

UV-B Exposure of Indoor-Grown *Picea abies* Seedlings Causes an Epigenetic Effect and Selective Emission of Terpenes

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Terpenoids are involved in various defensive functions in plants, especially conifers. Epigenetic mechanisms, for example DNA methylation, can influence plant defence systems. The purpose of the present study was to investigate the influence of UV-B exposure on the release of terpenoids from spruce seedlings and on needle DNA methylation. Ten-week-old seedlings grown indoors were exposed to UV-B radiation during 4 h, and the volatile compounds emitted from the seedlings were analysed. Analysis of the volatiles 1, 3, and 22 d after this UV-B exposure showed that bornyl acetate, borneol, myrcene, and limonene contents increased during the first 3 days, while at day 22 the level of emission had returned to the control level. UV-B exposure decreased the level of DNA methylation in needles of young seedlings, reflected in methylation changes in CCGG sequences. Exposure of young seedlings to UV-B radiation might be a way to potentiate the general defensive capacity, improving their ability to survive in outdoor conditions. UV-B-induced defence is discussed in the light of epigenetic mechanisms.

Key words: Defence, DNA Methylation, Volatiles

Introduction

Metabolites in plants are essential for defence and communication with the environment. Terpenes constitute the major part of the resin in conifers. The composition of terpenes as well as of volatile terpenes changes with tissue, age, and in response to various types of stress, for example insect or pathogen attacks (Persson *et al.*, 1993; Fäldt *et al.*, 2006) and UV-B exposure (Zavala and Ravetta, 2002). Jasmonic acid (JA) is a main signal-mediating compound in plants in response to mechanical and biotic stress, *e.g.* insect stress (Ballaré, 2011). Accordingly, treatment of conifers with methyl jasmonate (MeJA) increases the terpene emission as well as tissue levels of terpenes (Martin *et al.*, 2003). A change in terpene biosynthesis and emission is associated with increased defensive capacity in conifers against *e.g.* bark beetle (Franceschi *et al.*, 2005) and pine weevil (Heijari *et al.*, 2005). Interestingly, the response

to UV-B exposure in plants has similarities to the response to other types of stress, such as insect stress (Stratmann, 2003).

Excess UV-B radiation can cause direct damage to DNA and also increase the tissue level of reactive oxygen species (ROS), which in turn can damage DNA, proteins, and fatty acids in cellular membranes, as well as the photosynthetic apparatus. Accordingly, plants have to protect themselves from UV-B damage by production of UV-absorbing pigments, antioxidative metabolism, as well as DNA repair. The plant perception and signaling pathways in response to UV-B exposure are not completely known. Various signaling pathways including ROS, salicylic acid, JA, brassinolide, and ethylene have been suggested (A.-H.-Mackerness, 2000; Brosché and Strid, 2003; Sävenstrand *et al.*, 2004). Furthermore, we have earlier suggested that nicotinamide released from NAD during UV-B exposure, causing oxidative stress, could function as a signaling compound

in response to UV-B radiation (Berglund, 1994; Berglund *et al.*, 1996; Kalbin *et al.*, 1997).

There is a close connection between UV-B exposure and JA-based responses. Although UV-B radiation does not appear to increase the tissue levels of JA, UV-B exposure appears to increase the sensitivity of plants to JA-based signaling (Demkura *et al.*, 2010). Furthermore, antiherbivorous activity induced by UV-B radiation appears to be dependent on jasmonate signaling (Demkura *et al.*, 2010). UV-B exposure can also act in a synergistic manner with mechanical damage, with respect to defence mechanisms against herbivores (Stratmann, 2003), and maybe UV-B radiation could be considered a primer for JA signaling (Ballaré, 2011). To be prepared or primed means that a plant shows a more rapid, and maybe stronger, response when challenged by attackers or abiotic stress (Beckers and Conrath, 2007). Priming mechanisms could include epigenetic changes (Angers *et al.*, 2010; Jaskiewicz *et al.*, 2011) improving the availability of DNA for transcription.

Epigenetic modifications influence gene expression in eukaryotic cells. DNA encodes the genetic information. Together with proteins (histones), DNA is packed into chromatin. The way DNA is packed influences the availability of DNA for interactions with other molecules and transcription. Two main epigenetic mechanisms are DNA methylation and histone modification, which also influence each other. The pattern of DNA methylation can persist by a copying mechanism during many cell divisions and even lifelong. In plants these patterns may also be kept over generations. DNA methylation patterns can be influenced by the environment (Angers *et al.*, 2010; Law and Jacobsen, 2010; Sano, 2010; Verhoeven *et al.*, 2010). Epigenetics, reflected in DNA methylation, is closely associated with stress and defence in plants (Sano, 2010). Chromatin modifications may act as a memory for resistance (Jaskiewicz *et al.*, 2011). It is known that UV-B exposure can cause epigenetic changes in plants, particularly histone modifications (Fisher and Franklin, 2011). However, information on this topic is still limited in conifers.

Norway spruce [*Picea abies* (L.) Karst.] is frequently used for reforestation in Nordic countries. Generally it is grown in monoculture plantations, which increases the risk of comprehensive damage caused by various pests, for example the large

pine weevil (*Hylobius abietis* L.). To replace the environmentally toxic insecticides now used, new strategies are sought for which have to be non-toxic, efficient, cheap, and applicable in the form of seedling mass treatment.

A study by Danielsson *et al.* (2008) regarding spruce mini-seedlings (10-week-old seedlings) was pointing at an effect of outdoor growth relative to greenhouse conditions with respect to later protection against pine weevils during field growth. This raised the question whether UV-B exposure – missing in greenhouse – could influence the general defensive metabolism in spruce seedlings. A support for this theory is the well-known problem among growers of Norway spruce and Scots pine (*Pinus sylvestris* L.) concerning the stress response of seedlings moved from the greenhouse environment to outdoor storage. UV-B treatment in the greenhouse might be a way to improve the conifer seedling defensive capacity.

In this study we investigated the effect of UV-B exposure on the composition of terpenoid volatiles emitted from young spruce plants. We also analysed the effect of UV-B exposure on DNA methylation in needles from spruce seedlings.

Material and Methods

Plant material

The Norway spruce seedlings were produced from seeds derived from selected high-quality trees in a seed orchard where the mean location of the original sites of the trees was 59°12' N, 160 m above sea level. Seeds were sown in a greenhouse at Garpenberg, Sweden (60°15' N, 16°15' E) at the end of April 2008 in Jiffy 18 containers (Jiffy Products Sweden AB, Älmhult, Sweden; container volume, 13 ml; 1800 seedlings m⁻²). During germination the relative air humidity was kept at 75% and the air temperature at 22 °C. Fertilizing started 2 weeks after sowing and continued until 7 weeks after sowing, when the seedlings were delivered to KTH, Stockholm, Sweden, for further treatment. The fertilizer (N:P:K 100:13:65, w/v; Wallco; Cederroth International AB, Upplands Väsby, Sweden), a complete mineral nutrient solution, was added twice a week in the irrigation water rendering a total nitrogen supply of 24 g m⁻².

The experiments were performed when the seedlings were 10 weeks old.

Light conditions and UV-B exposure

The seedlings were kept at 23 °C in an 18 h light/6 h dark cycle under visible light ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$) from three fluorescent tubes (Philips SYLVANIA 840; Alfalux, Stockholm, Sweden) placed 33 cm above the seedlings. UV-B condition was achieved by complement with one UV-B tube (Philips TL 20W/12 RS SLV; Alfalux), giving an irradiance of 0.4 W m^{-2} between 250 and 320 nm. After UV-B exposure for 4 h, the seedlings were kept under control light conditions until analysis. Control seedlings were exposed to normal visible light without UV contribution during the entire experiment.

Volatile substances

Volatile substances were collected at different time points after the end of the UV-B exposure as shown in the Results section. Whole seedlings were used for the analysis. To enhance the amount of volatiles to detectable levels, groups of seven of the control seedlings and of the UV-B-exposed seedlings, respectively, were placed in two separate 500-ml glass beakers. The collection of volatiles from each group of seedlings lasted for 20 h, in order to avoid circadian effects. The results can be regarded as a mean of seven seedlings; thus individual differences are not considered, which may explain why the control levels in some cases differ between the groups of seven seedlings.

The volatiles released by the plants were collected by solid-phase micro-extraction (SPME) (fiber coating of $65 \mu\text{m}$ of polydimethylsiloxane/divinylbenzene; Supelco, Bellefonte, PA, USA). Before collection of the seedling volatiles, the fiber was heated in the GC injector for 10 min at 230 °C. The beakers were covered with aluminum foil, and the fiber was inserted ca. 2 cm above the seedlings. The amounts of the individual volatiles detectable by gas chromatography-mass spectrometry (GC-MS) ranged between 50 and 100 ng of a component, thus no saturation of the fiber was obtained and the collected quantities of the volatiles were in the linear range as discussed by Kännaste *et al.* (2013). After 20 h adsorption, the volatiles were analysed using an SSQ 7000 Finnigan GC-MS instrument (Termo Electron, Bremen, Germany) constituting a Varian 3400 gas chromatograph connected to a Finnigan SSQ 7000 mass spectrometer (electronic ionization, 70 eV; ion source temperature, 150 °C). A CB1 capillary col-

umn (30 m, 0.25 mm i.d., and 0.25 mm film thickness; Supelco) was used with a temperature program of 40 °C (1 min) increased with 4 °C/min to 190 °C (0.01 min) followed by a second increase of 10 °C/min to 230 °C (10 min), and with injector temperature at 215 °C and 30 s in split-less mode. Helium was used as the carrier gas.

DNA methylation

Seven d after UV-B exposure, seedlings to be analysed for DNA methylation were frozen at -20 °C. Needles from the seedlings were homogenized with a mortar and pestle under liquid nitrogen, and DNA was isolated with the DNeasy Plant Mini Kit from Qiagen AB (Solentuna, Sweden).

The analysis of DNA methylation was made by the luminometric methylation assay (LUMA) (Karimi *et al.*, 2006), using Pyro Gold Reagents (5 x 96 PSQ™ 96MA) purchased from Pyrosequencing AB/Biotage (Uppsala, Sweden) (later offered by Qiagen). The restriction enzymes *HpaII* and *MspI* cut the DNA in a methylation-dependent manner, and *EcoRI* was used as an internal indicator for DNA input. Obtained peak height for (C+G) addition in the Pyrosequencing part of the assay was proportional to cleavage by *HpaII* or *MspI* at CCGG sites and inversely proportional to the degree of methylation at this site. Peak height for A addition was proportional to cleavage by *EcoRI*. Values for (C+G)/A peak heights were used as a relative measure of DNA methylation.

Enzymes were purchased from New England BioLabs (Hitchin, United Kingdom).

Results

Norway spruce seedlings were exposed to UV-B light, after which the emission of volatile substances and changes in DNA methylation in needles were analysed.

Volatile substances

UV-B exposure resulted in elevated emission of terpenoids; the monoterpene hydrocarbons myrcene (retention time, 11.18 min), limonene (12.50 min), and borneol (17.28 min) and the monoterpene ester bornyl acetate (21.60 min). (-)-Limonene is dominating in spruce seedlings (Danielsson *et al.*, 2008). Enantiomers of borneol

could not be separated in this analysis. The ubiquitous α -pinene, β -pinene, and camphene did not increase compared to the above mentioned terpenes. Representative GC-MS chromatograms (total ion current) from control and UV-B-exposed seedlings are shown in Fig. 1.

Volatile collections from UV-B-exposed and control seedlings were made 1 d, 3 d, and 22 d after UV-B exposure (Fig. 2). The emissions of all four substances, bornyl acetate, borneol, myrcene, and limonene, increased 1 d after UV-B exposure, relative to the control. A further increase was observed after 3 d, but three weeks after treatment, emission of the volatiles had returned to normal.

DNA methylation

DNA methylation was analysed in needles from seedlings collected one week after UV-B exposure. It is important to point out that the method used gives a general picture of changes in DNA methylation and not a quantification of the methylation level. The method detects changes in methylation at 5'-CCGG-3' sites by use of the restriction enzymes *HpaII* and *MspI*, which can cleave DNA at these sites in a methylation-dependent manner. None of the enzymes can cleave the DNA strand if the outer C is methylated (*CCGG), while both can cut the DNA strand if CCGG is un-methylated. *MspI*, but not *HpaII*, can also cut if the inner C is methylated (C*CGG). Furthermore, *HpaII* can cut hemi-methylated DNA (Madlung *et al.*, 2002). Fig. 3 shows average changes in DNA methylation at 5'-CCGG-3' sites caused by UV-B exposure of the seedlings. *HpaII* cuts DNA from UV-B-exposed needles to a higher degree than DNA from control needles, which is indicative of a decreased DNA methylation in UV-B-exposed seedlings. Even though the result obtained with *MspI* cleavage may indicate an increased methylation after UV-B exposure, this difference is not so pronounced. Considering the differences between the restriction enzymes used, the result indicates that on the average, the inner C (corresponding to CG methylation) had been demethylated in response to UV-B exposure, while we cannot draw any conclusion about methylation of the outer C (corresponding to CXG methylation).

Discussion

The present investigation revealed that UV-B exposure of indoor-grown young spruce seedlings caused an increased emission of the volatile terpenoids bornyl acetate, borneol, myrcene, and limonene, compared to nonexposed seedlings. The dominating compound bornyl acetate is a typical substance in extracts and volatile emissions from spruce needles, and increases together with other monoterpenes, *e.g.* limonene, in needles (Martin *et al.*, 2003) as well as in bark and wood (Martin *et al.*, 2002) in MeJA-sprayed young spruce plants. There was also an increase in bornyl acetate in needle extracts from MeJA-sprayed pine (*Pinus pinaster*) plants (Sampedro *et al.*, 2010). These changes remind of the response in our UV-B-exposed spruce seedlings, and suggest a possible connection between UV-B exposure and jasmonate signaling. Limonene is known as a repellent (Nordlander, 1990) and bornyl acetate as an antifeedant (Klepzig and Schlyter, 1999) to the large pine weevil. Similar to the increase in myrcene emission after UV-B exposure in our study, MeJA spraying caused increased myrcene emission in Scots pine (Holopainen *et al.*, 2009) and in Norway spruce (Martin *et al.*, 2003), while in *Pinus pinaster*, MeJA spraying resulted in a decreased content of myrcene in the needles (Sampedro *et al.*, 2010).

The effect of UV-B exposure in conifers has been investigated in an outdoor long-term study (Turtola *et al.*, 2006). An increase of 30% of ambient UV-B radiation did not exert significant changes in extracted phenolics or terpenes. In contrast to that study, we exposed very young indoor-grown seedlings to strong UV-B radiation during a short period of time. To achieve an effect of UV-B exposure on frost hardiness and heat tolerance in young conifers, a considerable increase in UV-B radiation relative to ambient level was needed (L'Hirondelle and Binder, 2005). In line with this, it was shown that enhanced long-term UV-B exposure of outdoor-grown Norway spruce alleviated the negative effects of a prolonged drought period (Trošt Sedej and Gaberščik, 2008).

UV-B effects on DNA methylation in plants have been sparsely reported. Nevertheless, effects on histones and a general chromatin remodelling effect have been observed in maize (Casati *et al.*, 2008) and *Arabidopsis thaliana* (Fisher and Franklin, 2011; Lang-Mladek *et al.*, 2010). Analysis of

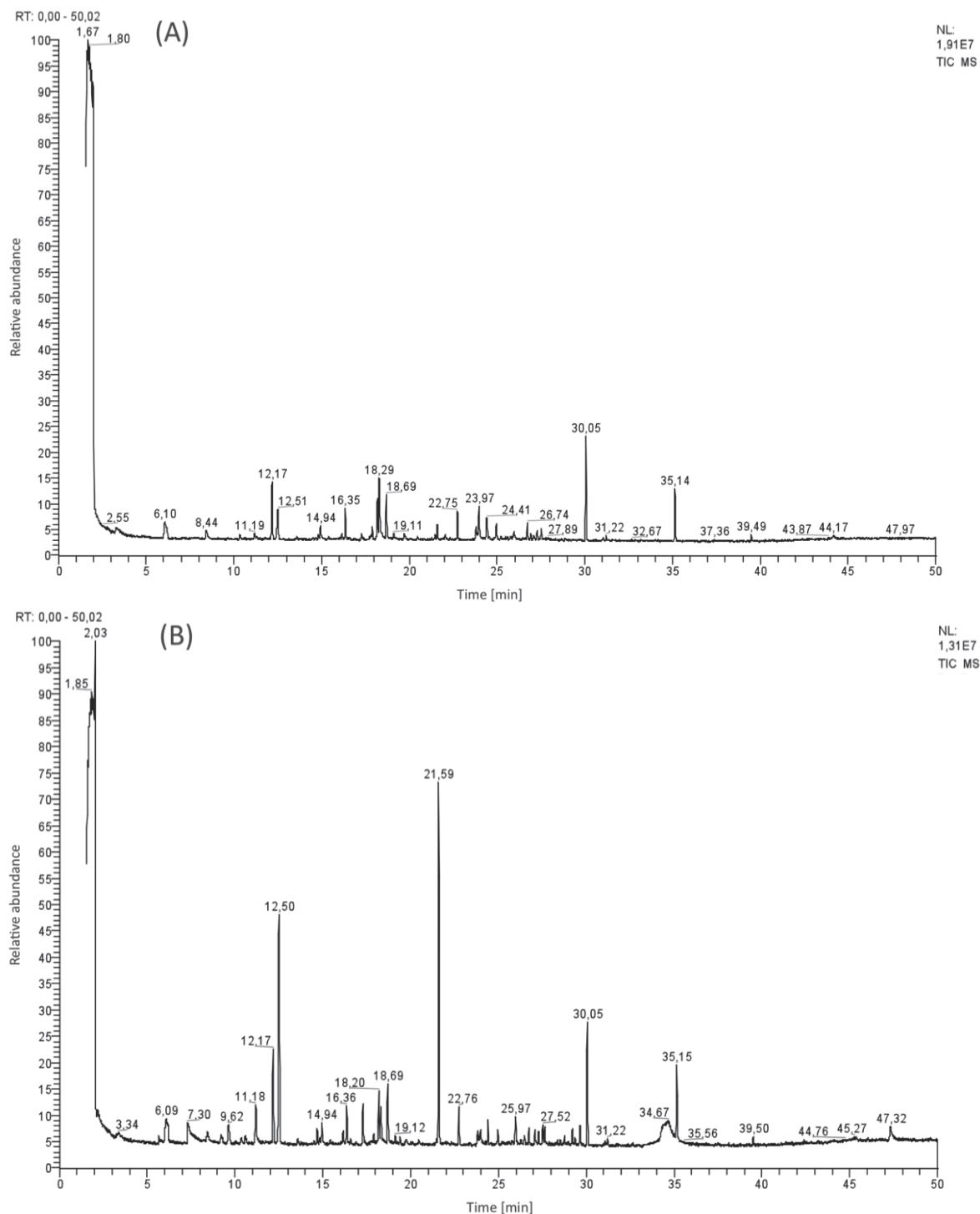


Fig. 1. Representative GC-MS chromatograms of the volatiles released from spruce seedlings, collected by solid-phase micro-extraction (SPME) 1 d after UV-B exposure. (A) Control seedlings; (B) UV-B-exposed seedlings. Retention times for investigated compounds: myrcene, 11.18 min; limonene, 12.50 min; borneol, 17.28 min; and bornyl acetate, 21.60 min.

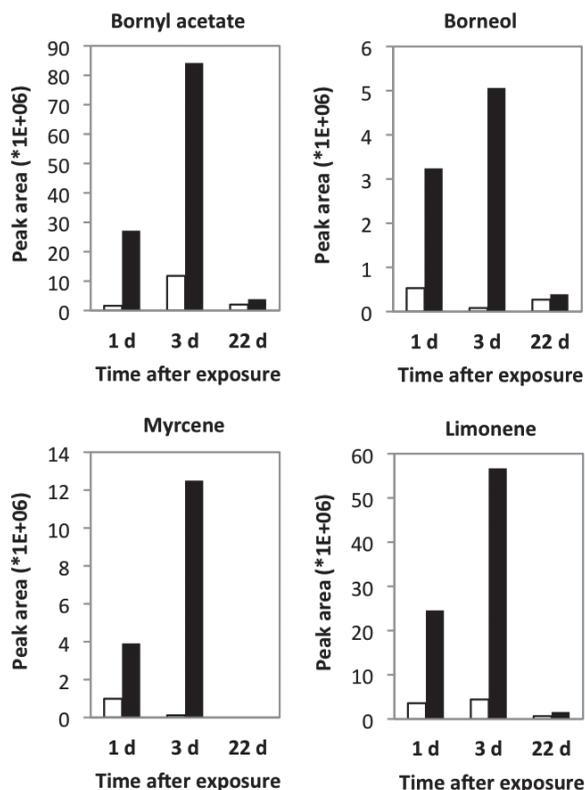


Fig. 2. Peak areas from GC-MS chromatograms of volatiles released from spruce seedlings. UV-B exposure lasted for 4 h. Volatile collection started 1 d, 3 d, or 22 d after UV-B exposure. □ Control; ■ UV-B exposure.

DNA methylation in one of these studies showed no effect (Lang-Mladek *et al.*, 2010). However, certain genes involved in epigenetic regulation, for example DNA methylation, were strongly upregulated in *Pinus radiata* 24–72 h after UV-B exposure (Valledor *et al.*, 2012). From studies in human leucocytes it is known that UV-B exposure can cause a decrease in global DNA methylation, despite the absence of a change in DNA methyltransferase gene expression (Wang *et al.*, 2009).

One purpose of our study was to explore the possibility that the degree of DNA methylation can be influenced by exposure to UV-B levels causing changes in the volatile emission. The method used for DNA methylation analysis detects methylation changes within the CCGG sequence (Karimi *et al.*, 2006). Animal cells are preferentially methylated at C in CG positions.

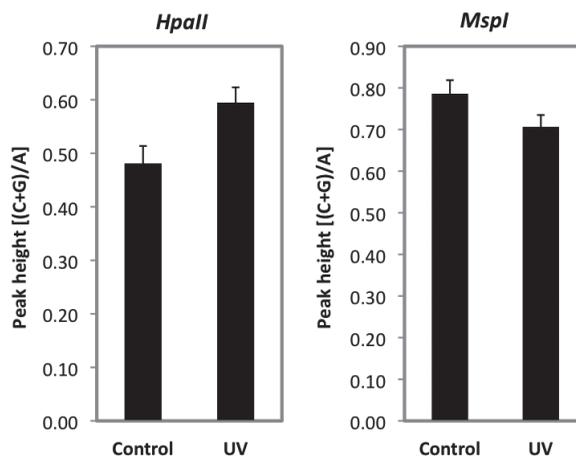


Fig. 3. Changes in DNA methylation of CCGG sites in needles from UV-B-exposed spruce seedlings, 7 days after a 4-h UV-B exposure. A higher value corresponds to a lower methylation level.

In addition, plants are also methylated in CXG positions, where X denotes any base. For example, in *Arabidopsis thaliana* approximately 20 to 30% of the 5'-CCG-3' trinucleotides were reported to be methylated at the outer C, and approximately 20% at the inner C (Jeddeloh and Richards, 1996). LUMA analysis of DNA cut by *HpaII* reflects changes in both CG and CXG methylation, while the results from *MspI* cleavage only reflect changes in methylation of the outer C in CCGG corresponding to a CXG sequence. Thus, our results indicate a general decrease in DNA methylation in CG (*HpaII* cleavage), but no clear change in CXG methylation (*MspI* cleavage). Our study shows that strong UV-B exposure of young spruce seedlings causes a pronounced increase in terpene emission, accompanied with a decrease in CG methylation in their DNA.

UV-B exposure can increase the resistance of plants against various types of stress, *e.g.* pathogens and herbivorous insects (Stratmann, 2003). There is a considerable overlap at the level of gene expression between UV-B radiation and insect-related stress, respectively (Izaguirre *et al.*, 2003). A synergistic interaction has been observed between UV-B exposure and drought regarding stress tolerance (Caldwell *et al.*, 2007). Accordingly, if the natural UV-B radiation is filtered out from outdoor living plants, there is a decrease both in secondary metabolite levels and their resistance to plant feeding insects (Zavala *et al.*,

2001; Caputo *et al.*, 2006). This exposure situation, *i.e.* lack of the UV-B part of the sun spectrum, can be found in many greenhouses, such as conifer nurseries. It has been shown in maize that there is a very different response to UV-B radiation between greenhouse-grown and field-grown plants (Casati *et al.*, 2011). We hypothesize that the lack of UV-B exposure in the greenhouse could be a drawback regarding the ability of very young spruce plants to cope with the outdoor environment, including insect attack. There is also a possibility that plants from the nursery can improve their general defensive capacity when treated with above ambient levels of UV-B radiation. Ambient or increased levels of UV-B radiation could speculatively promote the accumulation of secondary metabolites like terpenoids and phenolic compounds in the phloem or cambial tissue of their stems. This could in turn be important for their defensive capacity. In this way seedlings may be better prepared for the environmental change from greenhouse to outdoor growth in forest tree nurseries and in the field.

In future studies we will investigate the role of epigenetic mechanisms in the establishment of conifer resistance to biotic and abiotic stress as well as a role of terpenes in this process. Furthermore, we speculate that natural UV-B exposure is a basic generator of defence in trees. This would be a way to improve the general defensive capacity of greenhouse-grown plants during their time in the greenhouse and prepare them for outdoor conditions. We think that, from a technical point of view, UV-B exposure is a relatively simple procedure compared to many other treatments used for defence improvement in conifers.

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