Protection of spruce seedlings against pine weevil attacks by treatment of seeds or seedlings with nicotinamide, nicotinic acid and jasmonic acid

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Introduction

Forestry resources in Northern European countries are commonly replenished by reforestation, where new seedlings are planted soon after clearcutting of an older area of forest. In 2012, this method was utilized on 73 per cent of the regenerated forestry area in Sweden. This required a total of 374 million planted seedlings, of which spruce (Picea abies (L.) Karst.) seeds were treated with 2.5 mM nicotinamide (NIC), 2.5 mM nicotinic acid (NIA), 3 mM jasmonic acid (JA) or 0.2 mM 5-azacytidine (5-Aza), and 6-month-old seedlings grown from these seeds were planted at a reforestation area in central Sweden. Attack by pine weevils (Hylobius abietis) was reduced by 50 per cent by NIC treatment, 62.5 per cent by JA treatment and 25 per cent by 5-Aza treatment, when compared with seedlings grown from untreated seeds. Watering 18-month-old spruce seedlings with 2 mM NIC or 2 mM NIA did reduce attack during the first season in the field by 40 and 53 per cent, respectively, compared with untreated plants. Girdling was also reduced by the different treatments. Analysis of conifer seedlings treated with 5-Aza points at a possible involvement of epigenetic mechanisms in this defensive capacity. This is supported by a reduced level of DNA methylation in the needles of young spruce seedlings grown in a greenhouse from NIC-treated seeds. Seed treatment for seedling defense potentiation is simple, inexpensive and also a new approach for forestry with many potential applications.
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 com-
pounds to the ecosystem. The application method has primarily
been by spraying or fumigation. Trials of MeJA-sprayed conifers in-
dicate 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 elici-
tors 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 B3 (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 in-
fluence 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 ana-
logues 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 mo-

cular signal is needed, and DNA must be accessible for interaction
with the signal. In eukaryotic cells, DNA is packed together with pro-
teins (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 expres-
sion, 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 tran-
scription factors, as well as unidentified epigenetic mechanisms
(Pastor et al., 2013).

It is well-known that seed treatment can influence the perform-
ance 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 tempera-
ture, 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 potenti-
ate spruce seedling defense against pine weevils via a short seed
exposure or via seedling watering with defense potentiating com-
pounds, 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 com-
pounds 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 com-
pounds 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 con-
taining 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 sown with treated seeds at
the research station in Vassbo (60° 32’ N; 15° 33’ E). Before sowing, the con-
tainer 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 treat-
ments. 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

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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 cm⁻²) on 18 October by visual assessment.

**Experiment with watering**

Untreated spruce seeds were sown in July 2009 in a commercial nursery, Nassja 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 21 cm² 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 x 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 (CCGG). If the inner C is methylated (C(CGG), 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–Wilks 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
seeding 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 MspI. This illustrates a general decrease in methylation at the outer cytosine in CCGG sites, corresponding to CXX positions in DNA. There was no difference in cleavage with HpaII between the control and treated samples, however, suggesting that the overall methylation at the inner cytosine in CCGG 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) (Schraufstatter 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 nicotinamide ribosyltransferase (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

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

### 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 x treatments** |     |               |           |                         |
| Group 1 Nonattacked | 28  | 0.14          | 0.50      | 0.000                   |
| Group 2 Attacked   | 172 | 0.86          |           |                         |
| Total             | 200 | 1.00          |           |                         |
| **Number of girdled x treatments** |     |               |           |                         |
| Group 1 Nongirdled | 7   | 0.04          | 0.50      | 0.000                   |
| Group 2 Girdled    | 193 | 0.97          |           |                         |
| Total             | 200 | 1.00          |           |                         |

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

| Damaged area x 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.

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 behavior (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 (Krasa 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 defense 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 signaling 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 signaling 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.
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, 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).

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 B3), in addition to the better known defense-inducing compound JA, can promote defense against pine weevil attack in spruce. For practical use of the
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 insects when searching for suitable food sources.

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        | 134 | 0.81          | 0.50      | 0.000                  |
| Group 2        | 31  | 0.19          |           |                        |
| Total          | 165 | 1.00          |           |                        |
| Number of girdled × treatments |     |               |           |                        |
| Group 1        | 142 | 0.86          | 0.50      | 0.000                  |
| Group 2        | 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.

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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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