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ORIGINAL ARTICLE

Propagation through seed or somatic embryogenesis: comparing the effects of two methods and methyl jasmonate treatment on Norway spruce resistance to *Heterobasidion* infection

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Abstract

The clonal propagation method somatic embryogenesis (SE) has been shown to affect plant resistance to an insect pest. In a field trial, Norway spruce SE-plants (emblings) experienced less damage by the bark-chewing pine weevil compared to seedlings propagated through seeds from the same mother tree. Moreover, if emblings were treated with the defense-inducing hormone methyl jasmonate (MeJA), their resistance became much greater compared to the effects of SE and MeJA alone. Thus, we evaluated in a full factorial design if propagation method (seed or SE) and MeJA treatment (treated or not) can affect Norway spruce resistance to infection by the fungal pathogen *Heterobasidion parviporum*. Emblings and seedlings were half-sibs originating from four different Norway spruce families. We found that emblings and seedlings exhibited similar fungal lesion lengths (LL) and sapwood growth (SWG) in a constitutive state (no MeJA). Once treated, seedlings exhibited a 26% and 28% reduction in LL and SWG growth respectively, in line with previous studies. For emblings, it was the opposite. MeJA increased LL and SWG by 41% and 16% respectively. This is unexpected given the genetic relatedness of the material, and the previously documented effects of SE. It is possible that SE is causing changes in Norway spruce resistance that are effective against insects, but not pathogens. Also, MeJA treatment may be affecting embling terpene composition in ways that benefit fungal growth, as suggested from separate experiments. Further studies are needed to uncover the mechanisms behind the increased susceptibility of Norway spruce emblings to fungal infection.

Key message

Somatic embryogenesis (SE) does not confer greater resistance to a fungal pathogen, in contrast to effects documented for insect pests. If SE-plants are methyl jasmonate-treated, susceptibility to the fungus increases.

Keywords Clonal propagation · Conifers · Fungal susceptibility · Methyl jasmonate

Introduction

In Nordic forestry, reforestation after final harvest occurs mostly through planting. These plants are reared from seeds produced in commercial seed orchards, originating from so-called elite trees that exhibit superior attributes (Rosvall 2019). Approximately 400 million seedlings are planted each year in Sweden, and the dominating species are Norway spruce (*Picea abies* L. Karst.) and Scots pine (*Pinus sylvestris*) (Skogsstyrelsen 2023). However, Norway spruce and Scots pine seed orchards have limitations; for example, pollination occurs with pollen that blows in from surrounding stands (Heuchel et al. 2022), which limits the breeding gains in growth and wood quality. Furthermore,

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the deployment of elite trees in seed orchards also means that it can take 15 years or more before their progenies can be planted in the forest. Therefore, forest companies are currently considering clonal propagation, in addition to their traditional plant production chains, to be able to plant the best material on their most productive sites.

Somatic embryogenesis (SE) is a vegetative propagation technique that allows clonal multiplication of an individual, or its progeny, with superior traits. Since the late 20th century, SE has been used in agriculture and forestry to generate elite material in various economically important crops and tree species (Minocha et al. 1995). Norway spruce was the first conifer species to be regenerated in 1985 (Hakman and von Arnold 1985; Westin et al. 2013). In Norway spruce, the SE process involves inducing somatic cells or tissues often taken from immature zygotic embryos from seeds derived from controlled crosses. These zygotic embryos are induced to form proembryogenic masses (PEMs) in a controlled laboratory environment by using specific plant growth regulators (PGRs). The PEMs can be multiplicated or cryostored (Egertsdotter 2018). To stimulate embryo maturation, the initial PGRs are withdrawn and then replaced with abscisic acid (ABA). Under the right conditions, the mature embryos germinate into plantlets (von Arnold et al. 2005; Egertsdotter 2018).

Interestingly, it has been found that in vitro plant propagation can influence sapling and tree traits (Kvaalen and Johnsen 2008; Puentes et al. 2018; do Nascimento et al. 2021). In a field trial, Puentes et al. (2018) found that Norway spruce propagated via SE experienced about 10% lower attack frequency and 30% less feeding damage by the pine weevil (*Hylobius abietis*), compared to traditionally propagated seedlings from the same mother tree. These effects were corroborated once more by Berggren et al. (2023), indicating that SE plants may inherently exhibit greater resistance to pest damage. These findings represent a previously unknown plant protection advantage associated with SE propagation. The mechanism behind this phenomenon is not yet fully understood, but could be associated with several aspects of the SE process. For instance, the high levels of PGRs and extreme pH levels or heat shock sometimes involved in the SE process may cause stressful conditions for the embryos (Castander-Olarieta et al. 2019). Also, the transcriptome of somatic and zygotic embryos has been found to be highly similar when it comes to metabolism, cellular processes and embryo development; yet they differ strongly in the expression of a high number of stress-associated genes (Jin et al. 2013). Thus, early exposure to stress during SE (Fehér 2005) may prime (i.e., prepare) the plant's defense mechanisms for faster re-activation later in life, providing new opportunities for sustainable pest management strategies.

In addition to propagation methods, exogenous hormone application can be exploited to enhance plant resistance through direct activation of defenses or by priming the plants. It is known that damage triggers local and systemic plant stress responses, and plant hormones like jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and ABA mediate these responses (Bharath et al. 2021; Meena et al. 2022; Hewedy et al. 2023). Studies have shown that exogenous methyl jasmonate (MeJA) application can activate defense responses and reduce insect feeding damage (Senthil-Nathan 2019; Bhavanam and Stout 2021; Erazo-Garcia et al. 2021; Mouden et al. 2021; Zhang et al. 2022). In Norway spruce, MeJA has been shown to be effective in reducing damage by a major forest insect regeneration pest, the pine weevil (*H. abietis*), which attacks seedlings planted in clear-cuts after final harvest in Nordic forestry (Zas et al. 2014; Chen et al. 2021; Puentes et al. 2021; Berggren et al. 2023). MeJA confers greater resistance to seedlings likely due to its effects on triggering the formation of traumatic resin ducts, and the production of defensive compounds (Huynh et al. 2024).

As well as insects, conifer forests can be attacked by devastating pathogens. For Norway spruce, the major fungal disease in Europe is root and stem rot caused by pathogens in the *Heterobasidion annosum* species complex. The complex has five species globally and three occur in Europe, *H. annosum* s.s., *Heterobasidion parviporum* and *Heterobasidion abietinum*. These fungal pathogens cause annual losses exceeding €90 million (Garbelotto and Gonthier 2013; Lundén et al. 2015). *H. annosum* s.l. is known to spread from tree to tree and to subsequent rotations through root-to-root contacts in the ground (Garbelotto and Gonthier 2013). Currently there are management options that reduce the transmission of the disease to the planted seedling on sites where *H. annosum* s.l. is present, but it is believed that reforestation material with improved resistance is a way to reduce the spread of *H. annosum* s.l. This could be achieved through long-term genetic improvement in forest tree breeding, or potentially through biotechnological applications such as defense priming or plant propagation techniques (Chen et al. 2018; Puentes et al. 2018; Mageroy et al. 2020).

In this study we aimed to investigate if SE-propagated Norway spruce plants exhibit higher resistance to *H. parviporum* than their non-SE propagated half-siblings, given that they were more resistant to damage by the bark-chewing pine weevil (Puentes et al. 2018). We also aimed to test if such an effect was enhanced by the combined treatment with MeJA, as this was also observed previously (Berggren et al. 2023). We hypothesize that (i) SE-derived plants exhibit smaller lesions in the bark and shorter fungal extension in the sapwood than their non-SE propagated half-siblings, and (ii) MeJA treatment will reduce the lesion size in

Table 1 Sample size per Norway spruce plant type (emblings or seedlings), MeJA treatment (control or MeJA), and inoculation with *H. Parviporum* (control or inoculated)

Seedlings $n=79$				Emblings $n=77$			
Control $n=41$		MeJA $n=38$		Control $n=39$		MeJA $n=38$	
Control $n=21$	Inoculated $n=20$	Control $n=16$	Inoculated $n=22$	Control $n=17$	Inoculated $n=22$	Control $n=19$	Inoculated $n=19$

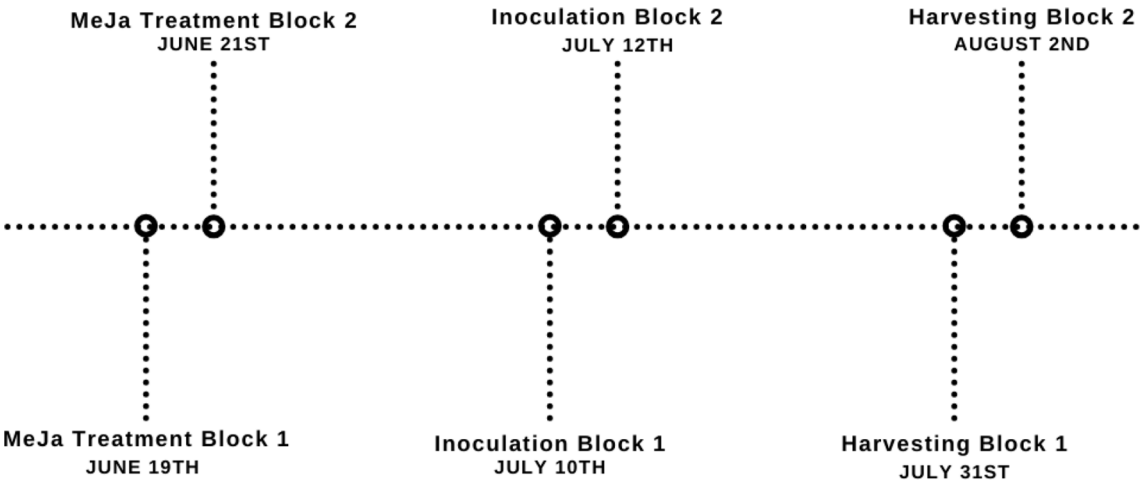

Fig. 1 Experimental timeline showing when treatment with methyl jasmonate (MeJA), inoculation with *H. parviporum*, harvesting and disease phenotyping occurred for each block

the bark and fungal extension in the sapwood irrespective of propagation method. To test these hypotheses, we used SE-derived (hereafter referred to as emblings) and seed-derived plants (hereafter referred to as seedlings) from the same mother (half-sibs) that were inoculated with *H. parviporum* (Chen et al. 2018) and treated with MeJA in a full factorial design.

Materials and methods

Plant material and treatments

Plants were generated through SE (emblings) according to the protocol described by Högberg et al. (2001), and from open-pollinated seeds (seedlings) collected from the same mother trees (non-SE plants). These trees are part of the clonal archive of the Norway spruce breeding population and are found at the Forestry Research Institute of Sweden in Ekebo. Plants were half-siblings originating from four mother trees (i.e., families); each family included varying number of embling genotypes and replicates per genotype (Table S1). However, each embling genotype was represented by at least one replicate in each of the treatments (4 treatment combinations of fungal inoculation × MeJA, see below). Plants were received at the Swedish University of Agricultural Sciences, Uppsala, in June 2022 and potted in plastic 1 L pots with commercial planting soil (P-JORD, Hasselfors Garden, Sweden). They were kept in a greenhouse under Nordic summer (16–18 L:4-8D, 20–24 °C day, 16–18 °C night) and fall (12 L:12D, 15 °C day, 10 °C night) conditions until November 2022, when temperature and light were slowly reduced for overwintering. In December 2022, they were placed in an unheated greenhouse with only natural light coming in. In April 2023, temperature and light were slowly increased to reach summer conditions from mid-May onward. In June 2023, when plants were three years old, emblings and seedlings were assigned to control or MeJA treatment, as well as no inoculation or fungal inoculation (Table 1). The experiment was conducted in two blocks (Fig. 1).

Methyl jasmonate treatment

Plants were treated with 20 mM MeJA. First, MeJA (95%, Sigma-Aldrich, ref. 392707) was dissolved in ethanol; this mixture was then added to deionized water 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 in a well-ventilated

greenhouse area, with plants placed beside each other in a row. The spraying nozzle was at a distance of about 30 cm from the plants, and the bottle was moved manually along the row while spraying. After a row was done, plants were rotated 180° to treat the opposite side. Every plant was sprayed for about one second each time, with all aboveground parts being covered with the solution except the current year's growth. We visually checked that all plants were fully covered by the solution. This methodology for treating plants with MeJA has been used in our previous studies, and we have consistently observed effects on resistance to insects and fungal pests (Chen et al. 2021; Puentes et al. 2021; Berggren et al. 2023). MeJA has been applied as early as 10 days and as late as 1 year prior to pest exposure (Chen et al. 2021; Berggren et al. 2023; here even emblings were included). However, most commonly we apply MeJA 15 days to 1.5 months prior to exposure, and (we and others) have obtained reliable results (Zas et al. 2014; Puentes et al. 2021). The treated plants were placed in a separate greenhouse to avoid contamination of non-treated plants. MeJA treatment was applied to the plants twenty days prior to inoculation (Fig. 1).

Preparation of fungal inoculum and inoculation of Norway spruce plants

Heterobasidion parviporum Rb175 was inoculated on Hagem agar (HA) (Stenlid 1985) medium and grown at 20 °C for one week. Thereafter, double-autoclaved dowels of Norway spruce sapwood (5 mm in diameter) were placed on the mycelium. The plates were incubated in darkness at 20 °C for four weeks to allow fungal colonization of the wood dowels. For each Norway spruce plant, a site for inoculation was chosen at least 10 cm above the soil surface and on a stem internode. The needles were removed from that internode and the diameter of the internode measured with a caliper. Thereafter, a 5 mm round piece of bark and phloem tissue was removed using a cork borer. A colonized wood dowel was inserted into the wound and secured with Parafilm®, following the method described by Chen et al. (2018). The use of uniform-sized dowels and incubation under consistent conditions ensured standardization of the inoculum across all experimental plants. For control plants, the inoculation wound was made with the cork borer and covered with Parafilm® (no wood dowel was inserted). The inoculated plants were kept in a greenhouse (18 L:6D, 24 °C day, 18 °C night) for three weeks in July (Fig. 1).

Scoring of disease phenotype

After three weeks of incubation, the disease phenotype was scored. At this time, the necrotic lesion length (LL) both upwards and downwards from the edge of the inoculation point on the inside of the bark, was measured (Fig. 2) (as described in Chen et al. 2018). The diameter at the point of inoculation was measured again. Fungal growth in the sapwood (sapwood growth, SWG) was measured according to established protocols (Stenlid and Swedjemark 1988; Arnerup et al. 2010). The inoculated stem was cut up into 5 mm discs using garden scissors and placed on moist filter papers in Petri dishes (Fig. 2). To avoid contamination, the stem was cut from the top towards the point of inoculation, and then from the bottom and towards the point of inoculation. Scissors were cleaned between every series. After one week

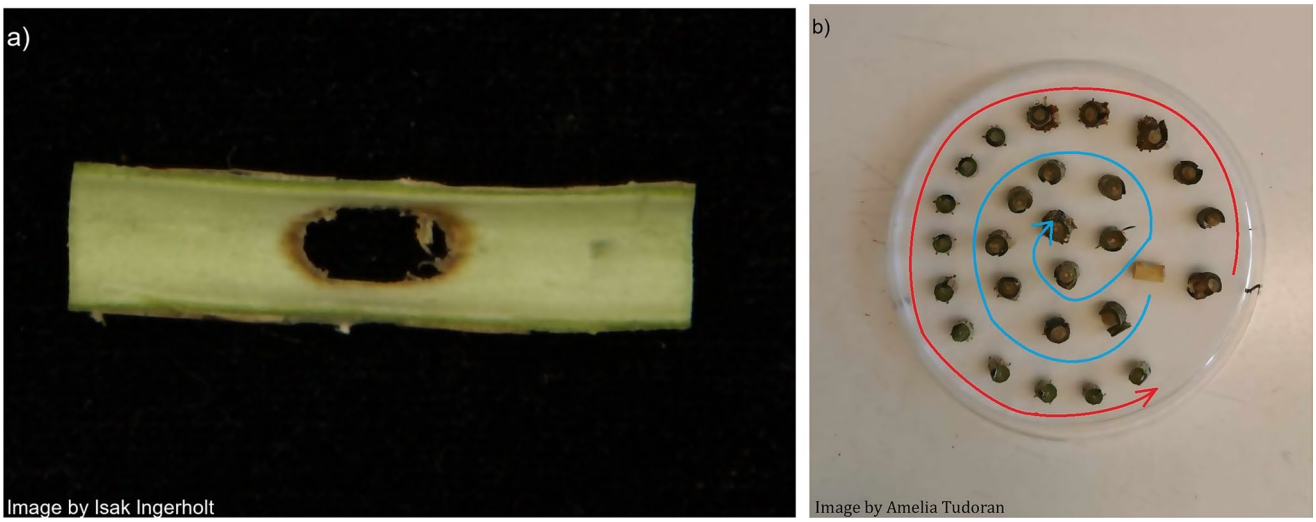

Fig. 2 (**a**) Lesion caused by *H. parviporum* on a seedling stem, showing necrotic tissue in the bark, and (**b**) cultured sapwood segments in a petri dish. The plug with inoculum is used as a starting point, with the red counterclockwise arrow on the outer part of the petri dish showing the upper part of the stem (total size 5 cm). The blue clockwise arrow on the inner part shows the lower part of the stem from the inoculation point

Table 2 Analysis of variance results (*F*: f-value; *df*: degrees of freedom; *P*: p-value) from models examining the fixed effects of plant type (Norway spruce embling or seedling), MeJA treatment (control or treated), plant diameter and experimental block on lesion length and sapwood growth of *H. Parviporum*. Standard deviation for the random effect of family is also shown

Fixed effects	Lesion length (mm)			Sapwood growth (mm)		
	F	df	*P*	F	df	*P*
Plant type	2.46	1	0.12	5.93	1	**0.01**
MeJA	0.01	1	0.93	0.32	1	0.57
Plant type × MeJA	3.44	1	0.06	4.90	1	**0.03**
Diameter	4.18	1	**0.04**	1.22	1	0.27
Block	3.17	1	0.07	1.4	1	0.24
Random effects	*Std. dev.*			*Std. dev.*		
Family	0.25			0.00		
Residual	0.78			24.0		

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

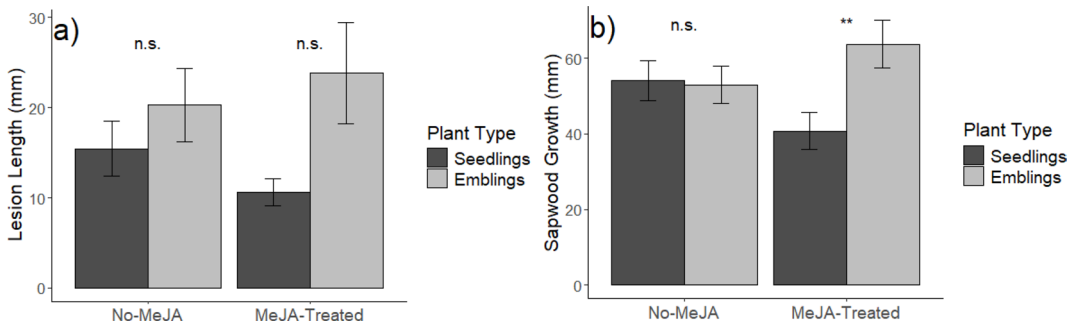

Fig. 3 Mean and standard error for (**a**) lesion length (mm) and (**b**) sapwood growth (mm) of *H. parviporum* in Norway spruce seedlings (dark grey bars) and emblings (light grey bars) when not treated (no-MeJA) and treated with MeJA

in the Petri dishes under humid conditions, the presence of *H. parviporum* on the discs was determined by observation of distinct conidia under the stereomicroscope (Stenlid and Swedjemark 1988; Arnerup et al. 2010). Samples with no conidia detected on the inoculation plug, and a total lesion length of 2 mm or shorter, were removed from the analyses as the inoculation was deemed as non-successful (Lind et al. 2014).

Statistical analyses

All statistical analyses were conducted using R (version 4.3.2). Linear mixed-effects models were fitted to assess the effects of plant type and MeJA treatment on LL and SWG separately (non-fungus inoculated plants were not used in the analyses). The models were fitted using the *lme4* package (Bates et al. 2015). For both LL and SWG, the fixed effects in the model included plant type (embling or seedling), MeJA treatment (control or treated), their interaction, and block (1 or 2), along with diameter at harvest as a covariate; family ($n = 4$) was included as a random effect. Residual diagnostics were performed using the *DHARMa* package (Hartig 2022) to assess model fit, ensuring the assumptions of normality and homoscedasticity were met. Lesion length, but not sapwood growth, was log-transformed. The significance of main effects and interactions was tested using the *anova* command in the *lmerTest* package (Kuznetsova et al. 2017). Pairwise comparisons of estimated means for main effects and interactions were conducted using the *emmeans* package (Lenth 2023).

Results and discussion

The present study aimed to investigate if SE-propagated Norway spruce plants exhibit higher resistance to *H. parviporum* infection than their non-SE propagated half-siblings, since it was previously reported that they exhibit greater resistance to the bark-chewing pine weevil (Puentes et al. 2018). We also examined if the effect was enhanced by MeJA treatment as observed by Berggren et al. (2023). We found that there were no significant main effects of plant type or MeJA treatment on LL in the phloem (Table 2). However, we found an almost significant effect ($P = 0.06$) for the interaction of plant type and MeJA (Table 2), indicating that emblings and seedlings differed in their response to the treatment. When untreated, emblings and seedlings had similar lesion lengths (Fig. 3a; Table 3). Once treated, MeJA reduced LL by about 26% for seedlings relative to untreated controls; while for emblings it was the opposite,

Table 3 Pairwise comparisons (t-ratio, p-value: *P*) between mean estimates of lesion length and sapwood growth of *H. Parviporum* for Norway spruce emblings and seedlings, when treated or not with MeJA

Comparison	Lesion length (mm)		Sapwood growth (mm)	
	t-ratio	*P*	t-ratio	*P*
No MeJA: Seedling vs. embling	0.09	0.93	-0.31	0.76
MeJA: Seedling vs. embling	-2.13	0.06	-3.27	**0.003**

Significant comparisons ($P < 0.05$) are in bold

MeJA increased LL by 41% on average relative to untreated controls (Fig. 3a). Irrespective of treatment, diameter significantly affected LL (Table 3). During model exploration, we did not find any significant interactions between treatments and diameter and were thus not included in the final model. We found that as diameter increased, LL decreased (model coefficient for diameter = -0.26, s.e. = 0.13, t-value = -2.05, p-value = 0.04). This contrasts with previous studies in Norway spruce and Scots pine, which have shown that *Heterobasidion annosum* infection can correlate positively with tree diameter (Swedjemark and Karlsson 2004; Mukrimin et al. 2019; Liu et al. 2022). Greater diameters are thought to be associated with a more extensive phloem area, and thus greater substrate available for fungal growth. However, greater diameters can also be associated with more vigorous individuals, that can better resist infection. Growth (diameter) and resistance phenotypes (such as LL) are quantitative traits, and the expression of quantitative traits can vary between environments (Coyne et al. 2000; Steffenrem et al. 2016,).

A similar pattern of treatment effects to that of LL was observed for SWG. When untreated, emblings and seedlings had similar spread of the fungus in the sapwood (Fig. 3b; Table 3). Once treated, MeJA reduced SGW by about 28% for seedlings relative to untreated controls; while for emblings it was the opposite, MeJA increased SGW by 16% on average relative to untreated controls (Fig. 3b). No significant effect of diameter was observed for SWG (Table 3). Overall, these results indicate that under a constitutive state, emblings and seedlings have similar resistance to *H. parviporum* infection, but induction through MeJA increases and decreases the susceptibility of emblings and seedlings, respectively.

The results from the study are contrary to our proposed hypotheses. Propagation through SE does not provide a plant protection advantage against a fungal pathogen, as it did for a bark-feeding insect. This suggests that the mechanisms conferring greater resistance to insect damage in Norway spruce emblings do not necessarily apply to fungal pathogens. The mechanisms by which conifers resist insects and pathogens share some common features, such as the production of terpenoid-rich oleoresins (resin) and phenolics that create physical and chemical barriers (Franceschi et al. 2005; Hammerbacher et al. 2020). However, there are also distinct differences due to the unique challenges each attacker presents. For example, insects like the pine weevil bore or chew though the bark, while fungal pathogens primarily infect through wounds, then kill cells and degrade cell walls. Resin flow and specific terpene compounds may be essential to deter or kill the insects, while compartmentalization with resin or lignosuberization may be more important for fungal pathogens (Franceschi et al. 2005; Hammerbacher et al. 2020). Moreover, conifers can fine-tune the concentration and type of terpenes depending on the threat, leading to resistance pathways optimized for each attacker (Raffa and Berryman 1983; Klepizig et al. 1996; Vázquez-González et al. 2020). In a recent study with the bark beetle *Ips typographus* and three major symbiotic fungi, it was found that the monoterpene compounds that were most toxic to beetles were the least inhibitory to fungal growth and vice versa (Zaman et al. 2024). Thus, it is possible that SE is causing changes in the resistance of Norway spruce plants that are effective against insects, but not against pathogens.

The second hypothesis we tested was that MeJA treatment would reduce fungal spread in Norway spruce plants irrespective of propagation method. Only seedlings responded as expected, in line with previous studies. In Scots pine, mean fungal growth of *H. annosum* was more than four times less in MeJA-treated than in control seedlings (Šņepste et al. 2021). Similarly, MeJA is known to reduce the spread of blue stain fungi in Norway spruce (Zeneli et al. 2006; Krokene et al. 2015; Puentes et al. 2021). None of the previous studies though have evaluated the effect of MeJA treatment on resistance against *H. parviporum*, so this is the first report. For emblings, MeJA treatment led instead to an increase in the spread of the pathogen both in the bark and sapwood. Interestingly, in separate experiments, we have found that after MeJA treatment, Norway spruce emblings produce none or few traumatic resin ducts and have lower concentrations of bark terpenes relative to seedlings (Berggren 2024, PhD thesis). It could be that after MeJA treatment, terpene composition in emblings is skewed in favour of compounds or concentrations that stimulate fungal growth; or even phenolic composition, which is known to be important for interactions with blue stain fungi (Hudgins et al. 2004; Malá et al. 2011; Li et al. 2012). Further studies are needed to fully understand why SE and MeJA provide syngerstic protection against an insect but increase susceptibility to a fungal pathogen. For instance, by examining gene expression patterns in emblings and seedlings in a constitutive and an induced state.

The differences we documented in the present study are indeed intriguing as emblings and seedlings were half-sibs, which means that they share some genetic components of

resistance. Due to their genetic relatedness, individuals among families are expected to exhibit similar levels of susceptibility or resistance to an attacker. On the other hand, differences among families are expected and often occur, as has been documented in studies examining genetic variation in resistance to *H. parviporum* in Norway spruce (Steffenrem et al. 2016; Chen et al. 2018; Capador-Baretto et al. 2022). In the present study, variation among families was not large; on the contrary, it was very small (Table 2). An important caveat is that we only examined four families, and that resistance to *H. parviporum* was evaluated at the seedling stage. Infection by this fungus occurs in mature trees. However, previous research has demonstrated that variation in resistance among individuals or treatments can be assessed in seedlings or saplings using the methodology employed in this study (Asiegbu et al. 2005; Arnerup et al. 2010; Chen et al. 2018; Terhonen et al. 2019). All in all, the results indicate that despite genetic relatedness, plants that have undergone SE differ from their siblings in attributes that can affect their defensive mechanisms later in life. Our understanding of how somatic and zygotic embryogenesis and plant development differ remains fragmented, but certainly deserves further attention (Juarez-Escobar et al. 2021).

In conclusion, while SE offers many advantages for plant propagation, in particular when it comes to delivering elite material for reforestation, our study highlights potential vulnerabilities that need to be addressed. The increased susceptibility of induced SE Norway spruce plants to *H. parviporum* compared to seedlings underscore the need for further studies addressing underlying mechanisms (e.g., by examining gene expression patterns, defensive metabolites such as terpenes). Moreover, it is important to examine if and for how long this vulnerability persists in the field (since this was a greenhouse study) in order to optimize the use of SE in forestry.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-024-02933-z.

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Author contributions The study was conceived and designed by AP and ME. AE was involved in plant production, and ME provided fungal cultures. Data collection and analysis were performed by AT and AP. The first draft of the manuscript was written by AT, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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