutive days (June 21-22, 2022) and half of the plants (taken randomly from each treatment) were wounded each day. Using a scalpel, a rectangular wound (height: 40 mm, width: 5 mm) was incised on the lower part of the stem (from ~1-2 cm above the soil surface and upwards) (Figure 14), avoiding branches (following Chen et al. 2023). All bark phloem within the wound was removed, completely exposing the xylem. To measure the initial wound size, a transparent plastic film was placed over it and the outline of the wound drawn with a black permanent marker. Drawings were scanned and the wound areas measured in QuPath image analysis software (Bankhead et al. 2017). After wounding plants were arranged in a semi-randomized block design on two greenhouse benches. We recorded the onset of healing (yes or no) two weeks post-wounding (July 5-6, 2022) by visually examining plants for signs of healing. Healing had initiated if new light green bark tissue was visible along the wound perimeter. Using a millimeter paper, the size (height and width) of each wound (i.e., the exposed xylem) was measured every second week (a total of 12 weeks post-wounding), to estimate wound closure in squared millimeters (mm^2) during the first year (Figure 14). Plants were randomized following the same block design as before at every two-week measurement. Size measurements of the wound was repeated during the second year, but only twice (May 19 and September 19, 2023). At the end of the experiment (September 19, 2023), we recorded if the wounds had complete closed or not (yes or no). Finally, we calculated wound healing rate (mm^2 growth of new tissue per day) by subtracting the final wound size from the first wound size measurement (two weeks after wounding) and dividing by the number of days in between. This was done for the first year, for the second year, and for the first and second year together (cumulative rate).

Figure 14. Stem wounding and healing over time. Photos and illustration: Kristina Berggren Nieto

3.6 Data handling and analyses

All analyses were conducted in R version 4.2.2. (R Core Team 2022) (**Paper I**) and version 4.3.1. (R Core Team 2023) (**Paper II**, **III**, and **IV**). In all experiments, we evaluated the effects of plant type (embling or seedling) and MeJA treatment (10 mM or deionized water), and their interaction. Various models were fitted. Linear and generalized linear models were fitted with the *lm*- and the *glm*-function, respectively, in the base stats R package; R Core Team 2023. Linear and generalized linear mixed effects models with the *lmer*- and *glmer*-function, respectively, in the *lme4* package (Bates et al. 2015). Generalized linear mixed models were also fitted with the *glmmTMB*-function in the *glmmTMB* package (Brooks et al. 2017). Binomial distribution was used for nominal categorical data (yes/no). Some linear models included the *varIdent*-function from the *gls*-function in the *nlme* package (Pinheiro et al. 2023) to account for when variances differed among plant types (i.e. variation among seedlings was either higher or lower than among emblings).

In all experiments, model validations were performed through inspection of residuals vs. predicted values, through simulation and plotting of scaled residuals and outliers using the *DHARMa* package (Hartig 2022), and by examining if assumptions of equal variances across treatments were met using the *LeveneTest*-function in the *car* package (Fox and Weisberg 2019). To meet model assumptions, some modes were log-transformed and some were square root-transformed. To test significance of main effects and interactions, analysis of deviance was used through the *Anova*-function in the *car* package (Fox and Weisberg 2019). For graph plotting and pairwise comparisons, estimated means for each treatment were obtained using the *emmeans*-function in the *emmeans* package (Lenth 2023).

4. Results and Discussion

Plants are constantly under attack from insects, pathogens, and mammals, but unlike animals, plants are sessile (immobile) and must defend themselves in place. Pest damage can disrupt growth, cause abnormal growths (excrescences), or even kill the plant, leading to poor harvests for farmers and foresters. Thus, increased pest resistance may be vital for plant health. This research investigated the consequences and mechanisms behind enhanced resistance to pine weevil feeding observed in Norway spruce plants propagated through somatic embryogenesis (SE), and factors that could moderate this effect. Our findings have the potential to aid forest regeneration by producing plants with innate pest resistance.

In summary, we found that treating SE plants with MeJA can substantially improve plant protection and reduce plant mortality (**Paper I**). SE propagation alone can reduce the number of pine weevils on plants and subsequently decrease pine weevil feeding damage, an effect enhanced by MeJA application (**Paper II**). Increased density of constitutive resin ducts in the phloem may explain increased pine weevil resistance in emblings, although the formation of traumatic resin ducts (TRDs) does not seem to play a role in the increased resistance observed in MeJA-treated emblings (**Paper III**). Finally, propagation via SE not only increases resistance in emblings, but also promotes their tolerance to wounding, with MeJA-treated emblings exhibiting the most pronounced effect (**Paper IV**). Our findings suggest that SE presents several plant defence benefits, and that combining SE with MeJA treatment offers the most beneficial approach for enhancing both plant resistance and tolerance against pine weevil damage. While the exact mechanisms behind the increased resistance in SE plants remain elusive, our data on pine weevil preference, and preliminary data on terpene chemistry (data not included in thesis), indicate a potential role of feeding stimulants or deterrents in reducing damage. Overall, this research reports promising methods for improving plant survival rates and promoting successful forest regeneration through more sustainable practices.

4.1 Combining methods for increase protection (**Paper I**)

4.1.1 Synergistic effects on pine weevil resistance

Increased knowledge on how different methods can be used together to enhance plant defence is crucial for future advancements in plant protection. This study aimed to investigate the potential of combining SE with MeJA treatment to achieve this goal.

As previously reported, SE (Puentes et al. 2018) and MeJA (Zas et al. 2014; Chen Y et al. 2020; Puentes et al. 2021) can separately enhance conifer resistance to pine weevil feeding; up to 50% reduction from MeJA and 30% from SE in field studies. This study investigated their combined effect on pine weevil resistance in a field and lab study. Plants have a limited amount of resources to allocate to defence or to growth and reproduction, including a trade-off between constitutive and induced defences (Herms and Mattson 1992). Given emblings' already elevated defences, we expected a limited or modest further increase from MeJA treatment. However, the results were surprising! MeJA treatment conferred a strong synergistic effect on reducing pine weevil feeding damage in emblings in the field. Treated emblings suffered 86% less damage in the field (Figure 15b) and 48% less in the lab (Figure 16b) compared to the untreated containerized seedlings. Notably, the weevils attacked all treatments equally in both the lab and the field (Figure 15a and 16a). A small effect on attack frequency following MeJA treatment aligns with results from Zas et al. (2014). Our findings suggest that the reduced damage observed in MeJA-treated emblings is not solely due to a lower risk of being attacked, but that differences in palatability play a more important role. Supporting this notion are the considerably smaller feeding wounds on the MeJA-treated emblings, suggesting that MeJA treatment may enhance chemical or other defensive mechanisms that deter pine weevils from extensive feeding. Previous studies have shown that plants propagated through SE exhibited higher levels of secondary compounds compared to conventionally grown plants (Lamhamedi et al. 2000; Fulzele and Satdive 2003). Similarly, MeJA treatment is known to induce traumatic resin duct formation and increases in terpenes and phenolic compounds (Martin et al. 2002; Krokene et al. 2008; López-Villamor et al. 2021; Puentes et al. 2021). Therefore, it is possible that SE and MeJA treatment have a combined effect on plant chemistry (or other traits), leading to a stronger defence response than either individual treatment. Furthermore, this dramatic decrease in phloem feeding damage resulted in a 98% reduction in mortality for the MeJA-treated emblings in relation to untreated containerized seedlings (Figure 15c). MeJA treatment alone has been shown to reduce the risk of girdling by pine weevils, leading to increased survival rates in conifer seedlings (Zas et al. 2014; Fedderwitz et al. 2016). However, in our field study the effect on mortality in MeJA-treated emblings was stronger than in MeJA-treated seedlings. The effect on mortality persisted over the following two seasons, resulting in MeJA-treated emblings exhibiting the lowest mortality after three years in the field (31%) (Figure 17). These findings could help improve the survival rate of young conifer plants over several years, which are vulnerable to pine weevil feeding in the first few years after planting, for example, by applying MeJA in nurseries before seedlings are shipped for planting.

Our findings strongly suggest a synergistic interaction between SE and MeJA in enhancing plant resistance, demonstrating a potentially robust plant protection strategy. Further research is needed to elucidate the underlying mechanisms responsible for this interaction.

4.1.2 Effects of plant size

In Sweden, two seedling types are mainly used in planted forest regeneration; containerized (grown in plug trays indoors/in greenhouses), and bare-root (sown/grown in cultivation beds outdoors). Containerized seedlings develop a smaller root system and above-ground parts due to limited growing space. These seedlings are easier to plant, but more vulnerable to pests compared to larger bare-root seedlings with thicker stem and bark, which is more difficult for the pine weevil to girdle. In our experiment, we saw an effect of size on mortality both in the first year, and cumulative over all three years (Figures 15c and 17). These findings suggest that MeJA-treated emblings offer comparable protection to larger bare-root plants despite their smaller size, providing several advantages such as easier planting, reduced greenhouse space requirements due to earlier field planting (especially beneficial for slow-growing species), and decreased economic losses due to field mortality.

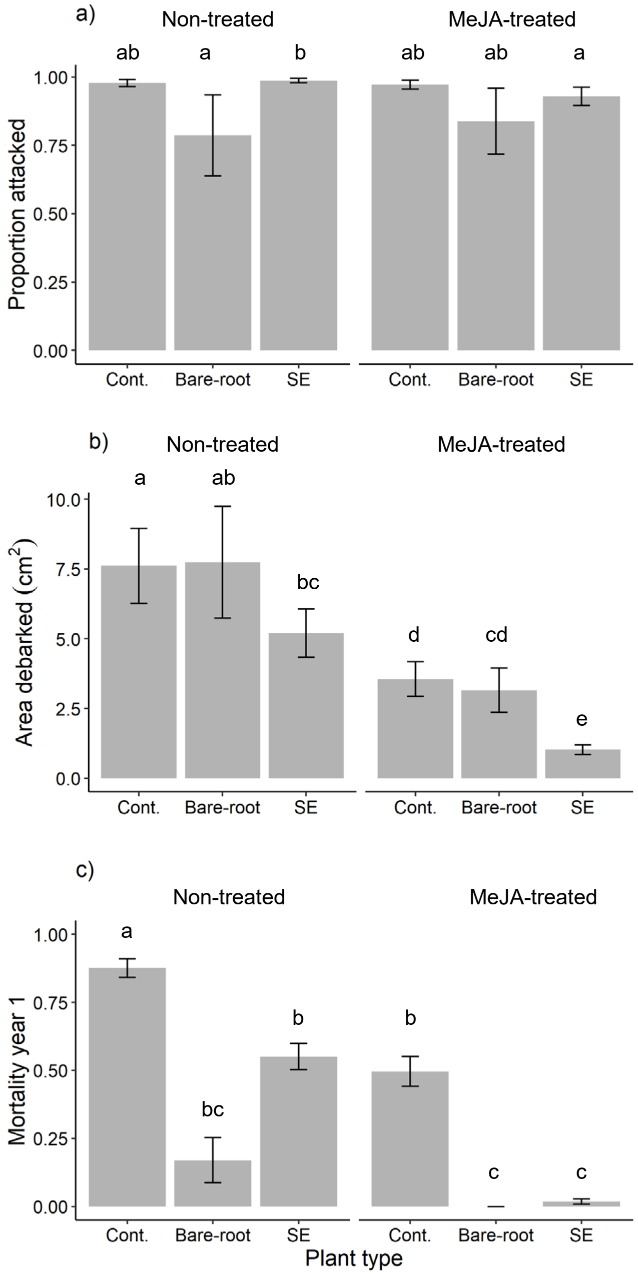

Figure 15. Estimated means (± SEM) of (a) the proportion of Norway spruce (*Picea abies*) plants attacked, and (b) area debarked (cm^2) by pine weevils (*Hylobius abietis*), as well as (c) plant mortality (proportion that died) by September 2019, year 1 in the field. Treatments represent plant types (Cont. = containerized seedlings, Bare-root =

bare-root seedlings, SE = emblings produced via somatic embryogenesis) treated with the plant hormone methyl jasmonate (MeJA; 10 mM applied once in June, 2019), or untreated (water application). Sample sizes from left to right: Non-treated Cont. n=114; Bare-root n=50; SE n=164; MeJA-treated Cont. n=114; Bare-root n=50; SE n=164. Different letters indicate significantly different means. (adapted from **Paper I**)

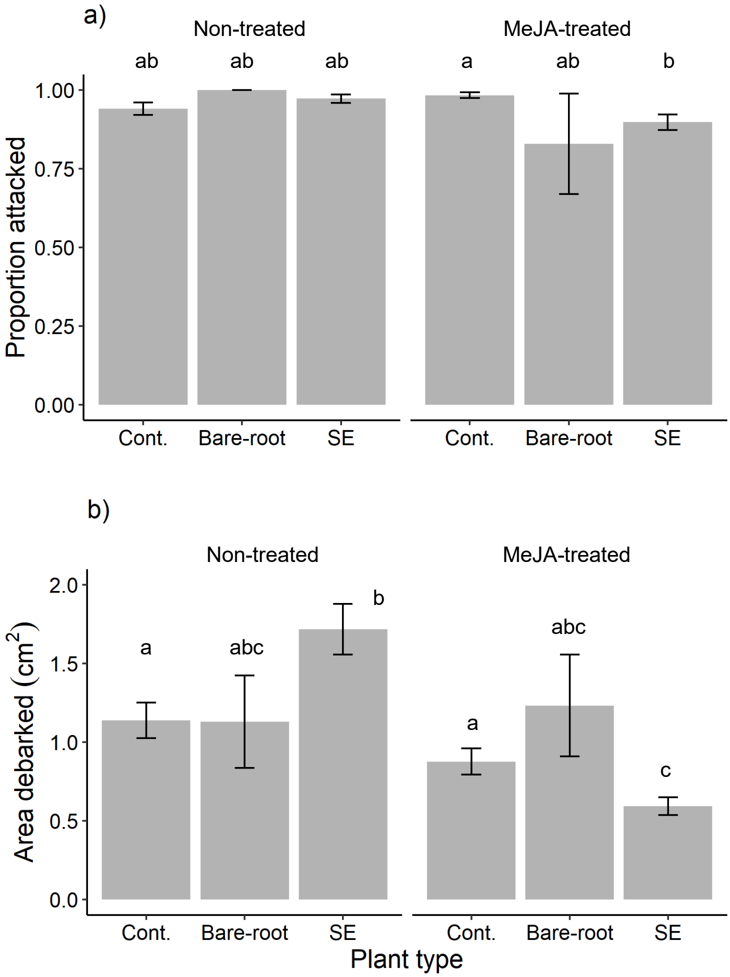

Figure 16. Estimated means (± SEM) of (a) the proportion of Norway spruce (*Picea abies*) plants attacked and (b) area debarked by pine weevils (*Hylobius abietis*) in the lab experiment (replicated in time between July-August 2019). Treatments represent plant types (Cont. = containerized seedlings, Bare-root = bare-root seedlings, SE = emblings produced via somatic embryogenesis) treated with the plant hormone methyl

jasmonate (MeJA; 10 mM applied once between July and August, 2019), or untreated (water application). Sample sizes from left to right: Non-treated Cont. n=150; Bare-root n=12; SE n=162; MeJA-treated Cont. n=150; Bare-root n=12; SE n=162. Different letters indicate significantly different means. (adapted from **Paper I**)

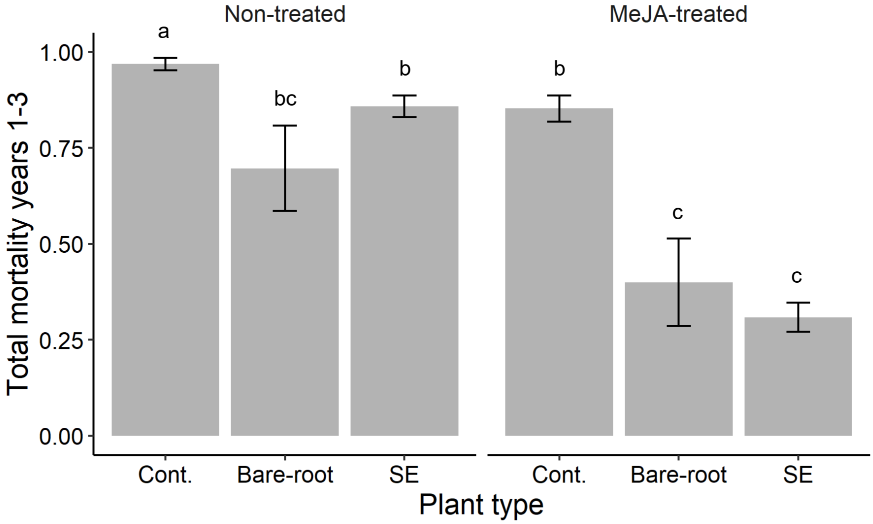

Figure 17. Estimated means (± SEM) of cumulative mortality of Norway spruce (*Picea abies*) plants over the entire experimental period in the field (years 1-3). Treatments represent plant types (Cont. = containerized seedlings, Bare-root = bare-root seedlings, SE = emblings produced via somatic embryogenesis) treated with the plant hormone methyl jasmonate (MeJA; 10 mM or water applied once in June, 2019), or untreated (water application). Sample sizes from left to right: Non-treated Cont. n=114; Bare-root n=50; SE n=164; MeJA-treated Cont. n=114; Bare-root n=50; SE n=164. Different letters indicate significantly different means. (adapted from **Paper I**)

4.2 Pine weevil preference over time (**Paper II**)

Understanding herbivore behaviour, particularly host detection and preference, are key components in developing effective, economically viable, and ecologically sustainable plant protection strategies. This study aimed to investigate pine weevil host selection and feeding preference over time to contribute to this knowledge.

As a continuation of the first study, we wanted to examine if the resistance observed in non- and MeJA-treated emblings was a result of changes in pine weevil behaviour. Insects rely on certain cues (volatile, visual, gustatory, tactile) to detect, select and accept a host for feeding or oviposition. To evaluate how pine weevil host preference is affected by SE and MeJA, we conducted a multi-choice experiment using circular arenas with plants from four treatments (embling, seedling, embling+MeJA, seedling+MeJA). Puentes et al. (2018) reported fewer attacks on emblings, but in our previous study (Figure 15a above), and in Fedderwitz et al. (2016), attack was similar across treatments. Neither did we observe any differences in attack between treatments in this study (Figure 19B), so reduced damaged might not be due to fewer attacks. Additionally, at the first time point (2 hours after the experimental start), no significant differences in the number of pine weevils on the plants were observed between treatments (Figure 18). This suggests that plant volatiles, at least initially, did not affect the behavior of the pine weevils. Instead, visual cues seemed to be important for host finding, which has been seen in other studies (Björklund et al. 2005; Björklund 2008; Brévault and Quilici 2010). Following host detection, gustatory cues are likely to determine host selection and acceptance. In our study, untreated plants were strongly preferred over the MeJA-treated, an effect visible already 7 hours after exposure to the plants (Figure 18). After 26 hours, untreated seedlings were preferred over untreated emblings. Due to feeding behaviour changing over time, gustatory cues seemed to be the most important for host acceptance. While less likely, herbivore-induced plant volatiles (HIPVs) might also have contributed to the change in weevil preference. Plants can distinguish herbivore feeding from mechanical wounding due to elicitors in insect saliva (Mattiacci et al. 1994; Paré and Tumlinson 1996), and modify the strength or composition of the volatiles they emit (Alborn et al. 1997; Giacomuzzi et al. 2017). Studies have shown that the pine weevil can be either attracted or deterred by HIPVs released from feeding by conspecifics (Zagatti et al. 1997; Bylund et al. 2004). Hence, it is possible that HIPVs also played a role in changing pine weevil preference over time. Finally, the overall number of pine weevils observed on the plants correlated with the amount of feeding damage (compare Figure 19A and C). This implies that diverting weevils from seedlings, through deterring odour or decreased palatability, could mitigate damage.

Our findings demonstrate that MeJA treatment and, to a lesser extent, SE alone can influence pine weevil behaviour and host preference over time. Furthermore, the gradual shift in weevil preference over time suggests a deterrent effect triggered by the taste of the plant rather than solely based on initial visual or olfactory cues. However, further research is needed to understand the exact mechanisms behind this resistance, suggesting changes in embling defensive chemistry are involved.

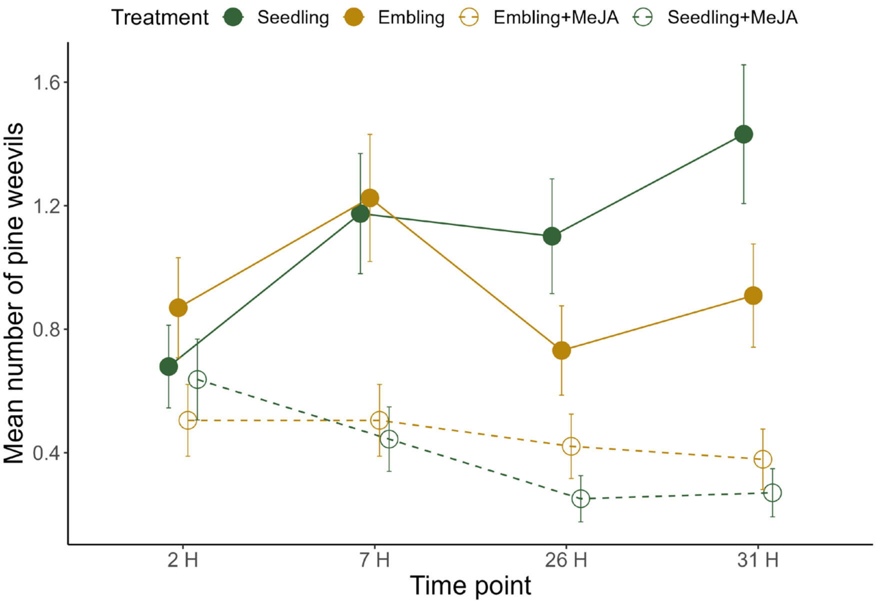

Figure 18. Estimated means (± SEM) of the number of pine weevils (*Hylobius abietis*) found on Norway spruce (*Picea abies*) plants in the lab arena. No. of pine weevils was recorded 2, 7, 26, and 31 hours after the start of a round, representing 11am and 4pm each day. Plant types represent seedlings (grown from seeds (green)) and emblings (produced via somatic embryogenesis (gold)), treated with the plant hormone methyl jasmonate (MeJA; 10 mM applied once, twenty days prior to the start of a round; dotted lines), or untreated (water application; filled lines). Sample sizes from left to right: non-treated seedlings n=40 and emblings n=40, MeJA-treated emblings n=40 and seedlings n=40. Note that the y-axis does not start at zero. (adapted from **Paper II**)

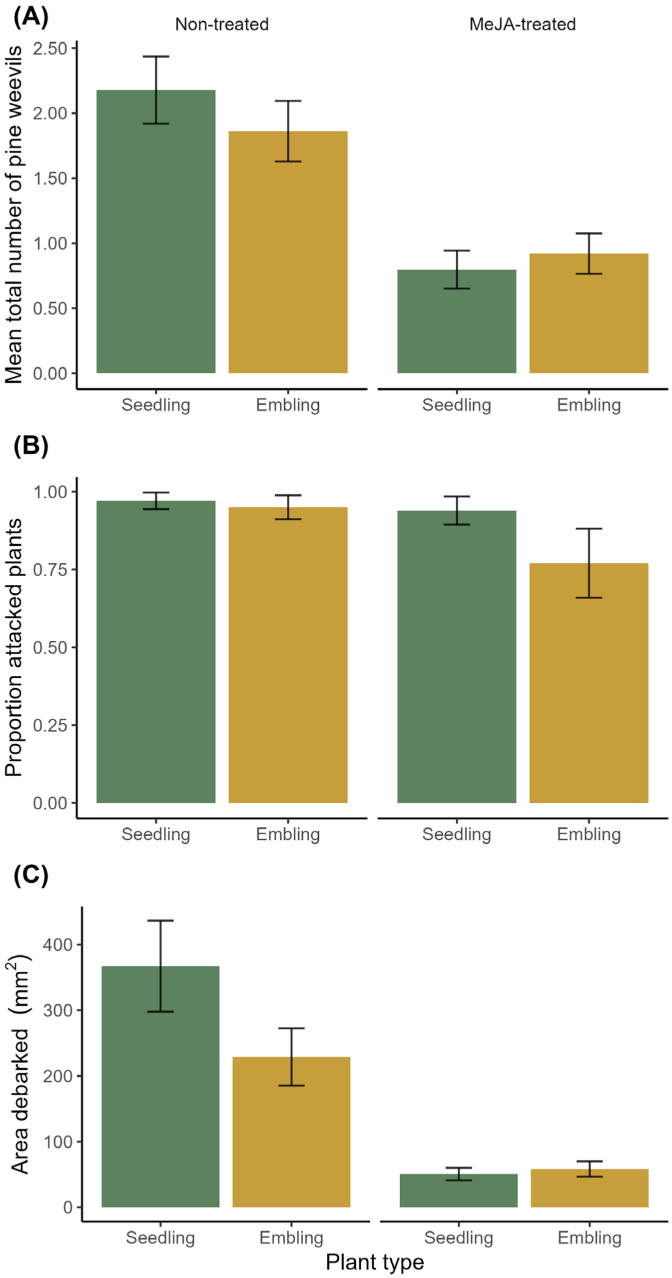

Figure 19. Estimated means (± SEM) of (A) the total number of pine weevils (*Hylobius abietis*) per plant, (B) the proportion of Norway spruce (*Picea abies*) plants attacked, and (C) area debarked (mm^2) by pine weevils (*Hylobius abietis*) in the lab arena. Mean

total number of pine weevils was the mean sum of all weevils per plant across time points. Attacked plants and area debarked were recorded at the end of the experiment (after 48 hours). Plant types represent seedlings (grown from seeds (green)) and emblings (produced via somatic embryogenesis (gold)), treated with the plant hormone methyl jasmonate (MeJA; 10 mM applied once, twenty days prior to the start of a round), or untreated (water application). Sample sizes from left to right: non-treated seedlings n=40 and emblings n=40, MeJA-treated emblings n=40 and seedlings n=40. Note the different scales on the y-axes. (adapted from **Paper II**)

4.3 Underlying mechanisms (**Paper III**)

4.3.1 Resin duct size and distribution

Unravelling the mechanisms behind the enhanced resistance observed in SE-propagated plants, both alone and in combination with MeJA, holds potential for advancing our knowledge of plant morphological and chemical responses to stress. This study aimed to elucidate this by investigate resin duct abundance.

After acquiring a deeper understanding on how emblings can affect pine weevil behaviour, we wanted to investigate the mechanisms behind their enhanced resistance. Conifers have evolved a complex resin duct defence system. Constitutive resin ducts (CRDs) are continuously produced in undamaged plants in the phloem and xylem, and traumatic resin ducts (TRDs) form in the outer margin of the xylem upon stress, such as damage or MeJA treatment. In our previous field study, we observed a higher secretion of resin on the stems of untreated, and especially MeJA-treated emblings. Hence, we hypothesized that emblings would have more, or larger resin ducts compared to seedlings, both constitutively, and induced after MeJA treatment. However, in the phloem, neither size nor conductive area of resin ducts differed between seedlings and emblings, even though untreated emblings displayed a 79% higher density of phloem CRDs (Figure 20C). Since the pine weevil feeds on the bark of young seedlings (Wallertz et al. 2014), this finding may contribute to explaining the previously reported enhanced resistance to pine weevil bark damage observed in emblings (Puentes et al. 2018, and **Paper I**). In addition, emblings exhibited significantly larger (34%) xylem CRDs (Figure 20B), and slightly increased xylem CRD conductive area and density (Figure 20D and F). However, xylem CRDs are generally small and few compared to the xylem area in

Norway spruce, thus, the role of xylem CRDs in pine weevil resistance remains unclear. The most surprising result was that MeJA-treated emblings formed both fewer (19%) (Figure 21A), and smaller (46%) (Figure 21B) TRDs than seedlings, contradicting our hypothesis of MeJA treatment inducing more and larger TRDs in emblings. This suggests that TRDs do not play a major role in the elevated resistance observed in MeJA-treated emblings, especially regarding bark feeders such as the pine weevil, and that other traits are behind this effect. This was observed by Tomlin et al. (1998), who reported a similar effect in white spruce (*Picea glauca*) resistant to the white pine weevil (*Pissodes strobi*). Additionally, Puentes et al. (2021) found a minimal response to MeJA application in constitutively resistant Norway spruce genotypes, suggesting a trade-off between constitutive and induced defences. Further, this observation of fewer and smaller TRDs in emblings raises questions about their long-term defence capabilities. Since TRDs are thought to be an adaptation to bark beetles and fungal pathogens (Berryman 1972; Nagy et al. 2000), could this potentially lead to increased susceptibility as the trees grow older?

As this is the first study of the potential role of TRDs in Norway spruce resistance against the pine weevil, it is difficult to draw any firm conclusions. Our results indicate a very weak, if any, link between TRDs and insect resistance. Although the results point towards some involvement of CRDs in resistance, this finding should be investigated using larger sample sizes and in several Norway spruce families. Other traits may be involved; for example, alterations in the chemical profile or lack of feeding stimuli could be the cause behind the results, rather than physical defence mechanisms such as size or abundance of resin ducts. More research is therefore needed to pinpoint the specific traits that contribute to the elevated resistance in MeJA-treated emblings.

4.3.2 Terpene chemistry

To further explore the underlying resistance mechanisms, we compared resin characteristics (terpene chemistry) between untreated and MeJA-treated emblings and seedlings. Terpenes consist of both volatile and non-volatile compounds, and are the main components in resin. We analysed terpene concentration and composition extracted from the lower stem bark. Due to time constraints, we are currently only able to present preliminary results, not included in the thesis chapters. Our initial analyses revealed both quantitative and qualitative differences in terpene chemistry between emblings and seedlings. We expected that emblings would exhibit higher terpene concentrations, or wider variety compared to seedlings. Surprisingly, emblings showed lower concentrations of terpenes considered to stimulate pine weevil feeding, which aligns with the feeding preference results (**Paper II**), but higher levels of others once treated with MeJA. However, it is important to emphasize that these are preliminary findings, requiring further exploration into the chemical defence mechanisms of emblings.

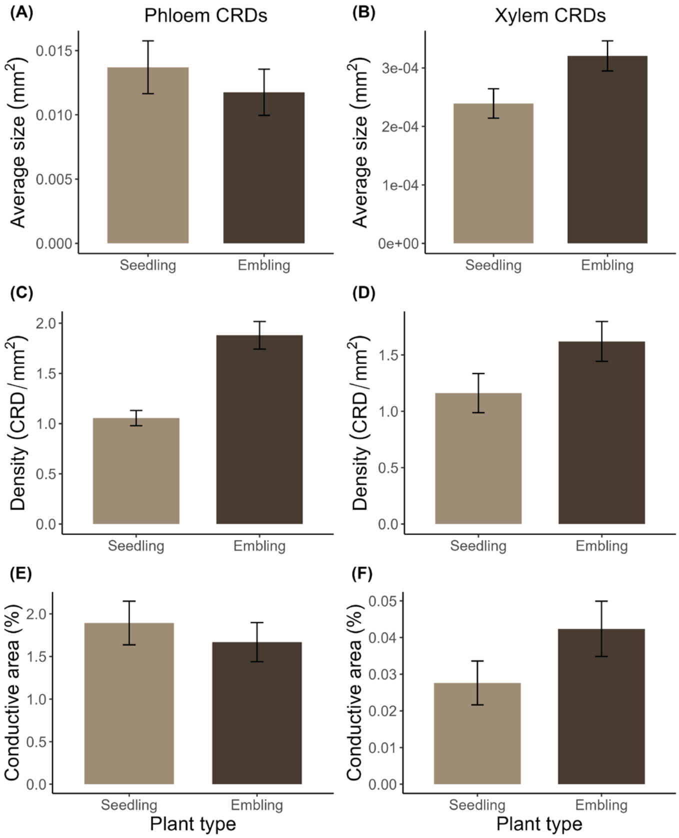

Figure 20. Estimated means (± SEM) of the (A)/(B) average size (mm^2), (C)/(D) density (CRDs per mm^2), and (E)/(F) conductive area (% CRD area (mm^2) of total phloem or xylem area (mm^2)) of Norway spruce (*Picea abies*) axial constitutive resin duct located in transversal stem sections. The left column represents CRDs in the phloem, and the right column CRDs in the xylem. Plant type represent seedlings (grown from seeds) and emblings (produced via somatic embryogenesis). Sample sizes from left to right:

seedlings n=30, emblings n=29. Note the different scales on the y-axes. (adapted from **Paper III**)

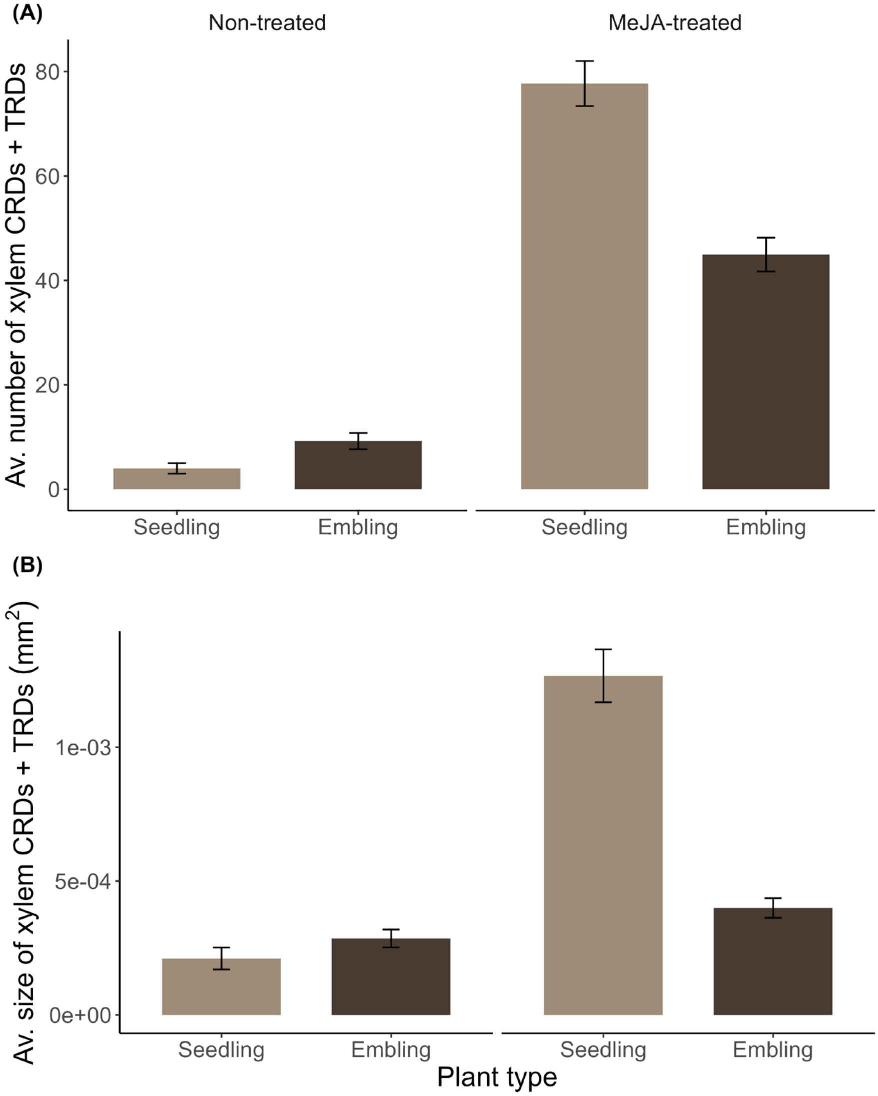

Figure 21. Estimated means (± SEM) of (A) number and (B) average size (mm^2) of Norway spruce (*Picea abies*) axial constitutive and traumatic resin ducts located in transversal stem sections. Plant type represent seedlings (grown from seeds) and emblings (produced via somatic embryogenesis), treated with the plant hormone methyl

jasmonate (MeJA; 10 mM applied twice with a 2-week interval in July, 2021), or untreated (water application). Non-treated plants contain only CRDs and MeJA-treated plants contain both CRDs and TRDs. Sample sizes from left to right: non-treated seedlings n=30 and emblings n=29, MeJA-treated seedlings n=29 and emblings n=30. (adapted from **Paper III**)

4.4 Wound healing ability of a resistant plant (**Paper IV**)

The two components of plant defence, resistance and tolerance, are interconnected. Evaluating how enhancing one might influence the other could not only advance our understanding of plant stress responses, but also pave the way for the development of better-defended plants in breeding programs. This study aimed to investigate wound healing ability in an already resistant plant to explore potential effects on tolerance.

In the last experiment, we wanted to examine if the emblings, apart from exhibiting increased resistance, also show increased tolerance-related traits. We conducted a wound healing experiment over two growing seasons to evaluate wound healing ability between untreated and MeJA-treated emblings and seedlings. In our previous field study, we observed a higher number of individuals that were healing their wounds among emblings. On the other hand, a lower healing rate has been documented for MeJA-treated Norway spruce seedlings when MeJA was applied after wounding (Chen et al. 2023). Thus, we expected untreated emblings to exhibit the greatest healing rate, and we expected lower healing in MeJA-treated emblings. We found that SE and MeJA indeed differentially affected healing abilities. While MeJA treatment (prior to wounding) increased the onset of healing with 48%, SE decreased onset, with 70% of emblings showing signs of healing two weeks after wounding, compared to 90% of the seedlings (Figure 22a). Due to this slow start, untreated emblings exhibited slightly greater wound sizes throughout the whole first season, especially early on (Figure 24). This means that changes in wound size over time during the first year were mainly affected by MeJA treatment. Moreover, MeJA slightly decreased the overall healing rate (9%) during year 1, while emblings exhibited the highest overall healing rate across both years (Figure 23a-c). In addition, emblings exhibited a greater proportion of completely closed wounds (68% more than the seedlings) at the end of the second season (Figure 22b). MeJA-treated emblings had the highest proportion of individuals exhibiting completely closed wounds. The study shows that SE and MeJA can have opposing effects on wound healing in Norway spruce plants.

We conclude that MeJA treatment may enhance the initiation of stem wound healing in Norway spruce but subsequently reduce healing rate, while SE appears to affect plants in the opposite way. The ability to recover from damage is essential to avoid negative effects and possible mortality from a weakened state (Neely 1988; Vasaitis 2012; Vasiliauskas 2001). A slow onset of healing may increase the risk of pathogen infection when the wound is fresh. As the wound heals, potential for fungal development and stem decay decreases (Biggs 1986; Romero 2014), and an increased healing rate and earlier closing of the wound contributes to the plant returning to a state where resources can be prioritized to growth and reproduction. Our findings suggest that MeJA-treated emblings offer the most beneficial traits with both increased onset of healing as well as healing rate and wound closure over two seasons. This knowledge can be used to develop strategies to promote faster wound healing and recovery in seedlings after planting.

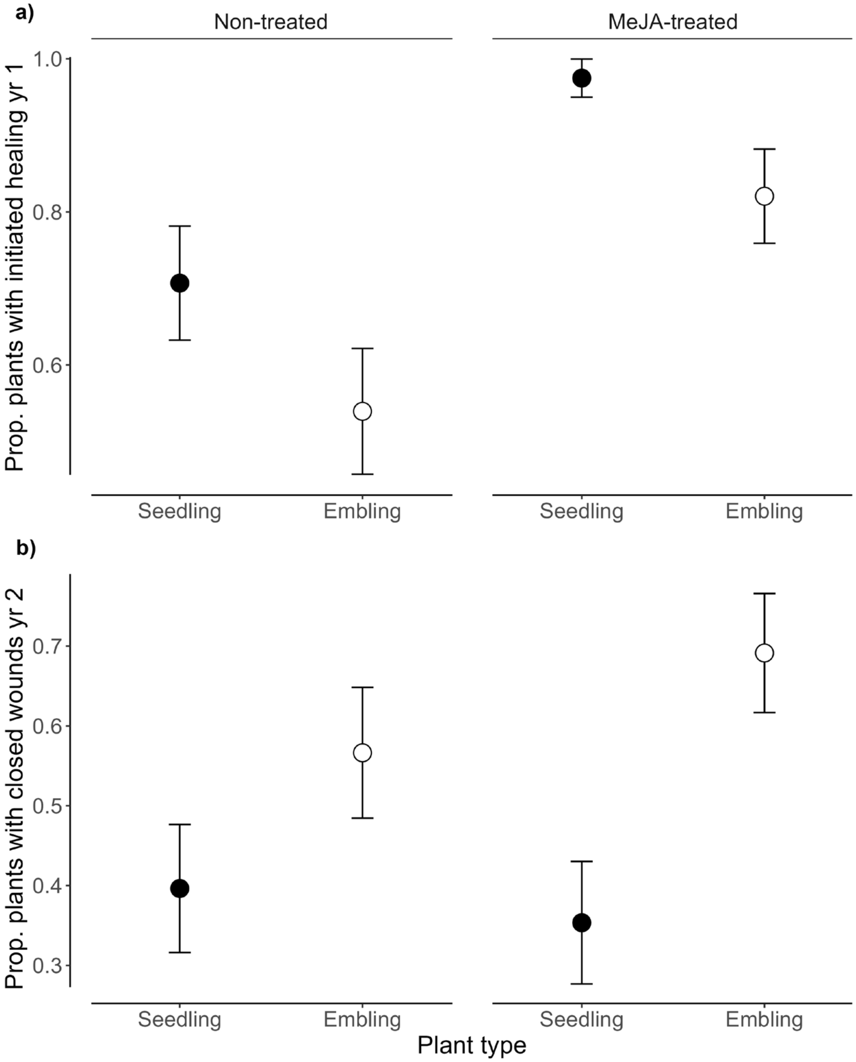

Figure 22. Estimated means (± SEM) of the proportion of Norway spruce (*Picea abies*) that had a) initiated healing (signs of light green new tissue growth in the wound perimeter) two weeks post-wounding in year 1 (July 2022), and b) completely closed their wounds (xylem covered with new tissue) in September year 2 (2023). Plant types represent seedlings (grown from seeds) or emblings (produced via somatic embryogenesis), treated with the plant hormone methyl jasmonate (MeJA, 10 mM applied once in June, 2022), or untreated (water application). Sample sizes from left to right: non-treated seedlings n=40 and emblings n=37, MeJA-treated seedlings n=40 and emblings n=39. Note that the y-axes do not start at zero. (adapted from **Paper IV**)

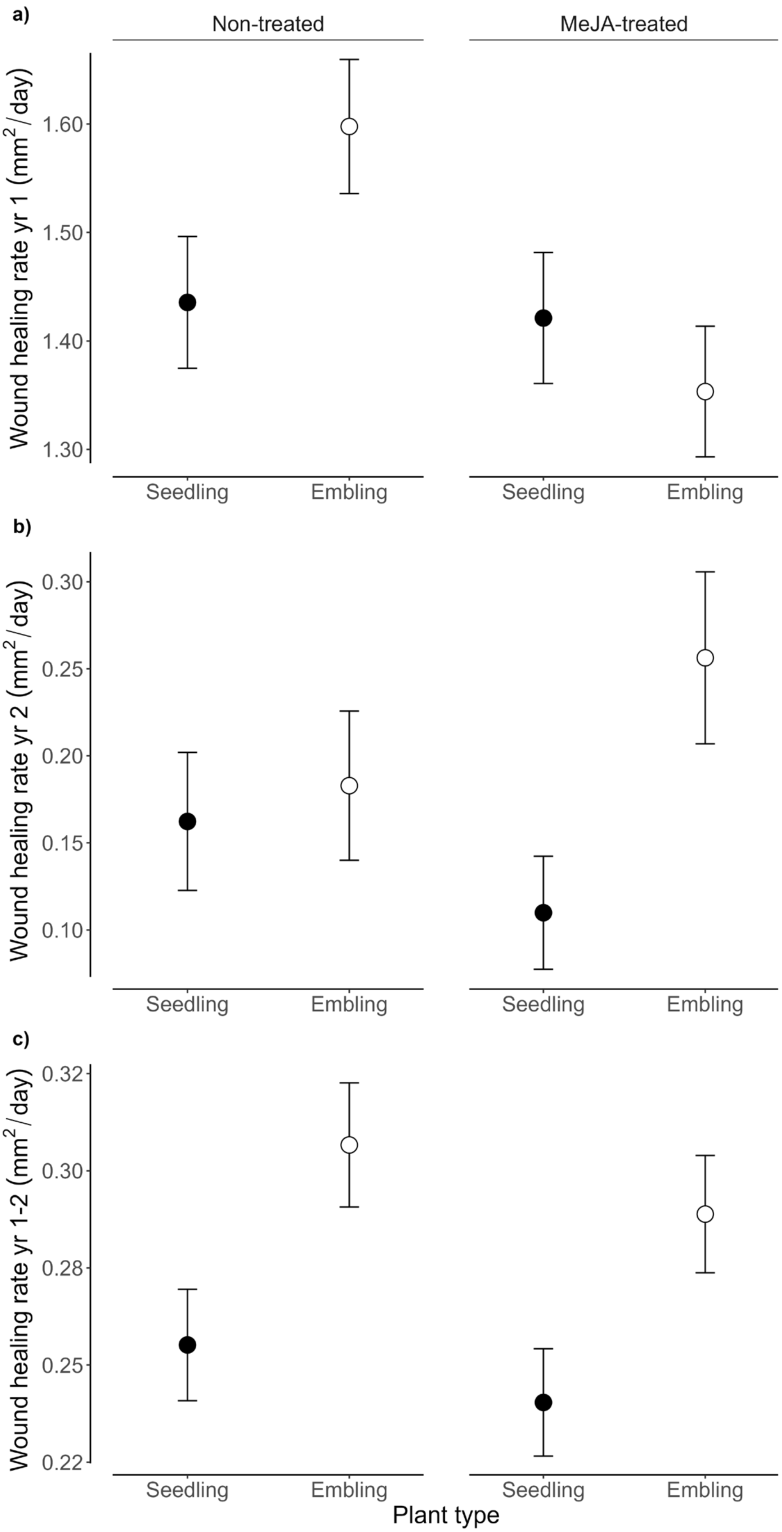
a)
Non-treated
MeJA-treated
Wound healing rate yr 1 (mm^2/day)
1.60
1.50
1.40
1.30
Seedling
Embling
Seedling
Embling
b)
Wound healing rate yr 2 (mm^2/day)
0.30
0.25
0.20
0.15
0.10
Seedling
Embling
Seedling
Embling
c)
Wound healing rate yr 1-2 (mm^2/day)
0.32
0.30
0.28
0.25
0.22
Seedling
Embling
Seedling
Embling
Plant type

Figure 23. Estimated means (± SEM) of Norway spruce (*Picea abies*) wound healing rate (mm^2 growth of new tissue per day) during a) year 1 (two weeks post-wounding until September 14, 2022; 70 days), b) year 2 (May 19, 2023 to September 19, 2023; 123 days) and c) years 1-2 (July 6, 2022, to September 19, 2023; 454 days). Plant types represent seedlings (grown from seeds) or emblings (produced via somatic embryogenesis), treated with the plant hormone methyl jasmonate (MeJA, 10 mM applied once in June, 2022), or untreated (water application). Sample sizes from left to right: non-treated seedlings n=40 and emblings n=37, MeJA-treated seedlings n=40 and emblings n=39. Note that the y-axes do not start at zero. (adapted from **Paper IV**)

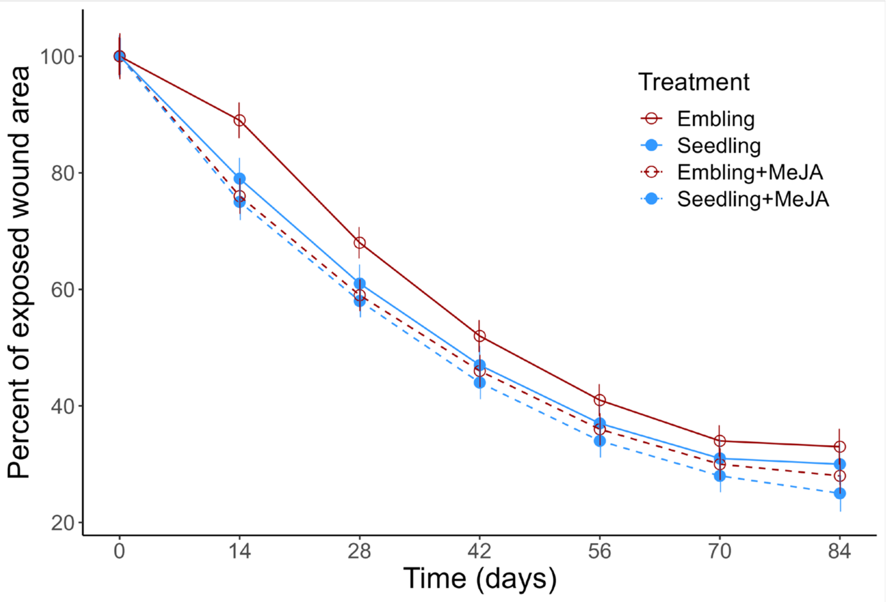

Figure 24. Percentage of the estimated mean wound area (mm^2) that remained open (i.e., exposed xylem) over time during the first year (day 0 = Wounds were inflicted, day 84 = last wound size measurement year 1; plants were measured every 14 days) for Norway spruce (*Picea abies*) plants. Treatments represent plant types (seedlings – grown from seeds, or emblings – produced via somatic embryogenesis), treated with the plant hormone methyl jasmonate (MeJA, 10 mM applied once in June, 2022), or untreated (water application). Sample sizes from top to bottom: non-treated emblings n=37 and seedlings n=40, MeJA-treated emblings n=39 and seedlings n=40. Note that the y-axis does not start at zero. (adapted from **Paper IV**)

4.5 Why do emblings display higher resistance?

This project aimed to investigate why emblings exhibit increased resistance to pine weevil feeding damage. We found that SE definitely acts together with MeJA to enhance resistance even further, it affects pine weevil behaviour, and plants' ability to heal wounds, but it affects to a lesser extent morphological defensive responses. The key question remains: why are emblings exhibiting enhanced resistance? If traumatic resin ducts are not explaining the further elevated resistance in MeJA-treated emblings, then what is? Identifying the specific mechanism requires further investigation, and as discussed above, we believe part of the answer lies in the terpene chemistry. Nonetheless, the results we have generated in this thesis have increased the knowledge of general plant defence responses and pine weevil behaviour in response to plants with varying levels of resistance. To further discuss potential factors that may influence embling resistance, we should return to the SE process itself.

4.5.1 The "SE selection"

SE is a highly complicated process due to the induction of embryo formation in somatic cells, and apart from the stress response that the process may cause in the somatic embryos, there are other consequences to consider. Somatic cells are all cells found in an organism that are not zygotic cells. Zygotic cells are the gametes, the reproductive cells, meaning the cells that normally form embryos that mature and grow into new individuals. Reprogramming somatic cells to behave as if they were zygotic cells is not straightforward, and not all genotypes can successfully undergo SE (as shown in Figure 25). This means that apart from the selection of favourable genotypes in the breeding program, there is a selection also at this stage. This inherent selection process may favour genotypes with already elevated defence mechanisms, and thus, better at tolerating stress.

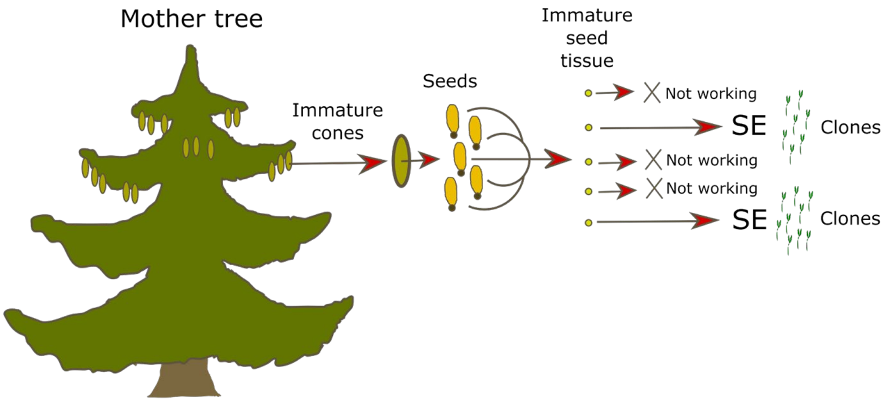

Figure 25. "SE selection": Seed tissue is taken from immature cones to produce SE clones. Some genotypes successfully go through the process, others not. Illustration: Kristina Berggren Nieto.

4.5.2 Do SE plants lack feeding stimulants?

Other potential causes for the increased resistance in emblings could be differences in bark toughness/lignin content, or the microbiome. However, the answer most likely lies in the chemical composition of embling resin. As preliminary bark analyses show that emblings contain lower concentrations of certain terpenes, it could potentially mean they are lacking specific feeding stimulants/attractant chemicals. This could in turn deter the pine weevils through reduced bark palatability (gustatory cues), rather than emblings displaying a higher toxicity. Thus, this possibility should be explored further by (1) identifying specific terpenes present in regular seedlings but missing in emblings, and (2) testing palatability through assessment of weevil feeding behaviour on selected genotypes with varying terpene profiles. It has been demonstrated that weevil feeding induces an increase of the monoterpene (+)-α-pinene (Lundborg et al. 2016), a pine weevil attractant (Nordlander, 1990). In addition, Lundborg et al. (2016) found that pine weevil feeding did not increase the levels of (+)-α-pinene in MeJA-treated seedlings, but instead increased the levels of the antifeedant 2-phenylethanol. The authors suggested that these changes in the chemical profile, such as the lack of an attractant, may explain the shorter feeding time on MeJA-treated seedlings (Lundborg et al. 2016). These results support the probability of a similar scenario in the MeJA-treated, and perhaps also in the untreated emblings.

4.6 Potential application of SE in forestry

4.6.1 Current and future forest practices

Through modern forestry practices, we have converted a considerable amount of natural forests into managed plantations consisting of monoculture stands of similar age. While this approach increases timber yield, it also creates ecosystems more vulnerable to disturbances like herbivore attacks and weather extremes. Due to the rising demand for wood products, the forestry sector is facing an increasing production pressure. Additionally, the phase-out of chemical pesticides in Swedish forestry and elsewhere necessitates a shift towards more sustainable management strategies. Seedling protection against the pine weevil historically involved insecticides like DDT, lindane and permethrin (Nilsson et al. 2010). Newer protection methods have involved integrated pest management (IPM), including soil scarification and physical stem protections such as shield covers (plastic, paper) and coatings (Dillon and Griffin 2008; Nordlander et al. 2009; Galko et al. 2022). However, the constant threat of insect outbreaks, and the risk of intensified future outbreaks with global warming, underscores the urgency for innovative solutions and a more sustainable approach to forest management. A potentially more economically viable approach involves triggering the natural defences of trees using defence elicitors (Krokene et al. 2023). Both MeJA, as a direct defence-inducing method, and SE, with its potential to produce plants that are inherently resistant to the pine weevil, offers promising approaches for future plant protection. However, potential drawbacks of these methods should also be considered.

4.6.2 Pros and cons of using SE

While using clones of selected genotypes can create a more efficient production, a limited number of genotypes reduces genetic diversity within a stand, potentially increasing vulnerability to disturbances such as pests and diseases (Burdon and Aimers-Halliday 2003; Bradshaw et al. 2019). Swedish forestry regulations address this concern by limiting the proportion of a stand that can be established from clones. Rosvall et al. (2019a) discuss how a decreased gene pool can be mitigated by using a mixture of clones, which are exchanged at certain time intervals. Additionally, a limited use of cloned stands in Sweden is likely to have a minimal impact on the overall gene pool, given the prevalence of naturally regenerating spruce (Rosvall et al. 2019a, Rosvall 2019).

Furthermore, studies have shown an initial reduction in growth for both SE-propagated spruce (Högberg et al. 2001; Puentes et al. 2018), and MeJA-treated conifers (Heijari et al. 2005; Zas et al. 2014; Puentes et al. 2021; Chen et al. 2023). This temporary growth reduction is likely due to resource allocation towards defence mechanisms (defences are costly) (Bonaventure and Baldwin 2010; Cipollini et al. 2014), creating a trade-off with growth and reproduction (Herms and Mattson 1992). However, these growth differences have been shown to decrease, as trees grow older (O'Neill et al. 2005; Zas et al. 2014; Puentes et al. 2018).

Another consideration is the increased cost associated with producing SE plants (Lelu-Walter et al. 2013; Bonga 2015) (or applying MeJA treatment), although efforts are being made to reduce costs, e.g. hedging emblings and rooting cuttings. On the other hand, there are many benefits of using SE as a propagation method. SE opens up possibilities for scaled-up, mass-clonal propagation of plants with desirable traits, commercially viable plant material, and faster genotype testing in tree breeding programs (Lelu-Walter et al. 2013; Tikkinen et al. 2017; Egertsdotter et al. 2019). Further, it offers opportunities for low-cost cryostorage (long-term preservation in liquid nitrogen) of the somatic embryos during the test time, which is normally both space-consuming, and poses the problem of deteriorating test material due to aging (Mo et al. 1995; Grossnickle et al. 1996; Högberg et al. 2001; Varis et al. 2017; Egertsdotter 2019).

Overall, while SE presents challenges, its potential benefits for plant propagation and resistance merit further investigation. The production costs may be outweighed by the potential benefits of reduced damage or mortality from pests. Ultimately, forest owners must weigh the economic and ecological factors involved to make informed decisions about forest management practices.

5. Conclusions and future perspectives

In conclusion, our study revealed different defence responses in emblings compared to untreated control seedlings, and the results often deviated from our hypotheses. MeJA treatment further altered these responses in emblings, differing from both untreated emblings and MeJA-treated controls. These findings highlight the complexity of plant defences and underscore the importance of continuous research into plant responses. They provide valuable insight into the increased resistance of emblings and open up novel ways of utilizing SE as a propagation method, as well as contribute to filling knowledge gaps. While the precise mechanisms behind this phenomenon were not revealed by our research, we have gained a deeper understanding of potential causes and proposed promising directions for future research. Overall, combining SE propagation with MeJA treatment appears to be the most effective strategy for enhancing both plant resistance and tolerance in this context, ultimately resulting in reduced plant mortality. We believe our research can contribute to the advancement of sustainable plant protection methods and encourage its use to protect conifers from pine weevils, or similar applications in other systems. Further studies of SE as a plant protection tool might open up possibilities beyond forestry.

Plants with innate resistance against abiotic and biotic stresses are valuable from a plant protection perspective. While increased knowledge on the underlying traits constitutes significant advances in understanding plant defence responses, it also opens up the possibility to manipulate and enhance them to our favour. In addition, understanding the interaction between herbivores and plant defence mechanisms, is crucial for developing target-specific and sustainable plant protection methods, and along with the predictions of novel insect threats due to global warming, it is necessary to continue the development of new protection strategies. This knowledge can further be implemented in plant breeding programs when selecting plant traits, to develop more pest-resistant plant varieties. Furthermore, MeJA has the potential to be applied to emblings at a young age, providing plants with good prospects of survival in the field.

In the future, these findings has the potential to contribute to achieving global goals of production, minimizing risks of economic losses due to pests, and promoting advancements in biotechnology and green solutions. Reduced reliance on chemical pesticides allows us to steer forestry towards more sustainable management strategies. However, safety evaluations of new methods are essential to avoid repeating past mistakes like the long-term negative consequences of DDT. At the same time, maintaining genetic diversity and healthy forest ecosystems is critical for building resilience against disturbances. Therefore, adopting alternative forestry practices such as mixed-species and uneven-aged stands, continuous cover forestry, selection forestry, gap/patch cutting, or retention forestry (the list of alternative methods to clear-cutting is long), might be the most effective long-term strategy for future plant protection. These approaches can potentially avoid or mitigate pest problems. In the end, the less we create a readily available food source and breeding material for herbivores, the fewer problems we will have with “pests”.

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Popular science summary

Imagine giving plants a built-in pest shield! In this thesis we explored how we can increase plants' own defences to fight back against harmful insects. One way is to use a plant hormone called methyl jasmonate, or MeJA, which acts like a distress signal in the plant when it is under attack by an insect to turn on its defences. If you spray a plant with MeJA, it can trigger it to turn on its natural defences and make it less attractive to pest insects. Because of this, the insect eats less on the plant. Another promising way to make insects less interested in a plant involves a plant production method called somatic embryogenesis, or SE. This method is used in the lab to produce many clones of a specific plant a lot faster than when you grow them from seeds, which is the most common way to produce plants. The cloned plants are called emblings and the plants grown from seeds are called seedlings. What is interesting is that these emblings seem to be less attractive to a pest insect called the pine weevil than the normal seedlings. Pine weevils are beetles that eat on the seedlings when they are planted on a clear-cut. The weevils are attracted to fresh clear-cuttings to reproduce and lay their eggs in stumps of newly felled conifer trees. The eggs become larvae and the larvae become new pine weevils. These new adult weevils also eat on the seedlings in the clear-cut, and this can go on for up to three years. They can eat so much that many of the plants die. In a place with a lot of pine weevils, up to 80% of the seedlings can die if they are not protected. This can be economically devastating for the forest owner. For some reason, the pine weevils do not like the emblings as much as they like seedlings, and we wanted to figure out why. In all experiments, we used Norway spruce emblings and seedlings that were 2-3 year old, and sprayed with MeJA or not. We then compared them to see if the results differed.

In our first experiment, we investigated what would happen if we combined SE and MeJA. By spraying emblings with MeJA we wanted to see if they would be even less attractive to the pine weevil compared to unsprayed emblings or MeJA-sprayed seedlings. The results were very exciting! Combining SE with MeJA appeared to be a powerful tool against weevils, and the MeJA-sprayed emblings got a lot less eaten by the weevils than the seedlings without MeJA (the control plants). Because of this, fewer emblings died and after three years in the field only 31% of the emblings with MeJA had died, which was the least of all plant types. Since this combination was so extraordinary, we decided to include a MeJA spray of half of the plants in all the coming experiments as well.

In our second experiment, we wanted to know if SE, with or without MeJA, could affect the behavior of the pine weevil. If the weevils, based on smell, looks or taste could tell the difference between plant types. And they could! First of all, it seemed like the weevils decide to go to a plant or not because they recognized the look of the plant and not because the plant smelled good or bad. Then, when they were on the plant, they took a test bite to see if it tasted good or not, before deciding to stay and eat or leave. Over the two days the experiment was going on, the weevils were less and less interested in eating on the plants that were sprayed with MeJA (both emblings and seedlings). Instead, they preferred the plants that were not sprayed, and they preferred the unsprayed seedlings the most.

In our third experiment, we wanted to look at the most important defence system in Norway spruce, the resin ducts. Resin ducts are canals filled with resin, a sticky mass that is more or less toxic to insects. Resin ducts can be found almost everywhere in the plant, but since the pine weevil kill the plants by eating on the stem bark, we only looked at resin ducts in the stem. To do that we cut horizontal stem slices and counted and measured the size of the ducts found in these slices. We found that emblings have more resin ducts in the bark than seedlings, which might be one of the reasons why the pine weevils like them less. When the plants are stressed, for example from an insect eating on them or when they are sprayed with MeJA, they create an extra wall of defence by forming something called traumatic resin ducts. Because the MeJA-sprayed emblings were so good at deterring pine weevils in our first experiment, we expected them to contain more or bigger traumatic ducts than the sprayed seedlings. Surprisingly, we found that the MeJA-sprayed emblings contained fewer and smaller traumatic ducts, making us wondering about the importance of traumatic ducts in the plants' defence against the pine weevil. Instead it seems like there are other reasons as to why the weevils do not like them.

In our fourth and last experiment, we wanted to see how well emblings could heal wounds on their stem. Since they are better at fighting off pine weevils, they might also be better at healing themselves. We discovered that SE and MeJA affects wound healing in two different ways. MeJA speeds up the start of the healing but slows down the further healing process. SE does the opposite; it slows down the healing start but speeds up the overall healing process. This meant that the MeJA-sprayed emblings were the best at healing their wounds since they both had the benefit of a faster healing start as well as faster overall healing! Furthermore, two years after the plants were wounded the MeJA-sprayed emblings had the most individuals with completely closed wounds.

In the end, we did not manage to find the exact reason why emblings get less eaten by pine weevils. Since pine weevils eat the spruce bark, they actually like some of the chemical compounds in resin that are toxic to many organisms. If these compounds are not there, they will like the bark less. Because of this, we believe that the reason they do not like emblings is because they contain less of the specific compounds that they like. They simply think they taste worse. But, to know for sure, we need to continue investigating these plants and to look more closely at the resin in emblings. Finally, it seems like combining SE and MeJA is the most promising way for boosting plant defences. What we discovered does not only make us understand plant stress responses and pest behaviour better, it might also help us improve the way we protect our plants and to do it in a more environmentally friendly way. This could be a game-changer for forestry, encouraging the use of more natural methods to keep our forests healthy! Our results also show how important it is to continue exploring how insects and pests interact with each other and how plants react to stress, so that we can find even better way to protect our plants.

Populärvetenskaplig sammanfattning

Tänk att ge växter ett inbyggt skydd mot skadedjur! I den här avhandlingen undersökte vi hur vi kan öka växternas eget försvar för att slå tillbaka mot skadliga insekter. Ett sätt är att använda ett växthormon som kallas metyljasmonat, eller MeJA, som fungerar som en nödsignal i växten när den attackeras av en insekt för att slå på sitt försvar. Om man sprejar en växt med MeJA kan det få den att aktivera sitt naturliga försvar och göra den mindre attraktiv för skadeinsekter. På grund av detta äter insekten mindre av växten. Ett annat lovande sätt att göra insekter mindre intresserade av en växt är en växtproduktionsmetod som kallas somatisk embryogenes, eller SE. Denna metod används i labb för att producera många kloner av en viss växt mycket snabbare än när man odlar dem från frön, vilket är det vanligaste sättet att producera växter. De klonade plantorna kallas SE-plantor och de plantor som odlas från frön kallas fröplantor. Det som är intressant är att dessa plantor verkar vara mindre attraktiva för en skadeinsekt som kallas snytbagge än de normala plantorna. Snytbaggen är en skalbagge som äter på plantorna när de planteras på ett kalhygge. De lockas till färska hyggen för att föröka sig och lägger ägg i stubbarna från nyavverkade barrträd. Äggen blir till larver och larverna blir till nya snytbaggar. Dessa nya vuxna snytbaggar äter också på plantorna på hygget, och detta kan pågå i upp till tre år. De kan äta så mycket att många av plantorna dör. På en plats med mycket snytbaggar kan upp till 80 procent av plantorna dö om de inte skyddas. Detta kan vara ekonomiskt förödande för skogsägaren. Av någon anledning gillar snytbaggarna inte SE-plantor lika mycket som de gillar fröplantor, och vi ville ta reda på varför. I alla experiment använde vi 2-3 år gamla SE-plantor och fröplantor av gran som sprejats med MeJA eller inte. Vi jämförde dem sedan för att se om resultaten skiljde sig åt.

I vårt första experiment undersökte vi vad som skulle hända om vi kombinerade SE och MeJA. Genom att spreja SE-plantor med MeJA ville vi se om de skulle bli ännu mindre attraktiva för snytbaggen jämfört med osprejade SE-plantor eller MeJA-sprejade fröplantor. Resultaten var mycket spännande! Kombinationen av SE och MeJA visade sig vara ett kraftfullt verktyg mot snytbaggar och MeJA-sprejade SE-plantor blev mycket mindre uppätna av snytbaggarna än fröplantorna utan MeJA (kontrollplantorna). På grund av detta dog färre SE-plantor och efter tre år i fält hade endast 31% av SE-plantor med MeJA dött, vilket var minst av alla planttyper. Eftersom denna kombination var så extraordinär beslutade vi oss för att inkludera MeJA-sprejning av hälften av plantorna i alla kommande experiment också. I vårt andra experiment ville vi veta om SE, med eller utan MeJA, kunde påverka snytbaggens beteende. Om snytbaggarna, baserat på lukt, utseende eller smak, kunde se skillnad på olika typer av plantor. Och det kunde de! Först och främst verkade det som att anledningen till att snytbaggarna bestämde sig för att gå till en planta eller inte berodde på att de kände igen utseendet av plantan och inte för att plantan luktade gott eller dåligt. När de sedan hade klättrat upp på plantan tog de sig en smakbit för att se om den smakade gott eller inte, innan de bestämde sig för att stanna och äta eller gå vidare. Under de två dagar som experimentet pågick var vivlarna mindre och mindre intresserade av att äta på de växter som sprejats med MeJA (både SE-plantor och fröplantor). Istället föredrog de plantorna som inte var sprejade, och de föredrog de osprejade plantorna mest.

I vårt tredje experiment ville vi titta på granens viktigaste försvarssystem, kådkanalerna. Kådkanalerna är fyllda med kåda, en klibbig massa som är mer eller mindre giftig för insekter. Kådkanaler finns nästan överallt i plantan, men eftersom snytbaggen dödar plantorna genom att äta på stambarken, tittade vi bara på kådkanaler i stammen. För att göra det skar vi horisontella stamskivor och räknade och mätte storleken på de kanaler som fanns i dessa skivor. Vi fann att SE-plantor har fler kådkanaler i barken än fröplantor, vilket kan vara en av anledningarna till att snytbaggarna gillar dem mindre. När plantorna stressas, till exempel av en insekt som äter på dem eller när de sprejas med MeJA, skapar de en extra försvarsmur genom att bilda något som kallas traumatiska kådkanaler. Eftersom de MeJA-besprutade plantorna var så bra på att avskräcka snytbaggar i vårt första experiment, förväntade vi oss att de skulle innehålla fler eller större traumatiska kådkanaler än de sprejade plantorna. Överraskande nog fann vi att de MeJA-sprejade plantorna innehöll färre och mindre traumatiska kanaler, vilket fick oss att undra över de traumatiska kanalernas betydelse i plantornas försvar mot snytbaggen. Istället verkar det som om det finns andra orsaker till varför snytbaggarna inte gillar dem.

I vårt fjärde och sista experiment ville vi se hur bra SE-plantor är på att läka sår på stammen. Eftersom de är bättre på att avvärja snytbaggar kanske de också är bättre på att läka sig själva. Vi upptäckte att SE och MeJA påverkar sårläkningen på två olika sätt. MeJA påskyndar starten av läkningen men saktar ner den fortsatta läkningsprocessen. SE gör tvärtom; det saktar ner läkningsstarten men påskyndar den övergripande läkningsprocessen. Detta innebar att de MeJA-sprejade plantorna var bäst på att läka sina sår eftersom de både hade fördelen av en snabbare läkningsstart och en snabbare övergripande läkning! Två år efter att plantorna skadats hade dessutom MeJA-sprejade SE-plantor flest individer med helt läkta sår.

I slutändan lyckades vi inte hitta den exakta orsaken till varför SE-plantor blir mindre ätna av snytbaggar. Eftersom snytbaggar äter granbark gillar de faktiskt vissa av de kemiska substanser i kådan som är giftiga för många organismer. Om dessa föreningar inte finns där kommer de att tycka mindre om barken. På grund av detta tror vi att anledningen till att de inte gillar SE-plantor är att de innehåller mindre av de specifika föreningar som de gillar. De tycker helt enkelt att de smakar sämre. Men för att veta säkert måste vi fortsätta att undersöka dessa plantor och titta närmare på kådan i SE-plantor. Slutligen verkar det som att kombinationen av SE och MeJA är det mest lovande sättet att öka plantornas försvar. Det vi har upptäckt hjälper oss inte bara att bättre förstå växternas stressreaktioner och skadedjurens beteende, det kan också hjälpa oss att förbättra vårt sätt att skydda våra växter och att göra det på ett mer miljövänligt sätt. Detta skulle kunna förändra spelreglerna för skogsbruket och uppmuntra till användning av mer naturliga metoder för att hålla våra skogar friska! Våra resultat visar också hur viktigt det är att fortsätta utforska hur insekter och skadedjur interagerar med varandra och hur växter reagerar på stress, så att vi kan hitta ännu bättre sätt att skydda våra växter.

Acknowledgements

After a few years travel on a both calm and stormy sea, I'm finally approaching the harbor. During these years, you realize you need many people to get through. Everyone you meet along the way have a role to play, big or small. I feel blessed to have met and to have so many amazing people in my life. I feel so much gratitude and have done my best to remember you all, but if I forgot some, I blame my end-of-the-PhD-mushy-brain.

First, I would like to give a huge thanks to my supervisors who made all of this possible! You have accompanied and helped me through one of the hardest and most rewarding parts of my life. **Christer**, I call you 'Finurliga Farbrorn' because you always have some fun inspiring thoughts to share. I have really enjoyed our time together and learnt a lot. **Adriana**, you are such an inspiration and I have learnt so much from you! You are very enthusiastic and meticulous, just like me ;) I think we make a good team and I have really enjoyed working with you on all the projects. **Maartje**, I am happy for the good times we had. Thanks for doing your best to help me when I had my one-year-of-pandemic-crisis. The only thing I regret is that we didn't talk more. **Helena**, I am really glad that you came into the group with your calm. You have such an awesome personality. Thanks for all the times you stopped by my office just for a quick chat. I really appreciated that.

I want to sincerely thank my family and friends for always being there for me. To my **parents** for everything they've done for me, contributing to making me who I am, and therefore indirectly affected my PhD work. I owe this to you. You taught me that when you do something you should do it properly. Tack **pappa** för att du gav mig ett enormt intresse för djur och natur, ett rejält tålamod, en stor portion knasig humor och självdistans (livet är roligare om man kan skämta om allt!), samt ett ypperligt minne och öga för detaljer. Tack **mamma** för att du gav mig en enorm envishet, en blandning av finsk sisu, norsk morsomhet och svensk innovationsförmåga, en förståelse för andra, en kärlek till ”hemma-djungel”, förmågan att se saker ur alla möjliga perspektiv, samt ett driv utöver det vanliga. Alla dessa egenskaper har varit mig mycket väl till hands under mina år som doktorand. I am grateful for growing up in northern Sweden, in Luleå. It contributed to my inner calm and taught me not to stress over small things – “there will be a day tomorrow as well”.

To my siblings for evoking the competitive spirit in me! Thanks **David**, for making me strong during my childhood, inspiring me to be (a bit, haha!) crazy, and for introducing me to metal and videogames, I’ve had a lot of use of it during my PhD ;) **Linda**, for being an inspiration and teacher, especially in plants and animals, for always listening, helping, giving the most reliable answers, for all relaxing summer activities. **Anna-Karin**, for teaching me about life, listening and giving the best advice, for everything you’ve helped me with and given me, especially during my undergraduate study-time in Sundsvall. **Anders**, for being someone I was (finally) stronger than as a kid, which drastically changed haha! Now for being the one I can ask when I need some extra muscles ;) For all the hospitality, movies, disc golf, berry picking, bonfire cooking, and fishing. **André**, for helping me live out my crazy-funny side as a kid (and being a Varan-TV buddy), for all the times you’ve joined our summer activities or been in charge of the bonfires at Långön. To the newer generation of “Berggrens”: **Adam**, **Miranda**, **Mira**, **Tyra**, **Wilma** and **Olivia** for all the joy and sharp questions you bring to my life! Furthermore, to **Anneli**, for seeing the potential in this shy teenage girl, for always being there, supporting me, all the advice, for caring so much about me, everything we’ve experienced together. You are an amazingly kind person. Jag ser fram emot att flumma-järnet på ålderdomshemmet när våra kroppar blir gamla och skröpliga (för våra hjärnor kommer aldrig att bli det!). **Sara**, I am very happy you decided to become a biologist in the same year and town as I did! For all the support, always giving good advice, for being there through my struggles and sharing all the happy moments. For inviting me to your family Easter trips to Åre (how I loved the egg hunts!). **Charles** & **Chatira**, you brought so much happiness into my life and kept me “sane” and company during these two hideous pandemic years when we had to work from home. I cherish you so much my adorable little furballs <3 To all my relatives who in different ways support me. **Linda Pettersson**, for always being supportive and encouraging, it means a lot even though we rarely meet. To my new friends who came into my life when I needed them the most: Thanks **Jimmy**, for opening your home to me, all the talks, teaching, horse driving, trips to race courses. It gave me so much calm and happiness. **Dan**, for opening what was also your home, all the talks, shared meals, and nice times. **Kinky**, **Nighty**, **Satine**, **Sugar Baby**, **OAO**, **QML**, and **Hot Mama**, for all the happiness, adventures, mind rest, excitement, and strong arms!! ;)

To my **biology teacher** at Åsö Vuxengymnasium, **Nils Ekelund**, **Svante**, and **Bege**, who all contributed to me ending up here. To all my **co-authors**: **Bob**, **Sam**, **Erika W**, **AKBK**, **Mikko**, and **Ulrika** for your contributions. To my **assistants** who have helped me to accomplished this: **Sebastian**, **Alessandro**, **Emma**, **Andrew**, **Erik**, **Matilda** and **the dog**, **Paul**, **Claes** and **Maria**, and **Bernabé**. Other people I met during my PhD: **Malin Elfstrand**, for support and teaching me about SE plants. **Paal Krokene**, for nice times at conferences and teaching me about induced defences. **Luis Sampedro** and **Raul**, for teaching me many useful things about plants and making my summer of 2021! Thanks!

I want to thank **my fellow forest entomologists** who make our unit so great! **Michelle**, for all the amazing help with statistics and talks about PhD life. **Yayuan**, for being so friendly and helpful, making me feel less socially intimidated when I was new, for all help with my experiments :) **Dragos**, for being a great office companion who listened to all my frustration whenever it decided to come out hehe, for always doing your best to help. **Amelia**, for all the talks, for being a great assistant and for so lovingly taking care of my furballs :) **Fredrika**, for being such a kind person and the talks we've had (especially in Lisbon). **Mats**, for organizing 'Ecologists on ice' and the amazing help identifying my insect samples or whatever beetle I find, your knowledge is truly impressive! **Simon**, for all nice talks and laughs, for being a fun and supportive company at the Lisbon conference (especially when I was almost dying before my talk). **Niklas**, for many nice and interesting talks, sharp questions and sarcastic jokes. **Helena H**, for everything you've helped me with during these years, for being such a nice, caring person. **Xiaoning**, for teaching me about China and plant-insect interactions. **Göran**, for being such a cool person and sharing your vast pine weevil knowledge. Many people praise you, I understand why. **Martin**, for interesting talks about the spruce bark beetle. Nice people who came recently or briefly to the unit: **Stephanie**, **Danja**, **Oliver**, **Matilda**, **Shermin**, **Naomi**, **Brook**...

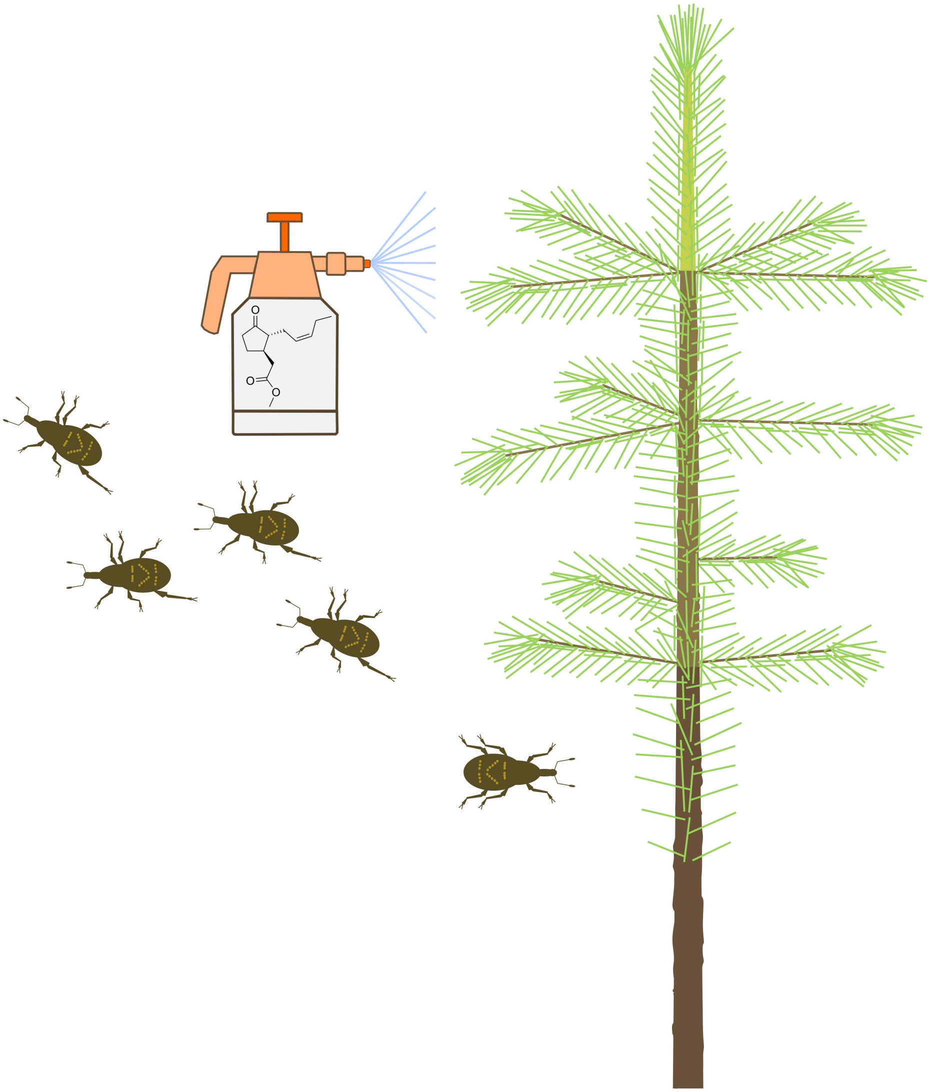
To all people in the **Ecology Centre** who have helped me, contributed to fun department symposiums, nice lunches/chats or just said hi. Thank you!

Velemir, for many great, fun, supportive and encouraging talks, for making me believe in myself and that I will succeed. **Tomas Pärt**, for all support and the many, funny lunch talks, especially about ice hockey and how amazing Luleå hockey is! ;) **Maria Viketoft**, for many nice chats. **Uffe**, for help with office equipment and nice pictures in the ekol-email. **Sirirat**, for everything you did for us. **Helena B**, for help with many different things. **Maria Kedmark**, for taking great care of my plants. **Lena**, for amazing administrative work. **Per Nyman**, for IT support and being a cross-country ski lover. **Johannes Forkman**, for all statistical help and nice chats. **Anna Lundmark**, for all media stuff, events and nice chats. **Åsa H**, for all admin help. **Robert Glinwood**, for quality checking my thesis work and progress.

To all previous and present PhD students and postdocs. We've had many great, fun, soul-soothing moments together. Thanks! **Julian**, for welcoming me, making me feel like I belonged, for all the many talks about forests etc. **Ineta**, for being such an awesome dude ;) all nice moments, all talks, everything you shared or helped me with, cat-stuff, for being a bit similar to me so I don't feel too much like the odd one out ;) I'll never forget the lake ice, so smooooth! **Guille**, for being one of the kindest persons ever, all nice moments, always cheering me up, for giving me hope that normal Spanish guys exist ;) **Juliana**, for all nice moments, for being a very fun person! **Eirini**, for always being so caring and kind, for recent help with inkscape! **Chloë R**, for being a nice cool person. **Adam**, for fun chats and the very nice trip to Leksand (especially when Luleå whipped butt haha!). The "bee-crew" **Piero**, **Anna**, **Herman**, and **Melissa**: for many nice pre-pandemic lunches and ping-pong! **Kaisa**, for nice chats and support, for bringing some pure Finnish sisu into this place! **Elsa**, for nice talks, hearing about your thesis struggle and successfully coming out on the other side gave me hope that I also could. **Tarquin**, **Merlin**, **Hannes**, **Carl**, **Holger**, five cool people that I had many nice talks with, keep up the good spirit! **Pablo**, for many fun talks, jokes, enjoyable moments! For supporting Cadiz ;) For understanding my fascination with beetles. **Tord**, for having the same amazing (slightly crazy) humor as me, for all the talks, I am happy to have laughed so much! For sharing the Kappa pappas packsäck writing and red art with me ;) **Svenja**, for nice talks and being a cool person. I really enjoyed our trip to Arjeplog, especially the helicopter ride ;) **Alessio**, for being a great assistant, a nice person, for help with statictics. **Rafaelle**, you are such a sweet person! For all the caring and peptalks. Never change! **Janina**, for being such a cool person and easy to talk to, all advice, support, cat-loving. **Valeria**, for being a nice mix: sweet, cool and kind ;) **Cassandra**, for being kind, for nice talks and stroopwafels! **Joel**, for being fun and easy to talk to, for our Norrland-Skåne battles ;) **Nick**, for being kind, caring, always yourself. Stay that way. **Sokha**, for all chats and teaching me about Cambodia. **Dimitrije**, for being a nice, funny guy, always up for a chat or giving advice. **Jonny**, for being a nice guy, for many great+fun times. **Fabian**, for being a human google search! ;) I don't know how you do it but I'm impressed. **Darwin**, for being a kind, funny person, for stepping by my office to chat, I really appreciated that. **Giulia**, for being a sweet person, helping me with R, introducing me to the botanical society :) **Electra**, for being sweet and caring. Never change. **Lorena**, for being easy to talk to, teaching me about Peru and plant responses. **Fede**, for being sweet + the fun Eurovision party at you place! **Sara Capitán**, a cool name on a cool person ;) for organizing the book club. **Mahmoud**, for being nice, for trying to help me with R. **Alwin**, for the crazy goofy talks ;) **Deanne**, for being a nice sleepover buddy in Norreda. To the other nice/cool people who's been around: **Anika**, **Laura**, **Kate**, **Andreas**, **Faranak**, **Sasha**, **Yuval**, **Arielle**, **Chloë M**, **Florence**, **Minh-Xuân**, **Sandra**, **Grace**...

The **BMC crew**: **Jens**, **Matilda** & **Valter**, for being "normal" people ;) For all great moments and LHF games watched together. **Mario** & **Sara**, for all great moments. **David**, for all times together, baking, dinners, visits, trips, fika, walks, talks, listening, for appreciating Sweden. **Weifeng**, for being kind and funny, all your help, taking care of C&C. The **Å (Ångström) crew**: **Christos**, **Evgenii**, **Anna**, **Serena**, **Johan**, **Teresa**, for many nice moments, especially our trips to Sälen and the High Coast. The **Contemporary** group at **Ekeby dansstudio**, especially **Julia** & **Natalie**, and **Louisa** for being the best teacher. To my extended family in Spain, especially **Maria** y **Juan**, sois como unos segundos padres para mí. Gracias por todo lo que habéis dado y hecho por mí, todos los viajes y visitas, por el apoyo y ánimo. To **Alberto**, **Paco**, **Selene**, **Ana**, **David** for all support and nice moments. To all **pine weevils** and **spruce plants** that were part of my project, for the opportunity to learn from you.

Finally, to **Carlos – El Toro de Cadi**. You were my inspiration to do a PhD. Thanks for everything you've helped me with, for being such a crazy person, you know I love it ;) For never making my life boring, making so many things happen, all the adventures, all goofiness, filling my life with so many people, for sharing my life journey, for being there. Te quiero <3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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 06 June 2023
DOI 10.3389/fpls.2023.1165156

OPEN ACCESS

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RECEIVED 13 February 2023
ACCEPTED 19 May 2023
PUBLISHED 06 June 2023

CITATION
Berggren K, Nordkvist M, Björkman C, Bylund H, Klapwijk MJ and Puentes A (2023) Synergistic effects of methyl jasmonate treatment and propagation method on Norway spruce resistance against a bark-feeding insect.
Front. Plant Sci. 14:1165156.
doi: 10.3389/fpls.2023.1165156

Synergistic effects of methyl jasmonate treatment and propagation method on Norway spruce resistance against a bark-feeding insect

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Utilizing plants with enhanced resistance traits is gaining interest in plant protection. Two strategies are especially promising for increasing resistance against a forest insect pest, the pine weevil (*Hylobius abietis*): exogenous application of the plant defense hormone methyl jasmonate (MeJA), and production of plants through the clonal propagation method somatic embryogenesis (SE). Here, we quantified and compared the separate and combined effects of SE and MeJA on Norway spruce resistance to pine weevil damage. Plants produced via SE (emblings) and nursery seedlings (containerized and bare-root), were treated (or not) with MeJA and exposed to pine weevils in the field (followed for 3 years) and in the lab (with a non-choice experiment). Firstly, we found that SE and MeJA independently decreased pine weevil damage to Norway spruce plants in the field by 32-33% and 53-59%, respectively, compared to untreated containerized and bare-root seedlings. Secondly, SE and MeJA together reduced damage to an even greater extent, with treated emblings receiving 86-87% less damage when compared to either untreated containerized or bare-root seedlings in the field, and by 48% in the lab. Moreover, MeJA-treated emblings experienced 98% lower mortality than untreated containerized seedlings, and this high level of survival was similar to that experienced by treated bare-root seedlings. These positive effects on survival remained for MeJA-treated emblings across the 3-year experimental period. We conclude that SE and MeJA have the potential to work synergistically to improve plants' ability to resist damage, and can thus confer a strong plant protection advantage. The mechanisms underlying these responses merit further examination.

KEYWORDS

emblings, forestry, *Hylobius abietis*, *Picea abies*, plant protection, regeneration pest, somatic embryogenesis (SE)

1 Introduction

Plants with enhanced resistance traits are in demand within plant protection against pests, given the need to replace adverse methods, such as chemical pesticides, with sustainable long-term strategies (Stenberg et al., 2015; Mitchell et al., 2016; Dreischhoff et al., 2020; Lalík et al., 2020; Hernández-Suárez and Beitia, 2021). Resistance is a vital part of plant defense, as it describes a plant's ability to avoid an attack or reduce the amount of damage received (Núñez-Farfán et al., 2007). It was recently discovered that a method used for plant propagation can make plants intrinsically more resistant to insect damage. In a study on 4-year-old Norway spruce (*Picea abies*), plants produced through somatic embryogenesis (SE) were more resistant to bark-feeding damage by the pine weevil (*Hylobius abietis*) than zygotic seedlings from the same Norway spruce families (Puentes et al., 2018). The authors found that plants propagated via SE were less frequently attacked, and received about 30% less damage by pine weevils than regular seedlings (Puentes et al., 2018). SE is a vegetative propagation method in which somatic cells or tissue is used to produce plants *in vitro* with the use of plant hormones (Mo et al., 1995; Klimaszewska et al., 2016; Egertsdotter, 2019). SE has been used for decades as a propagation method for many economically important crops (e.g., wine grapes, cacao trees, bananas) (Duarte-Aké and De-la-Peña, 2016; Etienne et al., 2016; López et al., 2022) and tree species (e.g., spruce, larch) (Lelu-Walter et al., 2013). Yet, its potential to produce conifer (and other) plants that are intrinsically more resistant to pests, has not been explored.

Given the different factors involved in producing SE plants, it is likely that the process itself affects plant resistance. For instance, initiation of the cell multiplication process and subsequent maturation of embryos requires high amounts of plant growth regulators (PGRs) such as ethylene and abscisic acid (von Aderkas et al., 2015; Méndez-Hernández et al., 2019). These plant hormones are also involved in responses to biotic stress (Müller, 2021). In some cases, somatic embryos may even be exposed to extreme pH and heat shock and, thus, often experience high levels of stress during development (Winkelmann, 2016; Méndez-Hernández et al., 2019). Such a stress stimulus early in life can prime or prepare plants for subsequent attacks, and result in faster or stronger activation of defenses (Conrath et al., 2006; Wilkinson et al., 2019). Moreover, studies have reported that plants produced via SE exhibit greater levels of secondary metabolites (which can be important for plant defense) when compared to plants produced through seeds or growing in the wild (Lamhamedi et al., 2000; Fulzele and Satdive, 2003; Domínguez et al., 2010). Producing plants via SE may, therefore, provide new opportunities to take advantage of plants' responses to stress and reduce pest damage.

Development of strategies to enhance plant resistance against pests have focused to a great extent on the use of chemical elicitors (e.g., Walters et al., 2014; Bruce et al., 2017; Siah et al., 2018; Yassin et al., 2021). One such elicitor is the plant hormone methyl jasmonate (MeJA). MeJA is an important signaling molecule mediating stress responses in plants, and it can activate resistance mechanisms (Yu et al., 2019). Exogenous application of MeJA prior to pest exposure has been shown to reduce feeding by insect herbivores, and can result in less plant damage for example in soybean, rice, strawberry and Andean lupin (Chen et al., 2018; Senthil-Nathan, 2019; Erazo-Garcia et al., 2021; Mouden et al., 2021). Moreover, it has been shown to enhance conifer resistance against insect pests such as the pine weevil (*H. abietis*) (e.g. Puentes et al., 2021), spruce bark beetle (*Ips typographus*) (Mageroy et al., 2020a) and Japanese pine sawyer (*Monochamus alternatus*) (Chen R. et al., 2020). Treatment of conifers with MeJA has been shown to result in e.g., traumatic resin duct production and increases in terpenes and phenolic-based compounds (e.g., Krokene et al., 2008; López-Villamor et al., 2021), which are important mechanisms of tree defense. Similarly to propagation through SE, treatment with MeJA also has potential to improve forest protection against detrimental pests.

Interest in using SE as a propagation method for conifer trees and induced resistance as a forest protection method is likely to increase. In Nordic European countries, production of conifers via SE is expanding (e.g., Lelu-Walter et al., 2013; Egertsdotter et al., 2019; Rosvall et al., 2019a; Rosvall et al., 2019b), as well as the potential to use MeJA in nursery seedling production (e.g., Chen Y. et al., 2020; Nybakken et al., 2021). Given the plant protection benefits that have been documented for SE and MeJA independently, it is timely to examine the combined effects of these two factors on plant resistance. If SE plants are primed or induced during production, a second stress stimulus from MeJA could provide an even faster response and/or greater levels of resistance relative to plants that have not undergone somatic embryogenesis. Alternatively, treatment with MeJA may generate little to no response in SE relative to non-SE plants, as SE plants could already be fully primed or induced. By testing these hypotheses, it would be possible to determine if SE is compatible with other plant resistance inducing strategies such as MeJA treatment.

In this study, we experimentally compared the effects of MeJA treatment on resistance of young Norway spruce plants produced via SE or from seeds. We examined resistance to the pine weevil (*H. abietis*) since exogenous application of MeJA to Norway spruce seedlings, and other conifers, has been shown to effectively reduce damage inflicted by this insect pest (e.g., Heijari et al., 2005; Zas et al., 2014; Fedderwitz et al., 2016; Lundborg et al., 2016; Puentes et al., 2021). Furthermore, in the study by Puentes et al. (2018), which documented the plant protection benefits of SE, damage inflicted by pine weevils was used as a measure of resistance. Therefore, the pine weevil-Norway spruce system provides a suitable starting point to examine the effects of SE and MeJA together. In this study, we addressed the following questions:

1. Do SE and MeJA together increase Norway spruce resistance to pine weevil damage to a greater extent than when these two methods are used separately (i.e., are effects on resistance synergistic)?
2. What are the separate and combined effects of SE and MeJA treatment on Norway spruce survival across years in the field?

We established a field and lab experiment in which MeJA-treated and non-treated Norway spruce plants (produced via SE and from seed in nurseries) were exposed to pine weevils. Plants were followed in the lab under one growing season, and in the field for three growing seasons. We quantified the proportion of plants attacked and stem area debarked by weevils, as well as plant mortality. The field experiment allows evaluation of resistance under actual forest regeneration conditions. The lab study allows evaluation of effects under controlled and non-choice conditions, which provides insight into whether the insect is avoiding the plant or it is simply not palatable.

2 Materials and methods

2.1 Study system

The pine weevil (*Hylobius abietis* L.) is a major forest regeneration pest in Europe (Nilsson et al., 2010). They lay their eggs nearby or inside the root bark of newly-dead or dying conifers, and are thus, attracted to the odors emitted by the stumps of freshly-felled trees (Nordlander, 1991; Nordlander et al., 1997). Once forest regeneration occurs through planting, adult weevils can feed extensively on the stem bark of several conifer seedlings (Wallertz et al., 2014), often removing an entire ring of bark phloem from the stem circumference (i.e., they girdle plants). Girdling often results in seedling mortality and, consequently, large economic losses (Långström and Day, 2004; Lalík et al., 2020). Feeding takes place during the plants' growing season (from spring till autumn in Nordic countries). Pine weevils are present in clear-cuts for up to three years after the forest is harvested, as new generations hatch after 1-2 years depending on geographical location (Bejer-Petersen et al., 1962; Nordenhem, 1989; Inward et al., 2012; Wainhouse et al., 2014), thus, feeding can occur on the same seedlings for more than one season. The parental generation stays at the clear-cut for the remaining part of their lives, but the new generation eventually leaves in search of oviposition sites (Nordenhem, 1989). Replanting due to loss of seedlings may be needed in sites with high pine weevil pressure, hence, causing increased regeneration costs (Leather et al., 1999; Mattsson, 2016).

2.2 Plant material

Plant material consisted of Norway spruce (*Picea abies* (L.) H. Karst) obtained from the Forestry Research Institute of Sweden (Skogforsk) and from commercial plant nurseries. Plants from Skogforsk were produced through SE (emblings hereafter), from trees belonging to the clonal archive used in breeding trials of Norway spruce. Plants were propagated via SE following the same methods as described in Puentes et al. (2018). A total of 652 emblings (~1 year old) originating from 19 full-sib families were produced, with varying number of clones per family. Zygotic seedlings (seedlings hereafter) were obtained from two commercial nurseries (Stora Enso Plantor AB in Nässja, and Södra Skogsplantor in Falkenberg, Sweden), and included seedlings of two types: smaller containerized seedlings (grown with roots in a soil plug) (n = 528, 1.5 years old) and larger bare-root seedlings (grown in an outdoor nursery bed with the opportunity to develop a larger root system) (n = 124, 3 years old). In Nordic countries, these are the two seedling types that are commercially available to forest owners for re-planting after harvest. SE plants were delivered frozen, as they were in winter storage, from Skogforsk to the University of Agricultural Sciences, Uppsala, Sweden, in May 2019. Plants were thawed by slowly increasing the temperature and then kept in a greenhouse (16h/8h light/dark and ~18/15°C day/night) until the start of the experiment. Containerized and bare-root seedlings, also previously frozen during winter storage, had already been thawed when they were received from the commercial nurseries a few days later, and placed in the same greenhouse as the emblings. Plants for the laboratory experiment were planted in 2L plastic pots, while plants for the field experiment were kept in plug trays (ø 6.5 cm per plug). After 3.5 weeks in the greenhouse, those plants intended for the field experiment were planted in the field and the remaining plants were kept in the greenhouse until laboratory trials started.

2.3 Experimental set-up

2.3.1 Methyl jasmonate treatment

For each plant type, half of the total number of plants were treated with 10 mM methyl jasmonate (MeJA). This concentration of MeJA has been used in our previous studies (Chen et al., 2021), and shown to effectively increase resistance against the pine weevil in conifer seedlings of similar sizes (height/diameter) as those in the present study. First, MeJA (95%, Sigma-Aldrich, ref. 392707) was dissolved in ethanol; deionized water was then added to this mixture to achieve a final ethanol concentration of 2.5% (v:v). This solution was shaken vigorously until a uniform milky emulsion was obtained, and then transferred to a plastic hand-sprayer bottle (Free-Syringe PC 1.5 liter, Jape Products AB, Hässleholm, Sweden). The bottle was pumped until it reached its inner air pressure limit (2.5 bar), and shaken again before each spraying occasion. Plants were sprayed outdoors, with plants placed beside each other in two rows. The spraying nozzle was at a distance of about 30 cm from the plants, and the bottle was moved manually along each row of plants. Each plant was sprayed for about one second, with all aboveground parts being covered with the solution. Non-treated plants were similarly sprayed but with deionized water. MeJA-treated plants were kept in a separate greenhouse to avoid contamination of non-treated plants. MeJA treatment was applied on the plants designated for the field study eight or nine days prior to being planted in the field, and ten or eleven days prior to the start of each round of the lab experiment.

2.3.2 Field experiment

The experimental site was located on a non-scarified clear-cut (7 ha, harvested autumn 2018, dominated by Scots pine (*Pinus sylvestris*)) near Tierp in central Sweden (60°21'N, 17°26'E) (see Figure S4 for details). A total of 328 emblings, 228 containerized and 100 bare-root seedlings were planted in the field on 18-19 June 2019. The number of plants from each type were represented equally in both MeJA treatments (0 mM and 10 mM MeJA), with each treatment including 164 SE, 114 containerized and 50 bare-root seedlings. Stem height and basal diameter of each plant was measured the day before transferring them to the field. Average height ± standard error (and ranges) were for emblings: 31.1 ± 0.4 cm (17.0 to 48.0 cm), containerized seedlings: 29.6 ± 0.3 cm (19.5 to 38.5 cm), and bare-root seedlings: 57.0 ± 0.7 cm (40.0 to 71.0 cm). Plants were planted in nine blocks (size 7 × 8 m) with 72 plants in each block (except one larger block with 80 plants, 7 × 9 m) spread over an area of the clear-cut spanning about 90 × 80 m. Each block consisted of nine columns, and each column contained eight positions; except the larger block that consisted of ten columns and eight positions. In each block, plants were placed with a one meter distance, and with a rolling positioning of the four MeJA-treatment and plant type combinations in columns (see Figure S1). Plants were assigned positions in blocks based on the following four treatments. 1: MeJA-treated embling; 2: non-treated embling; 3: MeJA-treated containerized or bare-root seedling, 4: non-treated containerized or bare-root seedling, with every treatment represented twice in each column (see Figure S1 for details). The design ensured that no plants belonging to the same treatment occurred beside each other in either a horizontal or vertical position. We also included a reference block (72 plants) with only non-MeJA-treated containerized seedlings, which allowed us to get an estimate of pine weevil pressure in the clear-cut without treatment interference. This reference block was located in close proximity to the experimental blocks.

The field experiment was a three-year study spanning from June 2019 to September 2021 (Figure S3 for a timeline). Plants were exposed to the natural light, temperature and relative humidity and precipitation conditions of the clear-cut throughout the whole experiment. Three variables related to plant resistance were recorded: if the plant had been attacked or not by pine weevils (0 = no, 1 = yes), pine weevil stem feeding damage (area debarked), and mortality (0 = alive, 1 = dead). Inventories took place late in the growing season each year: September 2, 2019 (11 weeks after planting; all three variables), September 15, 2020 (attack and mortality), and September 29, 2021 (attack and mortality) (see Figure S3 for an overview of the timeline and variables recorded). To estimate total area debarked per plant we measured the following variables: (1) debarked height - the height from the ground (right above the root collar) to the upper side of the uppermost pine weevil feeding scar on the stem, and (2) percentage debarked - the proportion of stem area damaged (%) in relation to the total surface area up to the debarked height described in (1). Using these measurements and the equation for the circumference of a circle (which estimates the perimeter of the plant stem), we calculated the debarked area (cm^2) for each plant as: Total area debarked = Circumference of the stem ($\pi \cdot d$) × (debarked height × percentage debarked). If the percentage debarked was found to be less than 10%, stem area debarked was calculated by measuring the area of each scar using graded millimeter templates and adding up these scars (cm^2) (see Figures S6, S7 for pictures of pine weevil feeding damage).

2.3.3 Laboratory experiment

A total of 324 emblings, 300 containerized and 24 bare-root seedlings were used in the laboratory experiment. The number of plants from each type were equally represented in both MeJA treatments (0 mM and 10mM MeJA), with each treatment including 162 emblings, 150 containerized and 12 bare-root seedlings. The experiment was replicated nine consecutive times (referred to as rounds), with a new set of 72 plants each round (i.e., plants were only used once; see treatment combinations per round below) during July-August 2019. Each round was three or four days long. Stem height and basal diameter of the individual plant was measured in the morning, or one day before the start of each round (see Figure S3 for an overview of the timeline and variables recorded). Average plant height ± standard error (and ranges) were for emblings: 40.8 ± 0.4 cm (17.0 to 60.0 cm), containerized seedlings: 33.7 ± 0.3 cm (18.0 to 46.0 cm), and bare-root seedlings: 58.5 ± 1.8 cm (36.5 to 73.0 cm).

In this non-choice test, plants were exposed to pine weevils that were collected during spring migration on May 21, 2019, at a sawmill (Balungstrands Sågverk AB) in Enviken, Sweden. Weevils were kept in a dark room at 10°C with access to water as well as stem pieces and branches of young Scots pine (*P. sylvestris*) to feed on. Seven days prior to each round, pine weevils were placed in a plastic box at room temperature and natural light (~25°C, light/dark: 16h/8h), for acclimatization, with Scots pine branches and water. Three to four days before the start of a round, food was removed in order to starve the pine weevils. During a round, each plant was obligatorily exposed to one starved pine weevil for three or four days, depending on how fast they started feeding. Note that plants in the same round were exposed to the same number of days to pine weevils, but the number of exposure days differed between rounds. A plastic transparent cylinder with mesh net on the top opening (h: 64 cm, d: 14 cm), enclosed each potted plant along with a pine weevil that had access to water (see Figure S5 for details). The experiment was conducted in a lab (Swedish University of Agricultural Sciences, Uppsala, Sweden) under room temperature conditions (~25 °C) with natural light coming in from the large windows of the lab (no artificial lamps were used). Plants were placed closely together in rows on tables, and the same within-block rolling treatment order as in the field was used (treatment 1: MeJA-treated embling; 2: non-treated embling; 3: MeJA-treated containerized or bare-root seedling, 4: non-treated containerized or bare-root seedling). Every round had a different order of treatments in columns/positions from the previous one. After each round ended, cylinders and pine weevils were removed, and the stem of each plant was cut right below where the lowest feeding scar was found on the stem (most often close to the root collar). Stems were kept in a refrigerator (5 °C) until damage was scored (maximum within 7 days), and then discarded.

We recorded whether the plant had been attacked or not by the pine weevil, as well as pine weevil feeding damage to the stem (area debarked) (see Figures S6, S7 for pictures of pine weevil feeding damage). The debarked area was calculated for each plant by measuring each feeding scar using graded millimeter templates, and adding all areas together (cm^2). Each plant in the laboratory experiment was only scored once.

2.4 Statistical analyses

All analyses were conducted in R version 4.2.2. (R Core Team 2022). Linear mixed models were fitted with the *lmer*-function and generalized linear mixed models with the *glmer*-function from the *lme4* package (Bates et al., 2015). Models were validated by inspecting residuals *vs.* predicted values, and using Levene's test for examining equal variances across treatments (*LeveneTest*-function; *car* package (Fox & Weisberg, 2019)) and by simulating and plotting scaled residuals using the *DHARMa* package (Hartig, 2021). Significance of main effects and interactions was tested with analysis of deviance using the *Anova* command from the *car* package (Fox and Weisberg, 2019). Estimated means for each treatment level and combinations were obtained through *emmeans* in the *emmeans* package (Lenth et al., 2020). Multiple comparisons were conducted between treatment means using the Tukey adjustment in the *emmeans* package.

2.4.1 Field experiment

To examine the effects of plant type and MeJA treatment on the proportion of plants attacked (0 = no, 1 = yes) by pine weevils and plant mortality (0 = alive, 1 = dead) by the end of the first year (September 2019), we fitted generalized linear mixed models with a binomial distribution. Similarly, to examine the effect of plant type and MeJA on area debarked we fitted a linear mixed model. Plants that had received zero damage were excluded from the model, and area debarked by pine weevils (cm^2) was log-transformed to meet model assumptions. For all these models, plant type (containerized seedling, bare-root seedling and embling), MeJA treatment (0 mM and 10 mM) and their interaction, were used as fixed effects. Initial plant height (height at the start of the experiment) was also included as a continuous covariate, and block was included as a random effect. The effects of treatment on the 19-full sib SE-families of Norway spruce used in the experiments were not examined separately, as these families responded similarly to MeJA treatment in the field (Figure S2).

Effects of plant type and MeJA on non-cumulative mortality in September 2020 (referred to as year 2), and non-cumulative and cumulative mortality in September 2021 (referred to as year 3) were analysed using generalized linear mixed models with a binomial distribution. These models included the same fixed and random effects as described above for attack, area debarked, and mortality. In analyses of non-cumulative mortality, plants that had died the previous year were excluded. Thus, these models examined mortality that occurred only that year (2020 or 2021). Moreover, since all containerized seedlings had practically died by the second year (97% mortality, 5 plants alive), these were excluded in the analyses of non-cumulative mortality for years 2020 and 2021 (i.e., plant type included only emblings and bare-root seedlings). On the other hand, analyses of cumulative mortality in 2021 represented the total plant mortality for the duration of the whole experiment (across 3 years) for all treatment combinations (i.e., no plant types were excluded).

2.4.2 Lab experiment

The effects of plant type and MeJA on proportion attacked were analysed using a generalized linear mixed model with a binomial distribution. Area debarked by pine weevils (cm^2) was log-transformed and effects were analysed with a linear mixed model. For both models, plant type (containerized seedling, bare-root seedling and embling), MeJA treatment (0 mM and 10 mM) and their interaction, were used as fixed effects. Plant height (height at the start of the round) was also included as a continuous covariate, and round (replication in time) was included as a random effect.

2.4.3 Calculations of additive, synergistic or antagonistic effects of SE and MeJA

To determine the magnitude and direction of the effect on plant resistance when MeJA and SE occur together, we calculated if the effect was additive, synergistic or antagonistic. An interaction is additive when their combined effect is the sum of each independent effect, and it is synergistic or antagonistic when their combined effect is greater or smaller (respectively) than the sum of each independent effect. Observed effects of seedlings and emblings exposed to pine weevils (i.e., actual values of area debarked per plant), were compared to expected effects obtained from the statistical model for area debarked, following the method used in Bansal et al., 2013 (see Supplementary Material, section 1.3 Supplementary Text). Calculations were only made for pine weevil damage recorded the first year in the field, and comparisons of observed and expected effects were conducted separately using the two types of control treatment plants (untreated containerized and bare-root seedlings).

3 Results

3.1 Field experiment (year 1)

3.1.1 Reference block

Overall, pine weevil pressure was high at the clear-cut where the experiment was located, as indicated by the levels of damage in the reference block. The reference block contained only non-MeJA-treated containerized seedlings of Norway spruce, and was situated close to the experimental blocks. The first year, late in the season (September 2019; 11 weeks after planting), 96% of the plants in the reference block had been attacked, resulting in 93% mortality. Stem area debarked ranged from 1.2 to 20.7 cm^2 (average wound size per plant ± standard error: 7.8 ± 0.9 cm^2) for plants in this block. By the second year, only two plants were alive in the reference block, and by the third year, all were dead.

3.1.2 Proportion attacked

In the first year, attack was in general high with 93% of all experimental Norway spruce plants being attacked by pine weevils. We found that the proportion of plants attacked differed significantly between treatment combinations (significant plant type × MeJA interaction, Table 1). Among non-treated plants, emblings were similarly attacked by pine weevils when compared to containerized seedlings (Table S1; Figure 1A). Yet, they were attacked to a greater extent (25% more) than bare-root seedlings (Table S1; Figure 1A). Even though the same pattern was observed

TABLE 1 Summary of results from models examining the effects of plant type and MeJA treatment on pine weevil attack and area debarked, and plant mortality, in the field experiment the first year (September 2019).

Field year 1	Attack			Area debarked			Mortality		
	χ^2	df	p-value	χ^2	df	p-value	χ^2	df	p-value
Plant type	8.71	2	**0.013**	11.55	2	**0.003**	37.38	2	**< 0.00001**
MeJA treatment	0.16	1	0.692	33.98	1	**< 0.00001**	32.97	1	**< 0.00001**
Plant type × MeJA	6.87	2	**0.032**	28.13	2	**< 0.0001**	10.43	2	**0.005**
Plant height	4.82	1	**0.028**	5.95	1	**0.015**	0.04	1	0.850

χ^2: Chi-square value; df: degrees of freedom; p-value; plant type (containerized seedlings, bare-root seedlings and emblings of Norway spruce); MeJA (methyl jasmonate) treatment (0 mM and 10 mM); attack (0 = no, 1 = yes); area debarked (cm^2); plant mortality (0 = alive, 1 = dead). Plant height (at the time of planting) was included as a covariate, and blocks in the field were included as a random effect (not shown). Significant effects ($p < 0.05$) are in bold.

for MeJA-treated plants (Figure 1A), differences in attack between treated plant types were not statistically significant (Table S1). Nonetheless, treatment with MeJA significantly reduced attack for emblings (6% reduction) relative to non-treated emblings (Table S1; Figure 1A).

3.1.3 Area debarked

We found that area debarked by pine weevils was affected by plant type and MeJA treatment, both separately and in combination (Table 1). Among untreated plants, emblings received the lowest levels of damage, 32% and 33% less than containerized and bare-root seedlings respectively (Figure 1B). However, these differences were statistically significant only when comparing emblings to containerized seedlings (Table S1). Treatment with MeJA reduced damage for all plant types, but damage reduction was much greater for emblings than for any other plant type (Figure 1B). Emblings, containerized and bare-root seedlings experienced an 80%, 53% and 59% reduction in damage, respectively, when each was compared to its own untreated plant group. Moreover, we found that SE and MeJA together resulted in an 86% and 87% reduction in damage, when MeJA-treated emblings were compared to non-treated containerized and bare-root seedlings, respectively (Figure 1B). Pairwise comparisons indicated that mean area debarked for MeJA-treated emblings was significantly lower than all other treatment means (Table S1). In addition to area debarked, we also noted that the average bark wound size inflicted by pine weevils for MeJA-treated emblings was much smaller than that of non-treated containerized seedlings (average wound size per plant type ± standard error, MeJA-treated emblings: 1.0 ± 0.2 cm^2, non-treated containerized seedlings: 7.6 ± 1.3 cm^2).

3.1.4 Additive, synergistic or antagonistic effects of SE and MeJA on area debarked

We compared the observed and expected effects of SE and MeJA on area debarked. We estimated these effects using the two types of control treatment plants, untreated containerized and bare-root seedlings, separately (see Supplementary materials). Relative to containerized seedlings, we found that the difference between the observed and expected effect of SE and MeJA on area debarked was positive (Obs − Exp = 0.127; Figure S8). Furthermore, the lower 95% confidence limit of the difference was greater than zero (lower CI: 0.091; Figure S8). Likewise, relative to bare-root seedlings, the difference was also positive (Obs − Exp = 0.083; Figure S8) and the 95% confidence limit was greater than zero (lower CI: 0.047; Figure S8). According to Bansal et al. (2013), this indicates that the effects of SE and MeJA together on plant resistance were synergistic, i.e., much greater than the sum of the independent effects.

3.1.5 Mortality

By September of the first year, late in the season, 39% of all experimental plants had died. However, mortality was significantly different among plant types, MeJA treatment and the combination of these two factors (Table 1). Among untreated plants, emblings experienced a significant 37% reduction in mortality relative to containerized seedlings, but died to a much greater extent (224% more) relative to bare-root seedlings (Table S1; Figure 1C). If plants were treated with MeJA, mortality was significantly reduced (Table 1). Relative to each untreated plant group, mortality was decreased by 97%, 43% and 100% for MeJA-treated emblings, containerized and bare root seedlings, respectively. Compared to plants receiving no treatment, SE and MeJA together significantly diminished mortality by 98% and 89% relative to untreated containerized and bare-root seedlings respectively (Table S1; Figure 1C).

3.2 Laboratory experiment

3.2.1 Proportion attacked

Similar to the field, the proportion of plants attacked by pine weevils was also high for the lab experiment, with 94% of all Norway spruce plants being attacked. We found that attack differed significantly between treatment combinations (significant plant type × MeJA interaction, Table 2). Among untreated plants, all plant types were similarly attacked (Table S2; Figure 2A). Among MeJA-treated plants, emblings experienced 9% less attack than containerized seedlings (Table S2), but were similarly attacked to bare-root seedlings. Bare-root seedlings experienced the greatest reduction in attack (17% less) compared to untreated plants of the same type (Figure 2A).

3.2.2 Area debarked

We found that pine weevil damage differed among treatment combinations (significant plant type × MeJA interaction, Table 2),

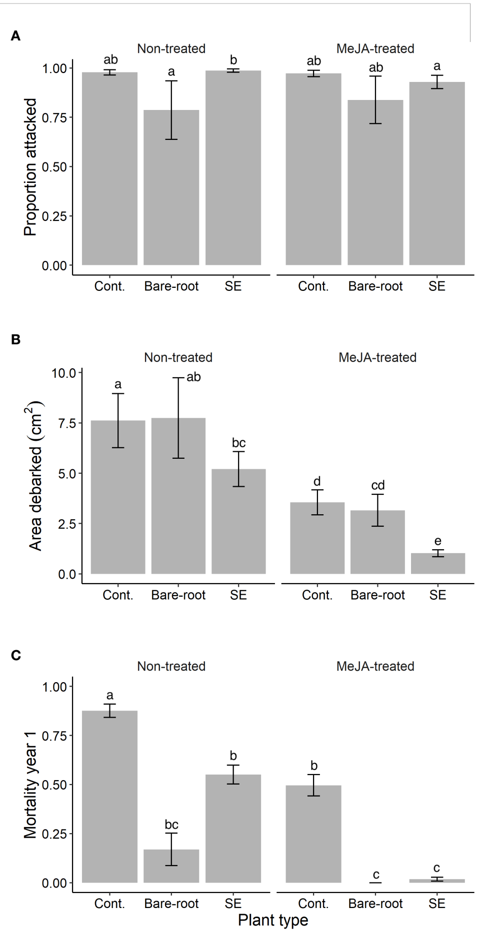

FIGURE 1
Estimated means (± standard errors) of **(A)** the proportion of Norway spruce (*Picea abies*) plants attacked, and **(B)** area debarked (cm^2) by pine weevils (*Hylobius abietis*), as well as **(C)** plant mortality (proportion that died) by September 2019, year 1 of the field experiment. Treatments represent different plant types (Cont. = containerized seedlings, Bare-root = bare-root seedlings, SE = emblings produced via somatic embryogenesis) treated (or not) with the plant hormone methyl jasmonate (Non-treated; MeJA-treated = 10 mM sprayed once in June, 2019). Sample sizes for each treatment from left to right: Non-treated containerized seedlings n=114; bare-root seedlings n=50; emblings n=164; MeJA-treated containerized seedlings n=114; bare-root seedlings n=50; emblings n=164. Different letters indicate significantly different means. A table with pairwise comparisons and p-values can be found in the Supplementary material (Table S1).

but the pattern of damage was somewhat different than that of the field experiment. Among untreated plants, emblings received the most damage, 51% and 52% more than containerized and bare-root seedlings respectively (Figure 2B). MeJA treatment significantly reduced damage levels for emblings and containerized seedlings by 66% and 23% respectively, relative to untreated plants of the same group (Table S2; Figure 2B). Damage to bare-root seedlings was slightly higher when plants of this type were MeJA-treated, but this difference was not significant (Table S2; Figure 2B). Similar to the field, SE and MeJA together resulted in the lowest plant damage levels relative to all treatments (Table S2). Area debarked was 48% lower for treated emblings when compared to either untreated containerized or bare-root seedlings (Table S2). No plants in the laboratory experiment died during the duration of each experimental round.

3.3 Field mortality years 2 and 3

During the second and third year of the field experiment, attack rate of Norway spruce plants by pine weevils remained high. Among those plants that were alive during the second and third year, 93% were attacked during year 2 and 71% during year 3. In addition, late in the season during the second and third year, 33% and 26% of the previous year's surviving plants had died. However, mortality differed among plant type and MeJA treatment combinations for year 2, but not for year 3 (Table 3). Note that since all containerized seedlings had practically died by the second year (97% mortality, 5 plants alive), these were excluded from analyses of non-cumulative mortality in years 2 and 3. In year 2, untreated emblings experienced 182% greater mortality than untreated bare-root seedlings (Table S3; Figure 3A). MeJA treatment significantly diminished mortality for treated emblings (77% less) relative to untreated plants of this group (Table S3). Together SE and MeJA resulted in 34% reduction in mortality when treated emblings were compared to untreated bare-root seedlings, resulting in these two groups having similar mortality levels (Table S3; Figure 3A). In year 3, mortality of plants that had survived the previous year was similar for plant type and MeJA treatment combinations (Table 3). MeJA treatment reduced damage for both emblings and bare-root seedlings by 30% and 44% respectively (Figure 3B), but these differences were not statistically significant (Table S3).

Overall, across the 3 years, 70% of all the experimental plants planted in year 1 had died. Cumulative mortality was significantly lowest for MeJA-treated emblings (Table S3; Figure 4). Of all treated emblings, 31% had died by the end of the experiment, which translated into a 68% lower mortality compared to untreated containerized seedlings. Treated bare-root seedlings experienced the second lowest mortality (40%), and the highest mortality was recorded for untreated containerized seedlings (97%) (Figure 4). Alone, SE significantly diminished mortality by 11% when comparing untreated emblings to untreated containerized seedlings (Table S3). Likewise, MeJA-treatment significantly decreased mortality by 64%, 12% and 43% for treated emblings, containerized and bare-root seedlings respectively when compared to each untreated group (Table S3).

4 Discussion

Our study found that producing plants via SE and subsequently treating them with MeJA can increase Norway spruce resistance to

TABLE 2 Summary of results from models examining the effects of plant type and MeJA treatment on pine weevil attack and area debarked in the lab experiment (July-August 2019).

Lab experiment	Attack			Area debarked		
	χ^2	df	p-value	χ^2	df	p-value
Plant type	1.78	2	0.410	19.21	2	**< 0.001**
MeJA	4.15	1	**0.042**	7.77	1	**0.005**
Plant type × MeJA	9.93	2	**0.007**	43.21	2	**< 0.00001**
Plant height	3.63	1	0.057	26.22	1	**< 0.0001**

χ^2: Chi-square value; df: degrees of freedom; p-value; plant type (containerized seedlings, bare-root seedlings and emblings of Norway spruce); MeJA (methyl jasmonate) treatment (0 mM and 10 mM); attack (0 = no, 1 = yes); area debarked (cm^2). Plant height (at the time of each experimental round) was included as a covariate, and round (replication in time) was included as a random effect (not shown). Significant effects ($p < 0.05$) are in bold.

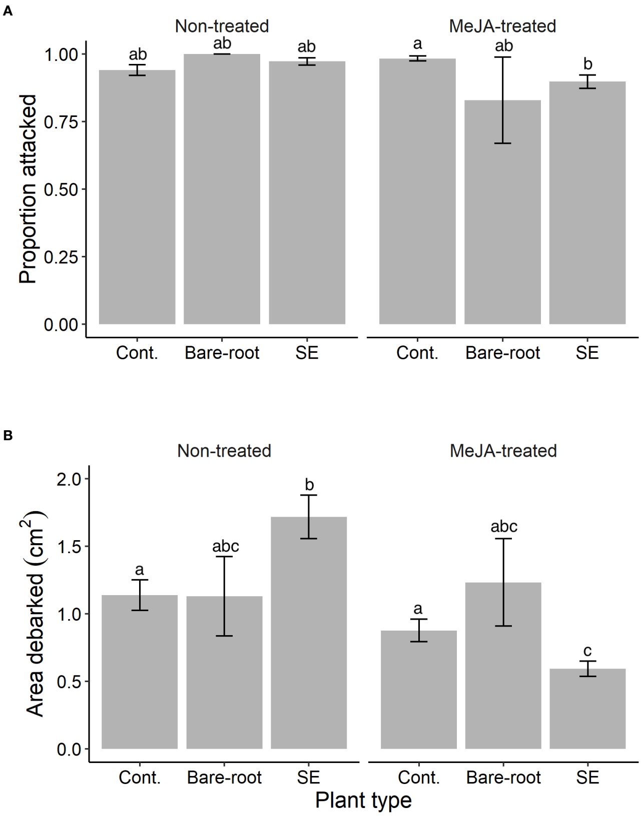

FIGURE 2
Estimated means (± standard errors) of **(A)** the proportion of Norway spruce (*Picea abies*) plants attacked and **(B)** area debarked by pine weevils (*Hylobius abietis*) in the lab experiment (replicated in time between July-August 2019). Treatments represent different plant types (Cont. = containerized seedlings, Bare-root = bare-root seedlings, SE = emblings produced via somatic embryogenesis) treated (or not) with the plant hormone methyl jasmonate (Non-treated; MeJA-treated = 10 mM sprayed once between July and August, 2019). Sample sizes for each treatment from left to right: Non-treated containerized seedlings n=150; bare-root seedlings n=12; emblings n=162; MeJA-treated containerized seedlings n=150; bare-root seedlings n=12; emblings n=162. Different letters indicate significantly different means. A table with pairwise comparisons and p-values can be found in the Supplementary material (Table S2).

TABLE 3 Summary of results examining the effects of plant type and MeJA treatment on plant mortality during years 2 and 3 in the field (September 2020 and 2021, respectively), as well as years 1 to 3 (September 2019 to 2021).

Mortality field	Year 2 *non-cumulative*			Year 3 *non-cumulative*			Years 1-3 *cumulative*		
	χ^2	df	p-value	χ^2	df	p-value	χ^2	df	p-value
Plant type	6.33	1	**0.012**	2.03	1	0.154	12.01	2	**0.002**
MeJA	1.46	1	0.228	3.05	1	0.081	8.55	1	**0.003**
Plant type × MeJA	9.89	1	**0.002**	0.58	1	0.445	7.54	2	**0.023**
Plant height	7.57	1	**0.006**	0.34	1	0.559	1.15	1	0.284

χ^2: Chi-square value; df: degrees of freedom; p-value; plant type (containerized seedlings, bare-root seedlings and emblings of Norway spruce) and MeJA (methyl jasmonate) treatment (0 mM and 10 mM); plant mortality (0 = alive, 1 = dead). Mortality for years 2 and 3 was analyzed as non-cumulative (i.e., only plants that were alive in September the previous year were included in analyses). Due to high mortality of containerized seedlings after year 1, this plant type was not included in analyses for years 2 and 3. For years 1-3, mortality was analysed as cumulative (i.e., total mortality across the three years for all plant types). Plant height (at the time of planting) was included as a covariate, and blocks in the field were included as a random effect (not shown). Significant effects ($p < 0.05$) are in bold.

pine weevil damage to a greater extent than when these two occur separately. Together, SE and MeJA decreased damage by 86-87% when treated emblings were compared to either untreated containerized or bare-root seedlings in the field, and by 48% in the lab. Moreover, survival in the field was positively affected by SE and MeJA together. MeJA-treated emblings experienced 98% and 89% lower mortality during the first year relative to untreated containerized and bare-root seedlings, respectively. These positive effects on survival remained for MeJA-treated emblings across the three years that plants were followed. Overall, we conclude that SE and MeJA have the potential to work synergistically to improve plants' ability to resist and survive damage, and can thus confer a strong plant protection advantage.

4.1 Effects of SE and MeJA on plant resistance and mortality in the field year 1

SE and MeJA, separately and in combination, affected to different extents the proportion of Norway spruce plants attacked by pine weevils, stem area debarked and survival across the experimental period. Among non-MeJA treated plants, emblings were attacked similarly to containerized seedlings, but attacked to a greater extent than bare-root seedlings (Table S1; Figure 1A). These results suggest that SE alone does not necessarily diminish the likelihood of plants being attacked by pine weevils. In contrast, Puentes et al. (2018) found a 10% reduction in attack for non-MeJA treated Norway spruce emblings relative to seedlings in one of their trials. It is important to consider that pine weevil pressure in the present study was very high (93% of plants were attacked the first year), which can make it harder to detect preferences among plant types (e.g., Tudoran et al., 2021). Indeed, Puentes et al. (2018) only found differences in attack between emblings and seedlings in the trial with lower pine weevil pressure (41% of plants were attacked), and no difference in the trial with almost 100% attack. On the other hand, when emblings were treated with MeJA, we found that the proportion of plants attacked decreased significantly by 6% compared to untreated emblings (Table S1). These positive effects were only seen for emblings, as containerized and bare-root seedlings had similar attack levels both in the untreated and treated groups (Table S1; Figure 1A). A lack of effect of MeJA treatment on the proportion of plants attacked is in line with Zas et al. (2014). The authors found that treating Norway spruce, Scots pine and Monterey pine seedlings with MeJA did not reduce the likelihood of being attacked by pine weevils in the field. Overall, it appears that SE and MeJA alone have little to no effect on plant attractiveness to pine weevils. Together, these two factors may lower the probability of being attacked, but the magnitude of these potential effects appears to be small. Evaluation of pine weevil preferences under controlled conditions (e.g., in an olfactometer), in addition to measuring volatile emissions for plants in each treatment, would be needed to disentangle the underlying causes of the observed pattern.

Even though there were small differences in the proportion of plants attacked, we found large differences in stem area debarked by pine weevils among treatment combinations (Figure 1B). In line with previous studies, we corroborated that MeJA treatment alone can effectively reduce pine weevil damage to conifer seedlings (Zas et al., 2014; Chen Y. et al., 2020; Puentes et al., 2021). On its own, we found that MeJA could decrease field damage the first year by about 50% on average for both types of Norway spruce nursery seedlings. Likewise, SE alone reduced damage to Norway spruce emblings by roughly 30% compared to seedlings produced by seed, as also reported by Puentes et al. (2018). Together, SE and MeJA acted synergistically to reduce stem area debarked (Figure S8), with treated emblings receiving 86-87% less damage than untreated containerized and bare-root seedlings. The traits and mechanisms underlying these effects need to be uncovered in subsequent studies, but a few explanations could be put forward and are discussed below.

Firstly, it seems that the lower levels of damage received by MeJA-treated emblings cannot be fully explained by a lower probability of being attacked (Figure 1A). Therefore, it is likely that differences in plant palatability, rather than attractiveness to pine weevils are more important. In line with this, we found that feeding wounds inflicted by pine weevils were much smaller on average (86% smaller) for MeJA-treated emblings relative to untreated containerized seedlings. Lower feeding rates may be due to enhanced chemical and/or other defenses in treated emblings, which deter pine weevils. For instance, plants produced

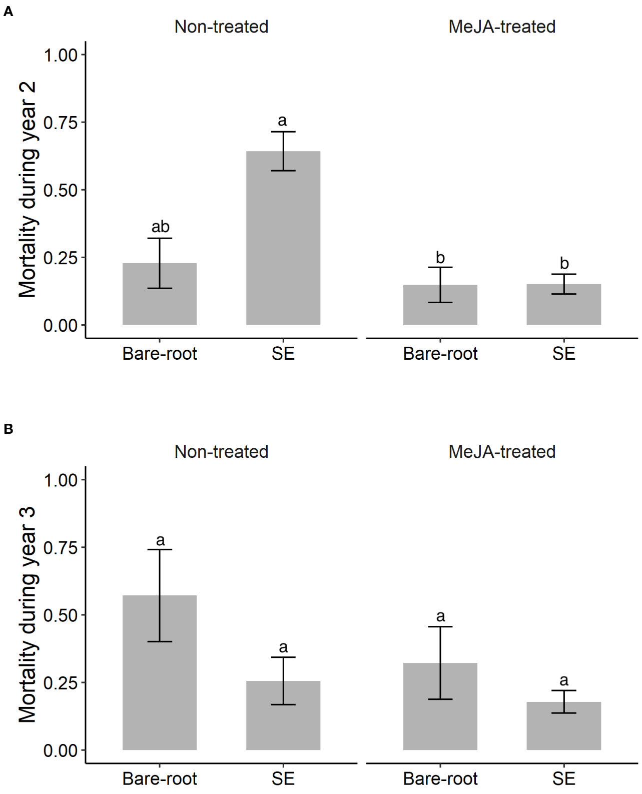

FIGURE 3
Estimated means (± standard errors) of non-cumulative mortality of Norway spruce (*Picea abies*) plants (proportion that died) by **(A)** year 2 (September 2020) and **(B)** year 3 (September 2021) of the field experiment. Due to the high mortality of containerized seedlings after year 1, this plant type was excluded from the analyses for years 2 and 3. Treatments represent different plant types (Bare-root = bare-root seedlings, SE = emblings produced via somatic embryogenesis) treated (or not) with the plant hormone methyl jasmonate (Non-treated; MeJA-treated = 10 mM sprayed once in June, 2019). Sample sizes for each treatment from left to right for years 2 and 3 respectively: Non-treated bare-root seedlings n=42, n=22; emblings n=73, n=35; MeJA-treated bare-root seedlings n=50, n=33; emblings n=161, n=143. Different letters indicate significantly different means. A table with pairwise comparisons and p-values can be found in the Supplementary material (Table S3).

through SE have been shown to harbor greater levels of secondary compounds than their non-SE counterparts (Lamhamedi et al., 2000; Fulzele and Satdive, 2003). Likewise, treatment with MeJA can result in traumatic resin duct production and increases in terpene and phenolic-based compounds (e.g., Martin et al., 2002; Krokene et al., 2008; López-Villamor et al., 2021; Puentes et al., 2021). Therefore, SE and MeJA may have a compounded effect on plant chemistry (and/or other traits), which exceeds the effect of each factor alone.

Greater resistance of treated emblings may occur due to a double-priming or induction of defenses; first early in life through SE (i.e., embryos are exposed to stress), and later through exogenous MeJA application. If plants have previously experienced stress, they can become more resistant to subsequent attacks through two mechanisms: 1) prolonged up-regulation of inducible defenses, and 2) priming of defenses (Wilkinson et al., 2019). In the first case, defenses are kept upregulated (i.e., active) for weeks or months following the stress stimulus. For example, newly-formed leaves of tomato plants have greater trichome densities in the weeks following MeJA treatment, relative to untreated plants (Boughton et al., 2005). However, such a strategy can be very costly for plants and is often not sustained for long periods of time. In the second case, defenses are primed and maintained at slightly induced levels, and become rapidly activated upon subsequent attack (Wilkinson et al., 2019). Since this strategy is less resource-costly, defenses can remain primed for longer periods of time. Our study does not allow us to distinguish if up-regulation and/or priming of defenses is responsible for the synergistic effect of SE and MeJA. However, it has been shown that MeJA can act as both an up-regulating and a priming agent in Norway spruce (Mageroy et al., 2020a). To

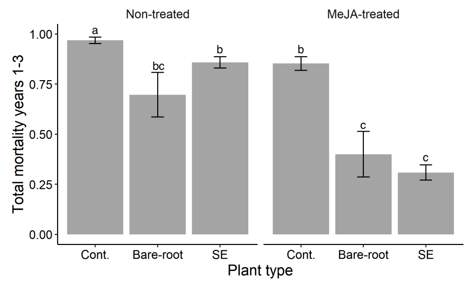

FIGURE 4
Estimated means (± standard errors) of cumulative mortality of Norway spruce (*Picea abies*) plants over the entire experimental period in the field (years 1-3). Treatments represent different plant types (Cont. = containerized seedlings, Bare-root = bare-root seedlings, SE = emblings produced via somatic embryogenesis) treated (or not) with the plant hormone methyl jasmonate (Non-treated; MeJA-treated = 10 mM sprayed once in June, 2019). Sample sizes for each treatment from left to right: Non-treated containerized seedlings n=114; bare-root seedlings n=50; emblings n=164; MeJA-treated containerized seedlings n=114; bare-root seedlings n=50; emblings n=164. Different letters indicate significantly different means. A table with pairwise comparisons and p-values can be found in the Supplementary material (Table S3).

conclusively determine the underlying mechanisms, a study on the effects of SE and MeJA on defense priming/induction, e.g., by examining defense gene transcription as in Mageroy et al. (2020b), would be needed.

Treating Norway spruce emblings with MeJA did not only reduce damage to a greater extent than the other treatments, but also significantly reduced plant mortality. During the first year, treated emblings experienced only 2% mortality compared to non-treated containerized seedlings, which experienced 88% mortality (Figure 1C). Such dramatic reduction in embling mortality was not expected, given the high pine weevil pressure at the field site (93% of plants died in the reference block), and that SE and MeJA individually decreased mortality by roughly 40% (Figure 1C). Mortality due to pine weevil feeding is often caused by removal of an entire ring of bark from the stem circumference (i.e., girdling). Girdling disrupts or hinders nutrient transport through the phloem (Romero, 2014), which can lead to plant death. Treatment with MeJA alone has been shown to reduce the likelihood of girdling by pine weevils, and therefore, increase conifer seedling survival (Zas et al., 2014; Fedderwitz et al., 2016). More specifically, Fedderwitz et al. (2016) showed that feeding scars are more spread out across the stem in MeJA-treated relative to untreated seedlings. Pine weevils often concentrate their feeding to the basal part of the stem, but treatment with MeJA appears to make seedlings less palatable, which changes their feeding behavior (Fedderwitz et al., 2016). In line with this, we also observed (but did not measure) that treated emblings tended to have shallower feeding scars (i.e., bark wounds did not always reach the stem wood) relative to untreated seedlings (K. Berggren, pers. obs.). Hence, the positive effects of SE and MeJA together on plant survival are probably mediated by the reduction in stem area debarked, and thus, lower likelihood of girdling for these plants.

Differences in mortality among treatments could also be a result of variation in size among plant types. Bare-root plants experienced the lowest mortality rates of all plant types (Figure 1C), and these plants were also the largest and thickest in terms of stem height and diameter. A previous study has shown that there is a positive relationship between Norway spruce basal diameter and survival to pine weevil damage (Thorsen et al., 2001), indicating that thicker stems can confer greater tolerance to damage. These positive effects could be mediated by physical bark properties that hinder girdling in thicker stems, and/or that larger and vigorous plants are better at recovering from stem damage (e.g., Neely, 1988; Boyes et al., 2019). In our experiment, bare-root plants received similar levels of pine weevil damage as containerized seedlings, both in the untreated and MeJA-treated group (Figure 1B). This indicates, firstly, that the lower mortality of bare-root seedlings relative to containerized seedlings is likely due to their size and not the amount of damage received. Secondly, that the effects of SE and MeJA on plant survival (and resistance) were not mediated by size differences since emblings were much smaller than bare-root seedlings (on average 30 cm *vs.* 50 cm, respectively; see Materials and methods). Yet, SE and MeJA together lowered mortality to the same extent as if a thicker and larger plant was planted. From a practical perspective, larger plants are less convenient to handle and can be more costly to produce (Berg, 1993). Thus, a plant smaller in size and displaying similar or higher resistance as a larger plant, would be preferred from a nursery and forest regeneration perspective.

4.2 Effects of SE and MeJA on plant resistance in the lab

Even though we found somewhat different trends, the results from the non-choice laboratory experiment complemented those of the field. Like in the field experiment, SE alone did not seem to affect the likelihood of plants being fed upon or not by pine weevils. Untreated emblings had similar attack levels as the other plant types in the untreated group (Figure 2A). However, MeJA diminished attack levels for emblings, and in line with the field, these effects were small in magnitude (9% less attacked than treated containerized seedlings; Figure 2A). In contrast to the field, MeJA reduced attack for bare-root seedlings by 17% (Figure 2A), and this resulted in treated emblings and bare-root seedlings having similar attack levels on average. Overall, both lab and field experiments consistently suggest that the probability of being damaged by pine weevils is not strongly affected by SE and MeJA together.

In terms of area debarked, the pattern of damage was somewhat different than that seen in the field. Among untreated plants, emblings were most damaged by pine weevils, while bare-root seedlings received once again similar levels of damage to containerized seedlings (Figures 1B, 2B). Thus, SE alone had no protective effect against damage under the lab experiment conditions. MeJA reduced damage once again for containerized seedlings and emblings but not for bare-root seedlings, which is in contrast to the field. Nonetheless, the lab and field results consistently showed that SE and MeJA together can decrease damage the most, relative to any other treatment combination (Figures 1B, 2B). A few factors could help explain some of the discrepancies between the lab and field experiments. In the lab, pine weevils were previously starved and restricted to feeding on only one plant type. Adult pine weevils usually walk around in search of food; they use visual and olfactory cues, and decide to feed (or not) in close proximity (< 2.5 cm) to the plant (Nordlander, 1991; Björklund et al., 2005). The lab set-up, with plants enclosed in large plastic cylinders, may interfere with their usual feeding behavior and thus affect levels of stem area consumed. For instance, Chen et al. (2021) found that MeJA was not as effective at reducing pine weevil damage to seedlings in a non-choice 48-hour lab experiment, compared to an earlier field experiment in which MeJA significantly decreased damage (Chen Y. et al., 2020). Moreover, plants were exposed to pine weevils for a short time in the lab compared to the field experiment. Once plants are attacked, treatment effects on induced plant resistance may take more than a few days to come into play. Despite these possible interfering factors, pine weevils fed the least on treated emblings, indicating that these plants were least palatable. Therefore, both lab and field experiments provide support for the conclusion that SE and MeJA can work together to synergistically enhance Norway spruce resistance.

4.3 Effects of SE and MeJA on plant mortality in the field years 2 and 3

We found that SE and MeJA together significantly affected Norway spruce mortality that occurred on year 2, but not on year 3 (Table 3). Important to note that almost all containerized seedlings died in year 1, and we examined non-cumulative mortality only for emblings and bare-root seedlings (see Statistical analyses). Among plants that survived in year 1, mortality of untreated emblings was much greater than that of untreated bare-root seedlings in year 2 (Figure 3A). Thus, the positive effects of SE alone on mortality observed in year 1 no longer remained the second year. Of the few studies that have examined SE-plants across years, Grossnickle and Major (1994) found that survival of Interior spruce (*Picea glauca* (Moench) Voss × *Picea engelmannii* Parry) emblings was just as high as that of seedlings (around 90%) by the second growing season. In Puentes et al. (2018), plant mortality was not followed across years. However, we revisited the sites from Puentes et al. (2018) and found no difference in embling and seedling mortality five years after planting (K. Berggren et al., unpublished data). Our results on the effects of SE alone are in contrast to previous work, but our study does not allow us to distinguish between possible causes of plant mortality. On the other hand, the effects of SE and MeJA together on plant mortality in year 2 were in line with those found in year 1. MeJA-treated emblings continued to exhibit very low levels of mortality, similar to those of treated bare-root seedlings (Figure 3A). This is in line with the findings that the beneficial effects of MeJA on conifer seedlings can persist two years after treatment (Zas et al., 2014; Chen Y. et al., 2020). Among plants that survived year 2, the same pattern of lower mortality for MeJA-treated plants was observed in year 3 (Figure 3B), but these differences were not statistically significant (Table 3).

All in all, across the 3-year experimental period, the highest survival was experienced by treated emblings. Only 31% of treated emblings had died after 3 years, while 40% of treated bare-root and 97% of untreated containerized seedlings had died after this time (Figure 4). These results suggest that SE and MeJA together can provide beneficial effects that persist several years after treatment. Future studies should examine if these two factors not only reduce damage by pine weevils, but can also positively affect other traits important to plant survival. From a plant protection perspective, greater survival of conifer seedlings is crucial in the early years after planting when seedlings are most susceptible. Seedling vigor and survival must be high to ensure establishment of future stands. Our results corroborate that planting without any type of seedling protection can compromise successful conifer forest regeneration, as pine weevil pressure is high during the three years after harvest (Örlander and Nilsson, 1999). Our study provides a sustainable way in which to protect seedlings, and incentivizes the development of practices that take advantage of our results. For example, MeJA could be applied to emblings in nurseries, even already before plants are packaged for winter storage (e.g. Chen Y et al., 2020). Although this study focuses on Norway spruce, SE is used in the production of other conifers and plant species. Hence, examination on the effects of SE and MeJA in other species may open up plant protection possibilities beyond forestry.

Data availability statement

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

Author contributions

AP, KB, CB, HB and MK conceived and designed the experiments. KB and AP conducted the experiments and collected the data. MN conducted the statistical analyses. KB and AP wrote the manuscript with input from the co-authors. All authors contributed to the manuscript and approved the submitted version.

Funding

This study was funded by the Nils and Dorthi Troëdssons Research Foundation (Stiftelsen Nils & Dorthi Troëdssons Forskningsfond) (project 1009/20).

Acknowledgments

We thank Karl-Anders Högberg at Forestry Research Institute of Sweden (Skogforsk) for providing SE-plant material, Södra Skogsplantor for providing bare-root seedlings, and Anders

Isaksson at the forest company Stora Enso AB for access to the clear-cut. We also thank Claes Hellqvist and Maria Jakobsson Hellqvist, Alessandro Nobile, Sebastian Pineda, Alessio Costa, Amelia Tudoran, Paul Eisenblätter and Emma Löfström for field and/or lab assistance. This study was funded by the Nils and Dorthi Troëdssons Research Foundation (Stiftelsen Nils & Dorthi Troëdssons Forskningsfond) (project 1009/20).

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1165156/full#supplementary-material

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Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2024:40

Utilizing or manipulating plant's own defence system, e.g. enhancing plant resistance against pests, has the potential to become a sustainable alternative within forestry. We explored the effects of somatic embryogenesis (SE) alone and in combination with methyl jasmonate (MeJA) treatment on plant resistance and tolerance, pine weevil behaviour, and the underlying mechanisms to their enhanced resistance. We show that SE together with MeJA may be a promising strategy for enhancing plant defences and offer a sustainable approach to plant protection.

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ISSN 1652-6880
ISBN (print version) 978-91-8046-344-7
ISBN (electronic version) 978-91-8046-345-4