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from insect attack. However, as long as broad clearcutting remains the dominant approach to forest regeneration, there will always be a need for the protection of individual seedlings from insect attack, as this cultivation method favours the development of large weevil populations on regeneration sites.

One promising new approach to seedling protection is the use of chemical elicitors, such as the well-known plant defense signalling compound methyl jasmonate (MeJA), which has been used in attempts to improve conifer seedling resistance to pine weevils (Heijari *et al.*, 2005; Holopainen *et al.*, 2009; Zas *et al.*, 2014). Exogenous application of such chemicals induces the plant's natural defensive capabilities, without introducing toxic new compounds to the ecosystem. The application method has primarily been by spraying or fumigation. Trials of MeJA-sprayed conifers indicate that the presence of stem resin(s) is an important feature of defense against pine weevil attack (Zas *et al.*, 2014), but little is generally known about the precise effect of other chemical elicitors on conifer defense against pine weevils. In this context, the defense-activating compound nicotinamide (NIC) (Berglund, 1994), also known as niacinamide, and its metabolite in plants, nicotinic acid (NIA), better known as vitamin B$_3$ (niacin), have here been tested for their ability to improve the defensive capacity of young spruce seedlings to pine weevil attack. NIC is known to influence a plethora of defensive activities in both animal (Surjana *et al.*, 2010; Canto *et al.*, 2013) and plant cells (Berglund *et al.*, 1993a,b; Berglund, 1994; Ohlsson *et al.*, 2008). NIC, and its plant metabolite NIA, have also been suggested to function as stress signal mediating compounds in eukaryotic cells (Berglund, 1994). Furthermore, isonicotinamide (Basson and Dubery, 2007) and 2,6-dichloro-isonicotinic acid (Metraux *et al.*, 1991), synthetic analogues of NIC and NIA, respectively, have been used to induce defense in various non-conifer plant species, which supports a role for the naturally occurring compounds NIC and NIA in native plant defense. Previous results indicated that NIC acts at a general level in plants as well as in animals, as various defense pathways and processes are activated, reflected in changes to gene expression patterns (Berglund and Ohlsson 1995; Surjana *et al.*, 2010).

Induced gene expression in plants and other eukaryotes depends on at least two factors: an endogenous or exogenous molecular signal is needed, and DNA must be accessible for interaction with the signal. In eukaryotic cells, DNA is packed together with proteins (histones) into a structure called chromatin, which must be unpacked to be available for interactions with other molecules. This process is influenced by features such as the level of DNA methylation and various histone modifications, which contribute to the so-called epigenetic regulatory mechanisms of gene expression, which can in turn be influenced by environmental factors and cellular signals (Cedar and Bergman, 2009; Bräutigam *et al.*, 2013; Kinoshita and Seki, 2014). Thus, it is not just the level of intrinsic or extrinsic inducing signals that determines the response, but also the state of chromatin packing. In the present study 5-azacytidine (5-Aza), a well-known inhibitor of DNA methylation (Yang *et al.*, 2010), was used as a reference substance for investigation of the possible influence of DNA methylation in defense activation.

Priming, also known as sensitization, is a strategy by which plants can accelerate and perhaps potentiate a defensive response when later exposed to a second occurrence of a certain kind of stress (Pastor *et al.*, 2013). This is an energy-saving mechanism which allows plants to mount a timely defensive strategy to biotic or abiotic stresses without the wasteful and unnecessary constitutive production of defensive molecules like proteins and secondary metabolites. The mechanisms behind priming are not well-known, but may involve increased levels of active transcription factors, as well as unidentified epigenetic mechanisms (Pastor *et al.*, 2013).

It is well-known that seed treatment can influence the performance of seedlings or mature plants within agriculture. For example, jasmonic acid (JA) treatments of seeds from tomato plants led to mature plants with a strong defensive capability against attack by arthropod herbivores and fungal pathogens (Worrall *et al.*, 2012). As far as we know, seed treatment has not so far been reported to promote insect defense in conifers, although it has been shown that spruce embryos treated by changes in temperature, sensitizing them to environmental fluctuations, can influence the ability of spruce plants to handle some abiotic parameters (Yakovlev *et al.*, 2011). We hypothesize that it is possible to potentiate spruce seedling defense against pine weevils via a short seed exposure or via seedling watering with defense potentiating compounds, and that epigenetic mechanisms are involved in this defense potentiation. The research questions in this study were: (1) can spruce treatment with the plant defense potentiating compounds NIC or NIA promote defense against pine weevil attack? (2) Can JA seed treatment give the mature plants protection against pine weevils? and (3) can treatment of spruce seeds with compounds known to generally decrease DNA methylation influence a plant's defense against pine weevils? It was hypothesized that these treatments would prime the seeds or seedlings, rendering the fully grown plants more capable of mounting a swift defensive response to signals arising from environmental stresses, primarily including insect attack.

Materials and methods

Plant material

All seeds utilized in this study were Norway spruce seeds, origin 57° 00′N, altitude 55 m, collected in the orchard of Öhn. The treatments described below were carried out either on these seeds or on seedlings grown from them. Seedlings were grown in various container types, all filled with Finnish peat (Kekkilä Oy, Tuusula, Finland). A complete mineral solution was used to fertilize the growing seedlings (Wallco, Sweden: N:P:K, 100:13:65 w/v).

Experiment with seed treated seedlings

Seeds were treated on 18 April 2010 with test substances in water solutions under gentle shaking for 4 h at 23°C in darkness. The test substances were NIC (2.5 mM), NIA (2.5 mM), JA (3 mM) and 5-Aza (200 μM). The surfactant Tween 80 (0.24 μl ml^{-1}) was added to the solutions to increase the contact between the substances and the seeds. To rule out the potential influence of the surfactant, in this study the control seeds were treated with water containing Tween 80, as defense-inducing effects have been observed for Tween (Moreira *et al.*, 2009). After treatment, seeds were allowed to dry on filter paper overnight before sowing. The following day 90 ml containers (Hiko V 90, BCC, Sweden) filled with peat were seeded with treated seeds at the research station in Vassbo (60° 32′N; 15° 33′E). Before sowing, the container units were split in half, resulting in 20 cavities per container unit. Each container was then sown with two seeds from each of the five treatments. In total, four container units (replicates) per seed treatment were sown. After sowing, the units were arranged in a completely randomized (CR) design in the greenhouse. During germination the relative humidity

was kept at ~70 per cent and the temperature at an average of 20° C. After 9 days, the germination results for the different treatments were investigated visually by assessing how far the germ had developed. Fertilization started the third week after sowing with a weekly nitrogen supply of 3 g N m⁻². After 3 weeks the seedlings were thinned so that only one seedling per container was left. Seedlings were kept in the greenhouse until the middle of June, when they were put outdoors for further growth.

On 20 August, the spruce seedlings were planted at Kann-Olles Heden (60° 38′N; 16° 13′E). This regeneration site was clear cut in 2008 and scarified with a harrow during late spring 2010. The site index is G 24, as 24 m is the dominant height of Norway spruce at the age of 100 years according to Hägglund and Lundmark (1982), and the soil type at the site is a sandy-loam till. In total, 200 spruce seedlings were planted in a CR-design with 4 replicate sets of 10 seedlings for each of the 5 treatments. Where possible, the seedlings were placed in the mineral soil, avoiding humus and the deeper parts of the harrow furrows. The heights of the seedlings were recorded when they were planted, while seedling vitality and the extent of pine weevil gnawing were registered for each seedling (gnawed bark area cm²) on 18 October by visual assessment.

Experiment with watering

Untreated spruce seeds were sown in July 2009 in a commercial nursery, Nässja nursery (60° 15′N; 16° 50′E), in 15 ml mini containers, and grew to seedlings which were to be treated via watering. In the middle of April 2010 the mini seedlings were collected and transported to the research station in Vassbo and immediately transplanted into larger 85 ml containers (Plantek 81, BCC, Sweden) for further growth. Before transplanting, the containers were filled with a peat growing medium. Three container units each containing 81 seedlings were randomly selected for one of three treatments: water (control), NIC and NIA. The container units were then randomly positioned for growth in a CR design. Seedlings were treated by watering (2 l m⁻²) twice a week with 2 mM NIC or NIA dissolved in the water from the middle of April until the end of June. The water also contained a dissolved complete mineral nutrient solution, and the weekly nitrogen supply was 3 g N m⁻². Seedlings were grown in a greenhouse at an average constant temperature of 20° C until the middle of June when they were put outdoors. Just before out-planting in the field, at the end of June, 30 seedlings from each of the treatments were randomly selected for measuring of height, stem diameter and root and shoot dry weight. Seedlings were then planted at Gettjärnsberget (60° 30′N; 16° 02′E), which had been clearcut in the autumn of 2008 and scarified with a harrow in the autumn of 2009. The site index is G 24 and the soil type is a sandy-loam till. The seedlings were planted in five randomized blocks, each containing three plots with 11 seedlings of each treatment. In early October, the seedlings were examined with respect to vitality and damage caused by the pine weevil.

Combination of seed treatment and watering with NIC for laboratory tests

Seeds were treated, and seedlings cultivated, as described above. To test the effect of a second exposure to NIC, seedlings grown from seeds treated with NIC were watered with NIC after 19 weeks of growth. Seedlings were watered to 2 l m⁻² twice a week with 2 mM NIC. This treatment lasted for 3 weeks. Tests with pine weevils and seedlings were carried out in the laboratory.

Six seedlings of each of the two treatments, the control (untreated) and the twice NIC-treated seedlings (seedlings grown from NIC-treated seeds and subsequently watered with NIC), were planted in plastic containers. The containers with seedlings were randomly lowered down through holes in the bottom of a rectangular box so that the soil surface was in line with the bottom of the box. The box had internal measurements of 1.0 × 0.7 and 0.2 m high walls, which were painted with fluon on the inside of the box to prevent pine weevils from climbing and escaping.

The top of the box was covered with a net for additional protection. The positioning of seedlings in the box was according to a CR design. Two tests were performed with 20 pine weevils placed in the box when starting the tests. The first test went on for 48 h, after which seedlings were examined for bark gnawing and the gnawed area of the seedlings was estimated. The first test was repeated with new seedlings and ran for 60 h.

DNA methylation

For analysis of DNA methylation, seed treatment and seedling cultivation were performed as described above, and 15-week-old seedlings were used for analysis. Needles were homogenized by pestle and mortar under liquid nitrogen, and DNA was extracted using the DNeasy® Plant Mini Kit from Qiagen AB (Sollentuna, Sweden). Changes in global DNA methylation were analyzed by the Luminometric Methylation Assay (Karimi et al., 2006), modified as described by Poborilova et al. (2015) and performed in a PyroMark Q24 instrument using Pyro Gold Reagents from Qiagen AB (Sollentuna, Sweden). In this assay, the restriction enzymes HpaII and MspI were used for methylation-dependent cleavage at CCGG sites. Unmethylated CCGG sites can be cleaved by both enzymes, while neither can cleave the DNA strand if the outer C is methylated (**C**CGG). If the inner C is methylated (C**C**GG), then MspI can cleave, but not HpaII. The resulting CG-overhangs were detected by pyrosequencing analysis with pyrophosphate (PPᵢ) as an internal standard. The result was expressed as changes in the ratio of peak heights for (C + G) and PPᵢ, [(C + G)/PPᵢ]. An increased peak height ratio corresponds to a decreased DNA methylation level. Note that the result was expressed as relative changes and not as a quantitative measure of methylation level.

Statistical analysis

Seedlings were grown in replicates and positioned in a CR design as described above. The statistical significance of the data was evaluated by analysis of variance using SPSS 20 software (SPSS Corporation). Microsoft Excel was used for computational analysis of the data. For parametric statistical tests, both Kolmogorov–Smirnov and Shapiro–Wilk tests of normality showed non-significance at the $P < 5$ per cent level, indicating that the distribution of data is normal. Analysis of variance (ANOVA) tests of the data were performed and the different treatment methods tested were compared using Student–Newman–Keuls (SNK) and Tukey's Honestly Significant Difference (HSD) multiple range tests at the $P < 0.05$ level to detect significant differences for seedling height and damaged area per attacked seedling, as well as Student's t-test for changes in DNA methylation level. For nonparametric statistical tests for number of total attacked seedlings and number of girdled seedlings, binomial tests were used to detect significant differences.

Results

Laboratory test

Initial small-scale laboratory tests suggested a potential protecting effect of NIC treatment against pine weevil feeding on spruce seedlings grown from seeds treated with NIC and subsequently treated with NIC via watering. The results, although not statistically strong, indicated that there was somewhat less damage on treated (NIC) seedlings, when compared with control (water) seedlings. In one test, lasting for 48 h, the total area of feeding was 0.6 cm² on treated seedlings and 1.8 cm² on control seedlings. In a second test, lasting for 60 h, the corresponding values were 9.6 cm² (treated) and 18.1 cm² (control).

Field tests

Two separate field tests were carried out, one test with spruce seedlings grown from treated seeds and a second test with

Table 1 ANOVA results for height of seedlings from treated seeds

Seedlings height × treatments	Sum of squares	df	Mean square	*F*	Sig.
Between groups					
(Combined)	7065.000	4	1766.250	1.456	0.217
Linearity	5184.000	1	5184.000	4.273	0.040
Deviation from linearity	1881.000	3	627.000	0.517	0.671
Within groups	236556.875	195	1213.112		
Total	243621.875	199			

Heights of 4-month-old greenhouse-grown spruce seedlings at the time of field planting. Variance of treatment between groups was not significant at the $P < 0.05$ level.
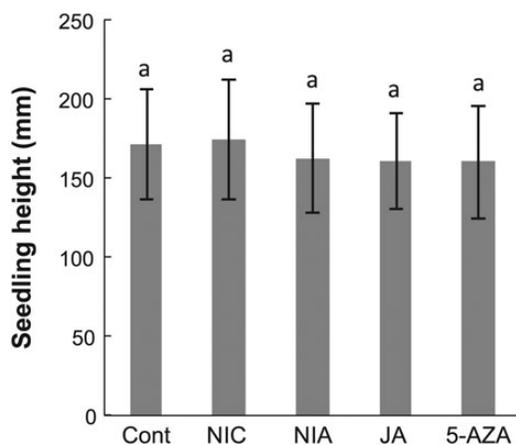

Figure 1 Heights of 4-month-old greenhouse-grown spruce seedlings at the time of field planting. Seedlings originated from seeds treated with water (Cont), 2.5 mM NIC, 2.5 mM NIA, 3 mM JA and 200 µM 5-Aza. Mean values based on 40 seedlings per treatment are shown. Error bars show standard deviation. Variance of treatment between groups was not significant at the $P < 0.05$ level.

seedlings grown from untreated seeds, but treated via watering. Seed treatment with NIC, NIA, JA or 5-Aza did not affect seedling growth, as analyzed after 4 months, when the plants were set out in the field (Table 1; Figure 1). Neither were any effects on the vitality of the seedlings, as indicated by bud and needle status, detected.

After 2 months in the field, the extent of pine weevil gnawing on six month old spruce seedlings was observed (Tables 2 and 3). The results showed that seed treatment with NIC or JA reduced the number of seedlings attacked, while the effect of seed treatment with 5-Aza was less pronounced and seed treatment with NIA probably did not have any effect. The number of attacked seedlings was reduced by 50, 62.5 and 25 per cent by seed treatment with, respectively NIC, JA and 5-Aza (Figure 2A). Among the attacked seedlings, some were more severely damaged by girdling. The number of girdled seedlings was reduced by all treatments, 75, 100, 100 and 50 per cent reduction by seed treatment with, respectively NIC, NIA, JA and 5-Aza (Figure 2A). Although this was not statistically verifiable, the damaged area per attacked

seedling showed a pattern similar to the number of attacked seedlings (Table 3), (Figure 2B).

Treatment of seedlings grown from untreated seeds by watering with NIC or NIA did not affect plant growth as determined by the height of the seedlings before they were set out in the field (Table 4; Figure 3).

The number of attacked seedlings was reduced by 40 per cent after NIC treatment and by 53 per cent after NIA treatment, and the number of girdled seedlings were reduced by 30 and 50 per cent after treatment with, respectively, NIC and NIA, compared with control seedlings (Table 5; Figure 4A). As in the case of seed treatment, water treatment with NIC or NIA did not reduce the damaged area per attacked seedling (Table 6; Figure 4B).

DNA methylation

The effect of seed treatment with NIC on DNA methylation at a global level in needles of 15-week-old spruce seedlings grown in a greenhouse was analyzed (Figure 5). Treatment with NIC had a reducing effect on DNA methylation, analyzed as increased cleavage of CCGG sites in DNA by the restriction enzyme *Msp*I. This illustrates a general decrease in methylation at the outer cytosine in **C**CGG sites, corresponding to CXG positions in DNA. There was no difference in cleavage with *Hpa*II between the control and treated samples, however, suggesting that the overall methylation at the inner cytosine in C**C**GG sites (CG positions) did not change.

Discussion

One of the most interesting aspects of stress in plants, in terms of the elicitation and priming of defensive strategies, is the question of what precise factor or condition is actually sensed by the plant, to induce a specific defensive response. Three important conditions in a plant which can be affected by stressors are oxidative stress, stability of DNA and energy state. Increased levels of reactive oxygen species arise during most types of stress, including herbivory (Kerchev *et al.*, 2012), and often cause serious physiological damage, including strand breaks to DNA. Free NIC can be formed in response to DNA strand breaks induced by oxidative or other kinds of stress via the action of the enzyme poly(ADP-ribose) polymerase (PARP) (Schraufstätter *et al.*, 1986; Berglund *et al.*, 1996; Kalbin *et al.*, 1997; Hunt *et al.*, 2004; Surjana *et al.*, 2010). In a negative feedback loop, NIC is also a potent inhibitor of PARP, so that NIC build-up leads to decreased NAD^+ cleavage (Canto *et al.*, 2013). In addition, NIC may itself be metabolized to NAD^+ via the NAD^+ salvage pathway (Ashihara *et al.*, 2005), a process in which the first step is metabolism of NIC to NIA by a nicotinamidase enzyme (Hunt *et al.*, 2004). Furthermore, NIC can induce the expression of genes involved in plant defense (Berglund *et al.*, 1993b). For instance, NIC is known to be a natural inhibitor of, but also a product of, a family of NAD^+-dependent protein deacetylases, the sirtuins (Denu, 2005), which have many regulatory functions in eukaryotic cells (Canto *et al.*, 2013). In short, an important feature of NIC in plants is that it is a key component of pathways involved in redox homeostasis as well as those involved with stress signalling and associated gene expression. Clearly, the full extent of NIC action in plant cells is highly complex, and so the precise mechanisms behind the defense-promoting properties of NIC and NIA are still unknown. Metabolism of NIC to NIA by a nicotinamidase may be an important step, and has been suggested to

Table 2 Binomial test of number of attacked and girdled seedlings from treated seeds

	Category	N	Observed Prop.	Test Prop.	Exact Sig. (two-tailed)
Number of attacked × treatments					
Group 1	Nonattacked	28	0.14	0.50	0.000
Group 2	Attacked	172	0.86		
Total		200	1.00		
Number of girdled × treatments					
Group 1	Nongirdled	7	0.04	0.50	0.000
Group 2	Girdled	193	0.97		
Total		200	1.00		

Differences between nonattacked and attacked seedlings, and between nongirdled and girdled seedlings were significant at the $P < 0.05$ level.

Table 3 ANOVA results for damaged area per attacked seedling from treated seeds

Damaged area × treatments	Sum of squares	df	Mean square	F	Sig.
Between groups					
(Combined)	9.062	4	2.266	1.873	0.150
Linearity	2.443	1	2.443	2.019	0.169
Deviation from linearity	6.619	3	2.206	1.824	0.171
Within groups	27.827	23	1.210		
Total	36.890	27			

Damaged area per attacked seedling by pine weevils on 6-month-old spruce seedlings. Variance of treatment between groups was not significant at the $P < 0.05$ level.

function as a negative regulator of the plant hormone abscisic acid (Hunt *et al.*, 2004). This could in turn promote defense mediated via another important plant hormone, JA (Asselbergh *et al.* 2008), a mechanism which is important in protection against insect attack.

In the present study, NIA treatment via root uptake in spruce seedlings showed an anti-pine weevil effect in terms of the number of attacked seedlings, while there was no such effect when NIA was used for seed treatment. This differential response to NIA could simply depend on differences in NIA uptake between seeds and seedlings, but it could equally hint at different modes of action for the two compounds: it is known that NIC and NIA have similarities and differences in their biochemical effects. One main difference between the actions of these compounds is in connection with PARP and the sirtuins, discussed above. These enzymes are inhibited by NIC, but have not been shown to be inhibited by NIA (Denu, 2005). The aforementioned inhibition of sirtuins by NIC may have a strong impact on gene expression, possibly via epigenetic mechanisms, which is not induced by NIA.

Epigenetic mechanisms, such as changes in levels of DNA methylation, are closely associated with stress and defense in plants. Various types of stress induce changes in DNA methylation levels, commonly causing hypomethylation, as well as certain chromatin modifications which may serve as a 'memory' of a particular stress, improving the chance for future resistance (Sano, 2010; Jaskiewicz *et al.* 2011; Bräutigam *et al.*, 2013; Kinoshita and Seki, 2014). We have previously discussed a potential role for NIC

in DNA methylation processes in plant tissue, particularly the hypomethylation of DNA (Berglund, 1994; Berglund and Ohlsson, 1995; Ohlsson *et al.* 2013). The decreased DNA methylation levels (Figure 5) and decreased damage by pine weevils (Figure 2A) following seed treatment with NIC, viewed alongside the similar observations of decreased pine weevil damage after seed treatment with the DNA methyltransferase inhibitor 5-Aza (Figure 2A), point at a potential involvement of general changes to DNA methylation levels in defense activation. Also supporting this connection are the results of an earlier study in which we demonstrated that UV-B exposure of indoor grown spruce seedlings caused decreased DNA methylation and increased emission of volatile terpenoids, known to influence pine weevil behaviour (Ohlsson *et al.* 2013). UV-B exposure has also been shown to increase the level of both NIC and trigonelline (*N*-methyl nicotinic acid) in plant tissue (Berglund *et al.* 1996). Trigonelline is formed from NIA, which in turn is formed from NIC by the action of nicotinamidase. It has been shown that trigonelline can promote anti-microbial defense in plants in association with a decrease in global DNA methylation (Kraska and Schönbeck 1993). In line with this, we consider it a possibility that the hypomethylation of DNA in plants grown from seeds treated with NIC could also serve to promote defense against herbivorous insects, resulting specifically in this case in a reduction in pine weevil attacks. In future studies, we would also like to include marker gene expression analysis to investigate a possible connection between DNA methylation and spruce defence induction.

Many reports point at the importance of the mother plant for resistance and adaptive responses in the next generation (Holeski *et al.*, 2012; Pastor *et al.* 2013), transmitted via effects on the embryo or seed (Yakovlev *et al.*, 2011; Worrall *et al.*, 2012; Bräutigam *et al.*, 2013). It is possible that exogenous application of native signalling compounds (or close synthetic mimics thereof) directly to the seed may mimic such information transfer from the mother plant to the embryo/seed, providing the young plant with the capacity to adapt to stressful changes in the environment. Although the nature of this signalling system is not yet well understood, epigenetic mechanisms are likely involved (Yakovlev *et al.*, 2011; Bräutigam *et al.*, 2013). Seed treatment over a matter of a few hours with for example NIC can influence the properties of the plant several months later, rather than inducing only transient physiological changes as might be expected. A plausible explanation is that physical changes to the plant's DNA have been made, thereby altering the epigenetic coding capacity of the organism, and that this information is therefore carried through many cell divisions.
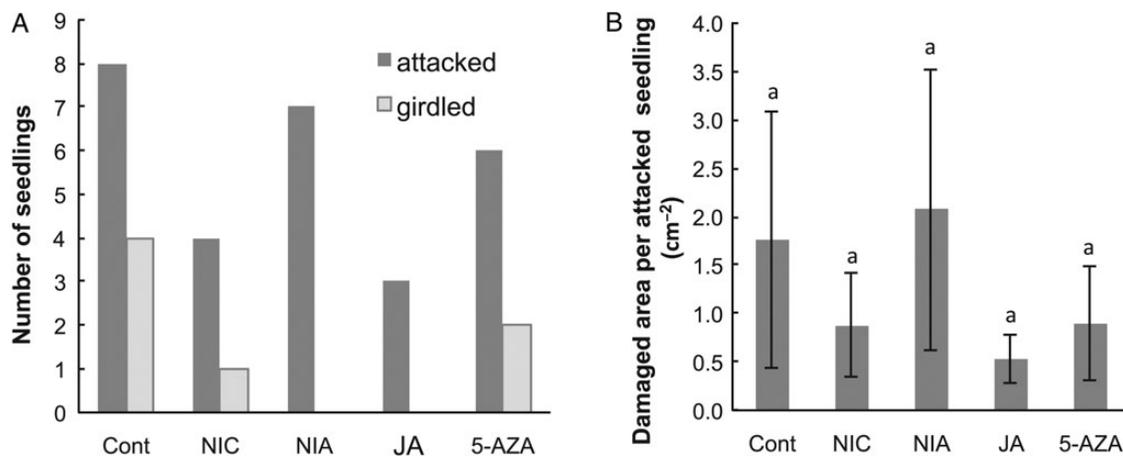
Figure 2 Attack by pine weevils on 6-month-old spruce seedlings in a field test. The graphs show the effects of seed treatment with water (Cont), 2.5 mM NIC, 2.5 mM NIA, 3 mM JA and 200 μM 5-Aza. Forty seedlings per treatment were examined. (A) Number of attacked and girdled seedlings. (B) Damaged area per attacked seedling; variance of treatment between the groups was not significant at the $P < 0.05$ level for any condition.

Table 4 ANOVA results for height of seedlings treated by watering

Seedlings height × treatments	Sum of squares	df	Mean square	F	Sig.
Between groups					
(Combined)	62.222	2	31.111	0.088	0.916
Linearity	6.667	1	6.667	0.019	0.891
Deviation from linearity	55.556	1	55.556	0.157	0.693
Within groups	30805.833	87	354.090		
Total	30868.056	89			

Heights of 1.5-year-old spruce seedlings at the time of field planting, after treatment via watering for 2.5 months. Variance of treatment between groups was not significant at the $P < 0.05$ level.

Figure 3 Heights of 1.5-year-old spruce seedlings at the time of field planting, after treatment via watering for 2 months. Seedlings were treated with water (Cont), 2 mM NIC or 2 mM NIA. Mean values based on 30 seedlings per treatment are shown. Error bars show standard deviation. Variance of treatment between the groups was not significant at the $P < 0.05$ level.

A major focus of research regarding induced defense against pine weevils in conifers has until now been concentrated on the effects of jasmonates sprayed onto plants (Holopainen *et al.*, 2009; Zas *et al.*, 2014). A recent field study showed that MeJA spraying of seedlings can promote defense against pine weevil attack in pine, and to a lesser extent also in spruce (Zas *et al.*, 2014). A major drawback of MeJA treatment via spraying appears to be a decreased plant growth, including decreased secondary growth, in conifer seedlings (Moreira *et al.*, 2012). However, watering of conifer seedlings with MeJA has also been studied and resulted in increased terpenoid levels, mainly in roots and stems (Huber *et al.*, 2005). An increased resin formation in stems has been shown to follow MeJA treatment, and stems or stem pieces from such plants presented to pine weevils are less extensively gnawed than stem pieces from untreated plants (Holopainen *et al.*, 2009; Moreira *et al.*, 2012; Zas *et al.*, 2014). However, an increased resin content of MeJA treated trees is considered a drawback regarding wood quality (Holopainen *et al.*, 2009). Interestingly, a study by Moreira *et al.* (2014) shows that in several pine species there is a trade-off between constitutive and inducible tissue non-volatile resin content, depending on geographical and climatic factors. With the seed treatment approach presented in
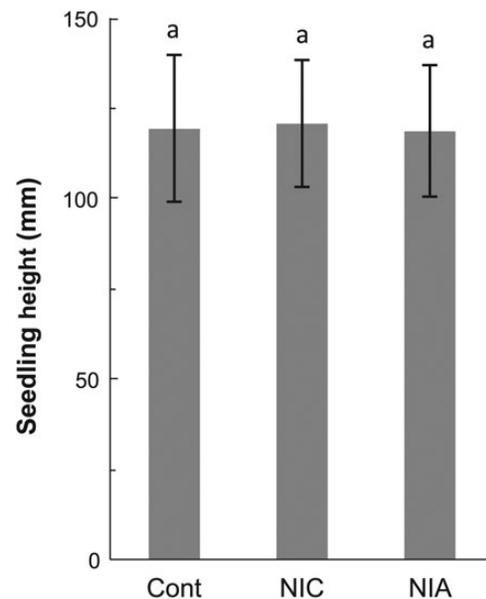
this report, we intended to develop a simple and mild treatment method that would have a minimal effect on growth. The indication that JA may be a defense-potentiating compound in spruce via seed treatment has to be investigated further in association with its metabolite, the long distance signal MeJA.

This study shows that seed treatment with the natural, non-toxic, and within this context novel, compounds NIC and NIA (also known as niacin or vitamin B$_3$), in addition to the better known defense-inducing compound JA, can promote defense against pine weevil attack in spruce. For practical use of the

Table 5 Binomial test of number of attacked and girdled seedlings treated by watering

	Category	N	Observed prop.	Test prop.	Exact Sig. (two-tailed)
Number of attacked × treatments					
Group 1	Nonattacked	134	0.81	0.50	0.000
Group 2	Attacked	31	0.19		
Total		165	1.00		
Number of girdled × treatments					
Group 1	Nongirdled	142	0.86	0.50	0.000
Group 2	Girdled	23	0.14		
Total		165	1.00		

Differences between nonattacked and attacked seedlings, and between nongirdled and girdled seedlings were significant at the $P < 0.05$ level.
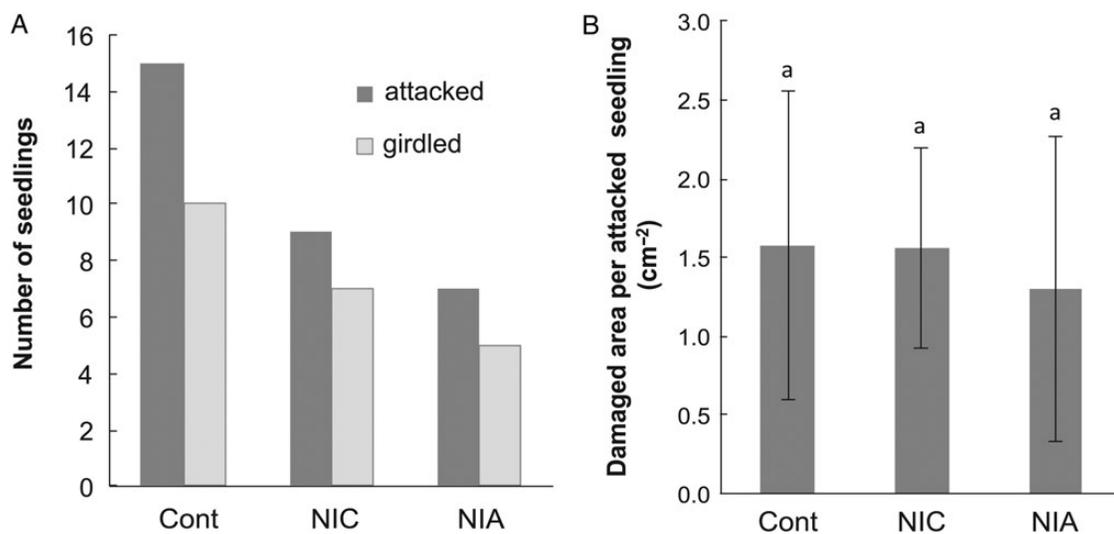

Figure 4 Attack by pine weevils on 1.5-year-old spruce seedlings in a field test after treatment of seedlings via watering for 2.5 months. Seedlings were treated with water (Cont), 2 mM NIC or 2 mM NIA. Fifty-five seedlings per treatment were examined. (A) Number of attacked and girdled seedlings. (B) Damaged area per attacked seedling; variance of treatment between the groups was not significant at the $P < 0.05$ level for any condition.

Table 6 ANOVA results for damaged area per attacked seedling treated by watering

Damaged area × treatments	Sum of squares	df	Mean square	F	Sig.
Between groups					
(Combined)	0.387	2	0.194	0.244	0.785
Linearity	0.301	1	0.301	0.378	0.543
Deviation from linearity	0.086	1	0.086	0.109	0.744
Within groups	22.252	28	0.795		
Total	22.639	30			

Damaged area per attacked seedling by pine weevils on 1.5-year-old spruce seedlings. Variance of treatment between groups was not significant at the $P < 0.05$ level.

compounds for spruce protection, toxicological evaluations have to be performed. Seed or seedling treatment with natural substances is an attractive alternative to the comprehensive use of toxic synthetic pesticides and genetically modified plants, although these strategies remain important.

It may be that the extent of pine weevil damage to seedlings depends on the attraction the insects feel towards the plants, as well as nutritional suitability of the stem tissue for feeding. We interpret the frequency of attacked plants as a measure of pine weevil attraction by volatile compounds, and the extent of damage as a measure of the tastiness of the stem cambium/ phloem for the weevils. The decreased number of attacked seedlings seen in the present study could be due to a lower attractiveness for pine weevils. The extent of girdling, which is detrimental to the seedlings, was lower in seedlings from treated seeds compared with the control in the present study. A low frequency of girdling may reflect that the insect, instead of staying at one place for continuous feeding, searches for a more tasty/attractive part of the stem. This behaviour would decrease the risk of girdling, even if the total area of damage may be considerable. This first trial regarding protection of spruce seedlings against pine weevil attack via seed treatment or watering only covers the first season in the field, a time which is decisive for the attractiveness of the plants to the
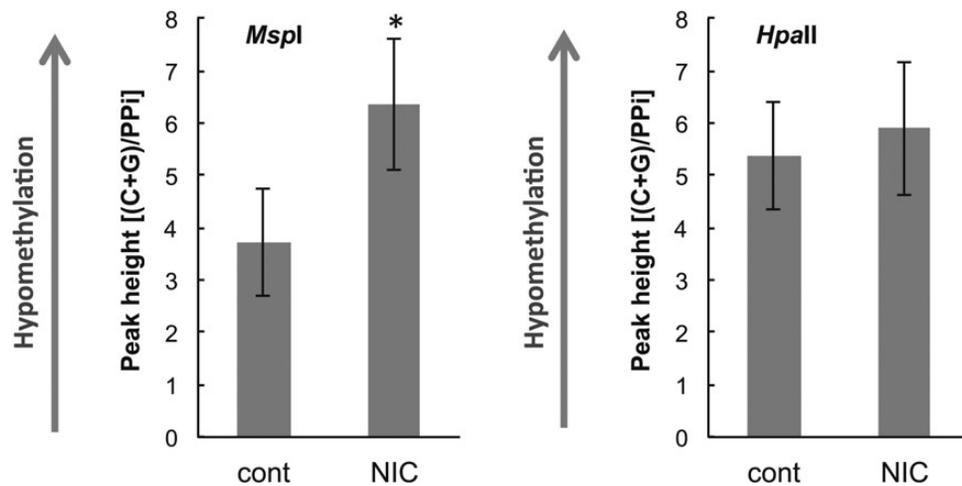
Figure 5 Effect of NIC (2.5 mM) seed treatment on DNA methylation changes in needles of 15-week-old spruce seedlings. DNA was cleaved by the restriction enzymes *Msp*I and *Hpa*II and analyzed by LUMA. A higher value for $[(C + G)/PP_i]$ ratio corresponds to a lower degree of DNA methylation. An asterisk (*) corresponds to a significant difference from the control at the $P < 0.05$ level, as determined by an unpaired t test.

insects, and thereby for the survival of the plants. Further optimization of the strategies outlined here, regarding the duration of seed treatment, the time period between treatment and sowing, the concentration of compounds used, and other factors, will hopefully lead to more improvements in seedling protection.

Therefore, the results of the present investigation should not be seen as a ready to use concept for conifer protection against pine weevils, but rather the opening of a door into new defensive strategies.

Conclusion

In the present study, we point at a new strategy for future research aiming at improved forest protection and environmentally friendly forestry. This investigation indicates that seed treatment and watering of young spruce seedlings with selected nontoxic plant compounds, especially NIC, can give protection against attack by pine weevils in the field. The results could point at a role for epigenetic mechanisms in this process. The results also support a potential importance of NIC and NIA as defense signal mediating compounds as originally suggested by Berglund (1994).

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Conflict of interest statement

None declared.

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