



Defence reactions of mature Norway spruce (*Picea abies*) before and after inoculation of the blue-stain fungus *Endoconidiophora polonica* in a drought stress experiment

Sigrid Netherer*, Magdalena Ehn, Emma Blackwell, Thomas Kirsits

Institute of Forest Entomology, Forest Pathology and Forest Protection, University of Natural Resources and Life Sciences, Vienna, Hasenauerstraße 38, A-1190 Vienna, Austria

Abstract

We performed an inoculation experiment using the blue-stain fungus *Endoconidiophora polonica* at the Rosalia Roof study site, which was set up to study drought effects on Norway spruce susceptibility to attacks by the European spruce bark beetle *Ips typographus*. Tree resistance parameters such as resin flow rate and extent of hypersensitive wound reaction in the phloem were investigated prior to and after fungal infection. Sample trees with different drought treatments (trees fully covered or semi-covered by roofs, control trees) were inoculated with *E. polonica* in July 2014. In order to check for areas of phloem necrosis, the outer bark around the inoculation holes was scratched off 6 weeks later. We recorded the amount of resin exudation within 12 hours overnight in August and September 2013 and 2014. Although wound reaction zones did not differ in size between tree collectives of the various treatments, fully covered trees tended to exhibit larger necrotic areas compared to control trees. The least water supplied trees showed lowest resin flow rates prior to fungal inoculation, but were the only group to show significantly enhanced resin flow five weeks after the evaluation of defence reactions. Our results agree with earlier findings that wounding and few fungal inoculations can increase tree resistance in the medium term given not too severe water stress. Further investigations will clarify how water stress affects the availability of non-structural carbohydrates for secondary metabolism, when depletion of resources eventually occurs, and at which point trees are most susceptible to bark beetle attack.

Key words: *Ips typographus*; conifer-pathogen interactions; tree resistance; resin flow; hypersensitive wound reaction

Editor: Tomáš Hlásny

1. Introduction

Conifer forests are increasingly prone to drought-induced physiological stress forcing up predisposition to bark beetle (Coleoptera, Curculionidae, Scolytinae) infestation (Marini et al. 2012; Gaylord et al. 2015; Seidl et al. 2015). Scolytines have adapted to their host trees, and vice versa, over a period of more than 100 million years (Hulcr et al. 2015). The majority of bark beetle species mainly attack sub-vital, dying or recently dead trees, thereby contributing to forest regeneration and succession (Martikainen et al. 1999; Winter et al. 2015). Some more aggressive species such as the European spruce bark beetle, *Ips typographus*, show the potential to colonise and kill still vitally defending Norway spruce (*Picea abies*) trees in the course of outbreaks, which are mainly provoked by extreme weather events (e.g. storms causing large-scale windthrows, prolonged drought and heat periods). Attack success of *I. typographus* is supported by mass aggregation via pheromones, high tolerance towards secondary metabolites secreted by host trees, and frequent association with the ascomycete fungus *Endoconidiophora polonica* (former name *Ceratocystis polonica*; de Beer et al. 2014). This species is mainly associated with *I. typographus* and some of its phoretic mites, but to a varying degree also with other spruce bark beetles (Kirsits 2004). It causes blue-stain in the sapwood due to its hyphae growing preferentially in wood

rays. *E. polonica* speeds up tree death through impairing and destroying the trees' vital functions of water and nutrient transport, by killing phloem and cambium, and causing occlusion of the tracheids (Kirsits 2004; Francheschi et al. 2005). The scolytines profit from a more rapid exhaustion of constitutive defences and the suppression or exhaustion of induced resistance of the host trees supported by the fungus (Christiansen et al. 1987; Rosner and Hannrup 2004; Lieutier 2004; Lieutier et al. 2009). *E. polonica* can be more easily manipulated for experiments on live trees than *I. typographus* beetles themselves. The fungal species therefore has been widely used to study the resistance of Norway spruce and its defence mechanisms in the phloem and xylem in low density and mass inoculation experiments (e.g. Christiansen & Glosli 1996; Krokene et al. 1999; Baier et al. 2002; Zhao et al. 2011; Skrøppa et al. 2015).

The efficiency of constitutive defence in conifers is largely determined by the density of the resin duct system and the intensity of resin exudation (Lieutier 2004; Boone et al. 2011). In Norway spruce, radial resin canals cross several layers of wood cells and directly lead to the secondary phloem, where *I. typographus* establishes its broods. Running vertically, the larval galleries cut the resin ducts, the repellent effects of which increase with size, number and total area (Baier et al. 2002; Rosner and Hannrup 2004). In addition, conifers are equipped with an extensive arsenal of

*Corresponding author. Sigrid Netherer, e-mail: sigrid.netherer@boku.ac.at, phone: +43 1 3686352 26

chemical, protein-based and anatomically inducible defences, activated in response to attack or wounding (Eyles et al. 2010). Encapsulating invading biotic agents and sealing wounded tissue, secondary metabolites such as terpenoids and phenolic compounds show also highly toxic effects on invading organisms (Franceschi et al. 2005; Mason & Singer 2015).

Constitutive and inducible defence characteristics of individual conifer trees are genetically determined and further depend on tree age, growing conditions, as well as past and present states of tree stress (Lombardero et al. 2000; Baier et al. 2002). Disturbances in the course of natural disasters such as storm or avalanches, which cause strong vibrations or heavy wounding, enhance the production of resin and terpene-related compounds in the bark (Baier 1996; Knebel et al. 2008; Blackwell 2011). While for slightly impaired trees a positive trade-off between primary and secondary metabolism is suggested (Bryant et al. 1983; Herms & Mattson 1992; Lorio et al. 1995), disposable carbon reserves required for repair and maintenance of life-sustaining mechanisms are increasingly depleted in case of severe stress (Niemets 2010). Such link between advanced physiological stress and limited constitutive defence capability in terms of less resin flow was recently demonstrated in response to drought-manipulation in stands of *Pinus edulis* (Pinyon pine) and *P. abies* (Gaylord et al. 2013; Netherer et al. 2015). Drought stress positively or negatively influences the capacity of conifers to prevent the spreading of microorganisms after invasion via hypersensitive wound reaction, which is characterised by the formation of necrotic areas and impregnation of tissues with resinous and phenolic compounds (Christiansen et al. 1999; Novak et al. 2014). Mild water deficiency causing only moderate stress even enhanced tree resistance of pines (*P. yunnanensis*) in response to inoculation of a bark beetle associated fungal pathogen (*Leptographium yunnanense*), which was indicated by limited fungus extension and reaction zone length in the phloem (Sallé et al. 2008). If the fungus however cannot be enclosed and killed rapidly enough due to severe tree stress, the first biochemical barrier is broken and the tree needs to extend reaction zones (Christiansen et al. 1987). Induced resistance in consequence of attack or wounding is argued to be even more influenced by resource limitation. Extended drought stress lasting several years eventually involves structural changes such as a reduction in number and size of resin ducts (Ferrenberg et al. 2015). Trees that are more resistant show significantly higher terpene levels following inoculation of *E. polonica* in the dark coloured zone around the entrance hole penetrated by fungal hyphae (Zhao et al. 2011). Concentrations and composition of particular phenols can even predict Norway spruce resistance to inoculation of *E. polonica* as shown by Brignolas et al. (1995, 1998). Interestingly, pre-treatment of spruce trees with this blue-stain fungus can itself enhance defence capacities via the formation of traumatic resin ducts and activation of polyphenolic parenchyma cells (Krokene et al. 2003).

Here, we present the results of an inoculation experiment with *E. polonica* performed at the *Rosalia Roof* study site in Lower Austria (Netherer et al. 2015), which was established to study the effects of drought on Norway spruce susceptibility to attacks by *I. typographus*. We assumed that our sam-

ple tree collectives differing in the extent of water deprivation would show variations in constitutive resistance as well as induced defence reactions prior to and after infection by *E. polonica*. For the trees fully sheltered by roofs, we expected the least capacity to defend the fungus, leading to the largest hypersensitive reaction zones and lowest amounts of resin exudation. We were further interested in the degree of correlation between the measured constitutive and induced defence reactions. Do higher constitutive resin reserves promote a more rapid control of the fungus, and is the induction of resin flow in response to fungal infection linked to the extent of hypersensitive wound reaction?

2. Material and Methods

2.1. Research site, field experiment, meteorological and soil water content measurements

The research area, a 90-year-old Norway spruce (*P. abies*) stand admixed with European beech (*Fagus sylvatica*), is located in the Rosalia mountains of Lower Austria at an elevation of about 700 m above sea level. Mean annual precipitation (1990–1999) amounts to 796 mm, mean annual temperature (1990–1999) to 6.5 °C (Gasch 1985). Air temperature and precipitation data of the study years 2013 and 2014 were recorded at a weather station set up at in an adjacent clearing. The experiments were performed at the *Rosalia Roof* drought-manipulation site that is described in detail by Netherer et al. (2015). Established at 1.20 m height from the ground in June 2012, the roof construction comprises six plots sized 18 m x 20 m within 3 different treatments: full cover, FC (plots P1, P4), semi cover, SC (plots P2, P3), and control, C (plots C1, C2). The drought manipulation study involved periodic recordings of soil volumetric water content (% SWC) in the main rooting horizons (0–30 cm and 0–60 cm soil depth) at the six plots using a portable Time Domain Reflectometry (TDR) measurement unit as described by Netherer et al. (2015). In this previous study, the sample trees located in the centre of the plots were periodically investigated for pre-dawn twig water potential by use of a pressure chamber (Scholander) during three seasons (2012, 2013, 2014). Here, we selected six Norway spruce trees per treatment, in total 18 trees, as illustrated in Fig. 1. The covered trees were located close to the edge but still influenced by the roofs.

2.2. Wound reaction of the sample trees following inoculation with *E. polonica*

A strain of *E. polonica* (accession no. RW/35/5-SHT of the fungal culture collection of IFFF-BOKU = CBS 109247 [Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands]), isolated in July 1997 from stained sapwood of a Norway spruce log that had been inoculated with an *I. typographus* beetle from Rothwald in Lower Austria about five weeks earlier (Grubelnik 1998), was used in the present experiment. The isolate was grown for about

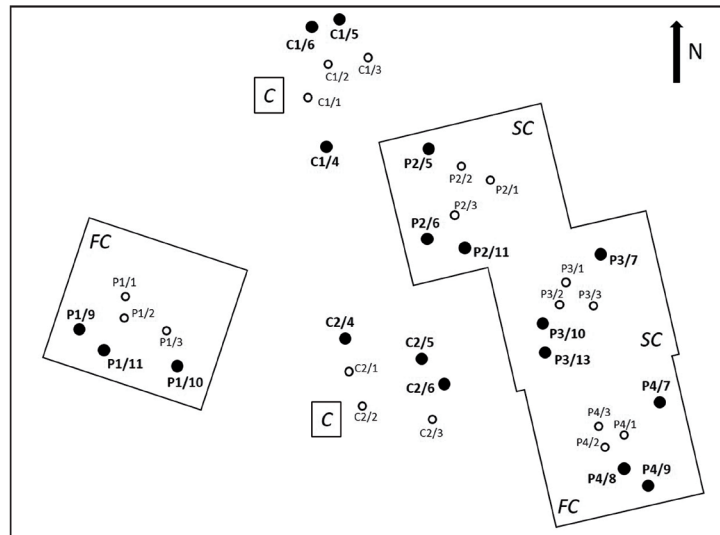


Fig. 1. *Rosalia Roof* study site comprising plots fully covered (FC) and semi-covered (SC) by rainout shelters, and control (C) plots. Norway spruce trees marked by black circles were investigated in this experiment; recordings for trees numbered 1–3 (white circles) in the centre of each plot were reported in Netherer et al. (2015).

seven weeks on ash leaf malt extract agar (AMEA, Kirisits et al. 2013, but without streptomycin sulphate) in 5.2-cm-diameter plastic Petri dishes. Sterile AMEA was used as control treatment. Wound reaction was tested on 6 FC, 4 SC and 6 C trees, two SC trees were left out in this experiment due to time constraints. On July 9, 2014, each of this 16 sample trees received eight inoculations with *E. polonica* and two control inoculations, which were evenly distributed in two rings at stem heights of 130 cm and 180 cm. In each ring, the four fungal inoculations were orientated towards the cardinal directions (N, E, S, and W), while the control inoculations were exposed to NE (at 130 cm stem height) or SW (at 180 cm stem height). With a 7-mm cork borer, holes were punched into the bark (up to the sapwood surface), in which inoculum was inserted, and the wounds were resealed with the bark plugs. Inoculum consisted of pieces cut with a 6-mm cork borer from fungal cultures or sterile AMEA. Between inoculations, instruments were flame-sterilised using alcohol and a Bunsen burner. Six weeks after inoculation, on August 26, 2014, wound reactions of the sample trees in response

to fungal and control inoculations were recorded. The outer bark around the inoculation holes was carefully scratched off, and the outlines of the reaction zones were sketched on transparent foils with a marker. The scanned sketches were subsequently analysed using DatInf Measure (ver. 2.2.2.48; DatInf GmbH, Tübingen) for the darker necrotic area formed by dead tree tissue and the lighter resin impregnated zone surrounded by wound periderm (Fig. 2). Mean areas in cm² of both zones summing up to total hypersensitive reaction area were calculated per tree, separately for fungal and control inoculations.

2.3. Resin flow rate

The amount of resin exuded within 12 hours overnight (19:00 to 7:00) was recorded at all 18 sample trees (6 FC, 6 SC, 6 C trees) in the year prior to the inoculation experiment, on two dates (August 20–21 and September 25–26, 2013). Measurements were repeated six weeks after fungal inocu-

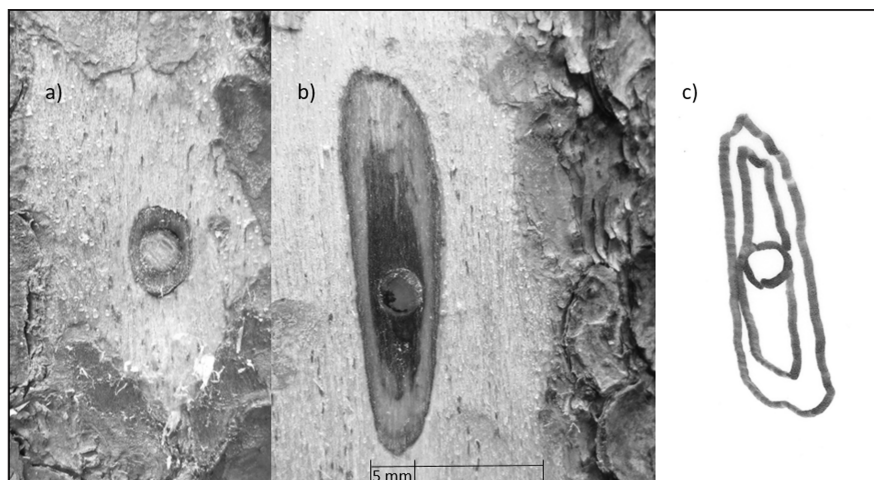


Fig. 2. Hypersensitive wound reaction in the bark of *Picea abies* following inoculation of (a) sterile AMEA, (b) the blue-stain fungus *Endoconidiophora polonica*; (c) sketch of reaction zone on transparent foil dividing necrotic from resin impregnated area.

lation, at the time secondary wound reactions were checked (August 26 – 27, 2014), and again five weeks after the evaluation of secondary defence reactions (September 30 to October 1, 2014). With a 4-mm cork borer, holes were punched into the bark on opposite stem sides at breast height (1.3 m above ground level). Glass tubes of 3 mm inner diameter were inserted, and vial sections filled with resin were recorded the next morning. Resin flow rate per tree was calculated as $\text{mm}^3 \text{resin hour}^{-1}$ averaged for both stem positions.

2.4. Statistical analyses

Statistical analyses were performed with IBM SPSS STATISTICS (ver. 19; IBM Corp., Armonk, NY, USA). Gaussian distribution of data was evaluated after Kolmogorov-Smirnov and Shapiro-Wilk, homogeneity of variance using the Levene-test. Paired-sample T tests were used to examine whether hypersensitive wound reaction caused by *E. polonica* and control inoculations as well as necrotic and resinous areas of the wound reaction zones generally differed in size. For the further analyses, data on hypersensitive wound reaction caused by *E. polonica* and resin flow was pooled according to plots or treatment. Overall effects of treatment (independent variable) and recording date (repeated factor) on resin flow were tested by MANCOVA. One-way analysis of variance (ANOVA) was used to test for differences in resin flow rates and reaction areas between plots and treatments (FC, SC, C) and differences in resin flow rates between different measurement dates within treatments. Means for treatments and dates were post hoc compared using the Bonferroni test. We investigated diverse relationships of resin flow rates and hypersensitive wound reaction using Pearson's correlation (two-tailed) and linear regression analyses.

3. Results

3.1. Weather, soil water conditions and tree water status

The study seasons 2013 and 2014 resembled each other in May to September mean air temperatures (15.5°C , 14.5°C) and precipitation sums (390 mm, 385 mm); yet, particularly

over the midsummer months, weather conditions differed between the years (Fig. 3). July and August mean temperatures were substantially high in 2013 (19.9°C , 18.9°C) and coupled with almost no rainfall in July (8.4 mm), while monthly air temperature means remained moderate in 2014 (July 18.3°C , August 15.9°C).

Simulated drought at the *Rosalia Roof* study plots started in June 2012. Roofing resulted in a significant reduction of soil volumetric water content and highly stressed sample trees located in the centre of the fully closed plots (average pre-dawn twig water potentials of -1.7 MPa in July 2013; comp. Netherer et al. 2015) in summer 2013. As shown in Fig. 3, SWC levels constantly remained on lowest levels (7 – 8%) for FC variants also during the season 2014, while soil water supply at SC and C plots fluctuated in response to air temperatures and precipitation. Water status of the central trees ranged between mean values of -1.2 MPa for FC and -0.6 MPa for C trees in June 2014 (unpublished results).

3.2. Size of the wound reaction zones according to treatment

The trees responded to control inoculations with sterile AMEA in a very limited way, showing significantly smaller wound reaction areas (necrotic area $0.062 \pm 0.04 \text{ cm}^2$; resin impregnated area $1.408 \pm 0.13 \text{ cm}^2$) than those caused by fungal infection ($T = 5.395$; $df = 15$; $p \leq 0.01$). Inner parts formed by dead tree tissues in response to inoculations with *E. polonica* were much smaller than the lighter areas where fungal growth was stopped by resin ($2.16 \pm 1.5 \text{ cm}^2$ compared to $7.02 \pm 4.6 \text{ cm}^2$) (Fig. 4). This difference was significant ($T = -5.439$; $df = 15$; $p < 0.01$). Total hypersensitive wound reaction did not differ in size between the investigated tree collectives, neither between plots, nor between treatments. The lack of significant differences is partly due to high variability of tree reactions within treatments, in particular at the roofed plots. A trend is, however, visible, with FC trees showing largest ($10.69 \pm 3.4 \text{ cm}^2$) and C trees exhibiting smallest and most homogenous total wound reaction zones ($7.79 \pm 1.4 \text{ cm}^2$), while values for SC trees were in-between ($8.99 \pm 2.7 \text{ cm}^2$) (Fig. 4).

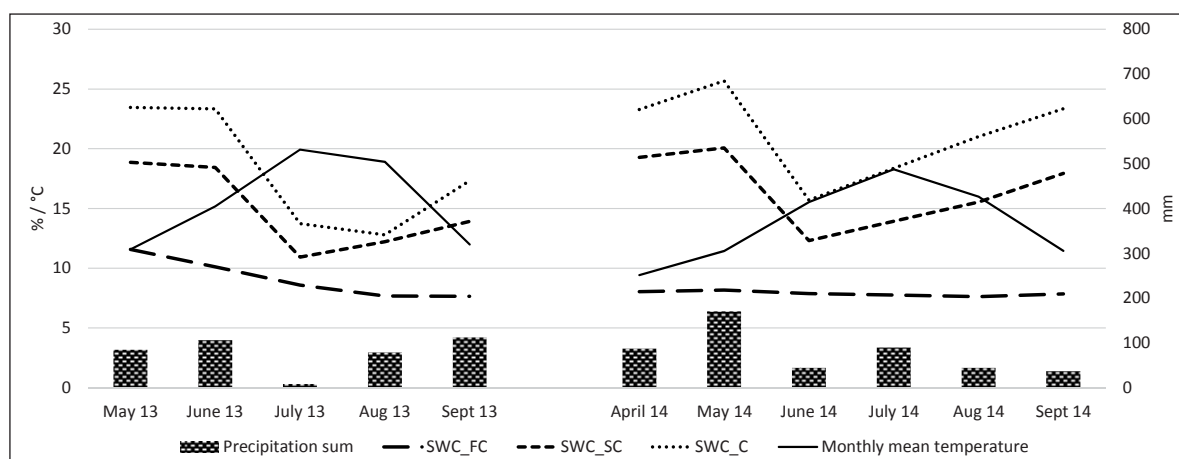


Fig. 3. Mean monthly volumetric soil water content (SWC) per treatment (full cover plots, FC; semi-cover plots, SC; control plots, C), monthly mean temperature ($^\circ\text{C}$) and monthly precipitation sum (mm) during the study seasons 2013 and 2014.

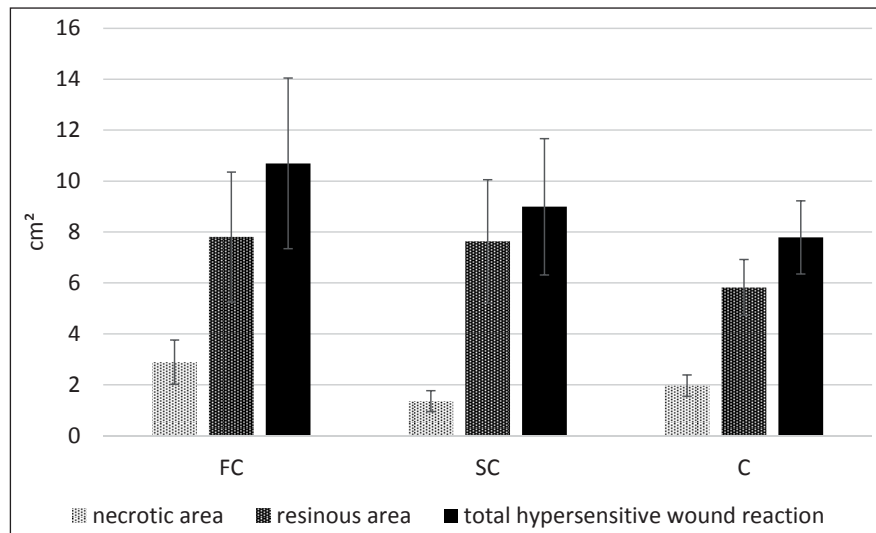


Fig. 4. Mean necrotic and resinous areas adding up to total hypersensitive wound reaction ($\text{cm}^2 \pm \text{SE}$) in the bark of *Picea abies* trees following inoculation with *Endoconidiophora polonica* according to treatment (full cover, FC; semi-cover, SC; control, C).

3.3. Resin flow rates according to date and treatment

Temperatures at the time of resin flow measurements (mean air temperature recorded between 22:30 and 7:00) were slightly lower in 2013 (August 20–21, 11.7 °C; September 24–25, 10.3 °C) compared to 2014 (August 26–27, 15.4 °C; September 30–October 1, 12.9 °C). Within treatments, resin flow did not differ significantly between any two plots on any recording date, which justified the pooling of data. Overall, MANCOVA showed that date of measurement did not affect resin flow significantly ($p=0.005$), nor did the interaction of treatment and date ($p=0.099$). On all sampling dates from August 2013 to August 2014, resin flow rates remained homogeneously low at FC and C trees (Fig. 5). Being significantly highest among all treatments on August 21, 2013 ($F=4.497$, $df=2$, $p=0.030$), SC tree resin flow rates showed higher variation and decreased continuously, yet not significantly, in the course of the study (Fig. 5).

In comparison (see Netherer et al. 2015), constitutive resin flow was similarly low for centrally located FC trees (P1/1-P1/3, P4/1-P4/3, 5.7 ± 7.1 and 6.5 ± 4.5 mm^3 resin hour^{-1} in August and September 2013, respectively). Yet, we did not see such correspondence among control trees (C1/1-C1/3, C2/1-C2/3, 17.3 ± 2.5 mm^3 resin hour^{-1} in September 2013).

In September 2014, eleven weeks after inoculation of *E. polonica* and five weeks after wounding due to evaluating defence reactions, a clear and significant increase in resin exudation at FC trees ($F=4.199$, $df=3$, $p=0.019$), but not in C and SC trees, set in. Considering all sampled trees, weakly or moderately positive, but not significant correlations were observed between resin flow rates measured in August 2014 and September 2013 (Pearson's $r=0.442$; $p=0.086$) or September 2014 (Pearson's $r=0.334$, $p=0.190$).

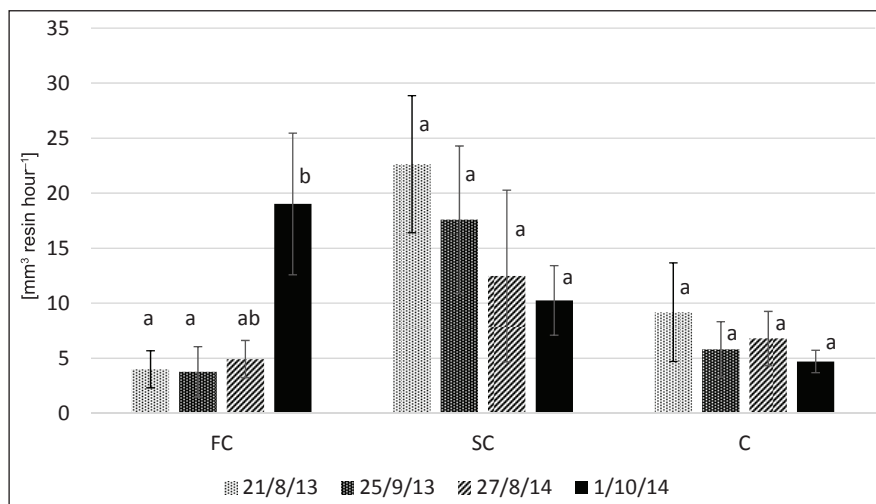


Fig. 5. Mean resin flow rate (mm^3 resin $\text{hour}^{-1} \pm \text{SE}$) at *Picea abies* trees according to treatment (full cover, FC; semi-cover, SC; control, C) prior to (August, September 2013) and after fungal inoculation (August, October 2014); different letters within treatments indicate significant differences in resin flow between dates of measurement.

3.4. Relationship between hypersensitive wound reaction and resin flow

Hypersensitive wound reaction was positively (though not significantly) correlated with August resin flow rates, and even significantly positive with those recorded in September (Table 1). This means that trees previously showing a strong response to fungal inoculation also increased resin flow rates after wounding due to evaluation of necrotic lesions in the bark. Pearson's correlation of difference in resin flow rates (delta RF) between September and August sampling dates and size of hypersensitive reactions, however, turned out to be low and not significant. Exclusion of the two outliers exhibiting either highest decrease (P2/11, SC) or strongest increase (P4/7, FC) in resin flow rate finally revealed clear linear relationships between delta RF and total wound reaction zones (Table 1, Fig. 6).

Table 1. Two-tailed Pearson's correlations (*significant, $p < 0.05$, **highly significant, $p < 0.01$) of total hypersensitive wound reaction (HWR) and resin flow rate (RF) recorded on two dates after fungal inoculation as well as change in resin flow rates between the measurement dates (delta RF; without outliers, delta RF_wo) in 2014.

RF	HWR
Six weeks after fungal inoculation: August 26/27, 2014	$r = 0.342$ $p = 0.194$
Eleven weeks after fungal inoculation: September 30/October 1, 2014	$r = 0.540^*$ $p = 0.031$
delta RF 2014	$r = 0.216$ $p = 0.422$
delta RF_wo 2014	$r = 0.814^{**}$ $p < 0.001$

4. Discussion

Exclusion of rainfall is a frequent approach to research chemical, physical and tree physiological effects of drought in forest ecosystems; yet, linkage to tree resistance and pre-

disposition to forest pests is rarely considered in such experiments (Anderegg et al. 2015). The *Rosalia Roof* experiment was the first drought manipulation study performed in a mature Norway spruce stand to correlate indicators of increasing water stress with susceptibility of trees to bark beetle attack. Increasingly negative pre-dawn water potentials of twigs and limited resin reservoirs to repel attacks enhanced both attractiveness of spruce trees for *I. typographus* and boring success of beetles (Netherer et al. 2015). In the present study, we examined further individuals of Norway spruce included in the *Rosalia* experiment, which were located not directly in the centre of the plots, yet, were just as influenced by the differential water supply treatments. Despite strongly reduced water availability of the tested FC trees compared to SC or C trees, part of their roots were presumably located outside the roofed soil area and could gather some water. After severe water deficiency in the dry and hot season 2013, we therefore considered the trees to be only moderately stressed in the wetter and cooler season 2014. We focused on a set of constitutive and induced defence responses of the trees to blue-stain fungal infection. Inoculation of *E. polonica* is a frequently applied method for testing the capacity of trees to avert biotic invaders and to mobilise defences (Lieutier et al. 2003; Krokene et al. 2012). The long co-evolution of this pathogen with Norway spruce is substantiated by local and systemic induction of resistance marker genes in response to infection (Fossdal et al. 2012). Effectiveness of trees in defending the fungus therefore allows for an evaluation of actual tree susceptibility as well as further disposition to biotic attacks.

Norway spruce generally exhibits a low and very variable potential for successful control of invaders by constitutive mechanisms at the time of attack (Christiansen et al. 1987). Resin reservoirs prior to fungal inoculation were clearly limited at the fully covered trees due to the strongly reduced water availability. Such relationship was expected and is well-known since the water manipulation experiments performed on loblolly pine (*P. taeda*) (Dunn & Lorio 1993; Lorio et al. 1995). Resin flow rates were however comparably low for

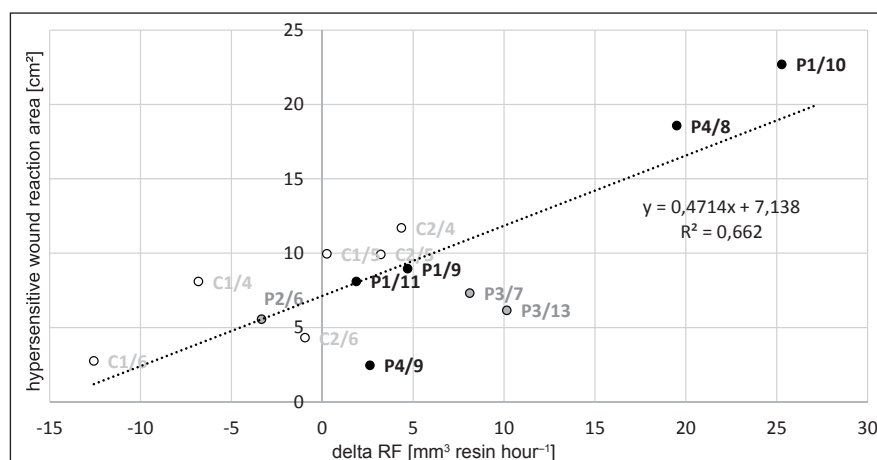


Fig. 6. Linear relationship of changes in resin flow rates ($\text{mm}^3 \text{resin hour}^{-1}$) within 5 weeks after the control of inoculations (delta RF) and size of hypersensitive wound reactions (cm^2) of 14 sample trees according to different treatments (black circles, full cover, FC; grey shaded circles, semi-cover, SC; white circles, control, C). Outliers P2/11 (SC, strongest decrease in RF) and P4/7 (FC, highest increase in RF) are not included.

sufficiently water-supplied control and clearly higher for partially covered trees. Likely, genetic variability in defence capacity apart from differences in water supply played an important role in constitutive defence characteristics of the sample trees. In response to fungal infection, conifers develop necrotic phloem lesions by impregnating affected tissues with resin, increasing monoterpene levels and changing the composition of phenols in the secondary phloem (Lieutier et al. 1996; Novak et al. 2014). In various inoculation experiments, resistant and more susceptible spruce clones were shown to differ in the stimulation of metabolic pathways leading to particular phenolic monomers after wounding and fungal attack (Brignolas et al. 1995, 1998).

In the case of an increasingly strong attack, the hosts fail to mobilise adequate resin and phenolic concentrations in the hypersensitive reaction zone, enabling the invading microorganism to overcome this first barrier (Christiansen et al. 1987). In our experiment, variability between trees in sizes of necrotic, dark coloured areas penetrated by fungal hyphae and of the lighter zones where fungal growth was finally stopped by resin impregnation was high. Nevertheless, despite similar low constitutive resin flow rates, the control trees consistently tended to exhibit smaller lesions pointing to a slightly increased capacity of defending *E. polonica* by resinous reaction. Although results remained ambiguous, probably due to the limited number of sample trees, largest wound reaction areas were found in the fully covered trees. Previously, Croisé et al. (2001) observed decreased resistance of severely stressed Scots pine (twig water potential less than -2.0 MPa) to *Leptographium wingfieldii* associated with *Tomicus piniperda*; yet, the potted trees were only 5 years old. On the other hand, mild water stress increased the critical threshold of inoculation density for fungi associated with Scots pine and Norway spruce in young plants (Christiansen & Glosli 1996; Dreyer et al. 2002). Similarly, moderately stressed *P. yunnanensis* (minimum twig water potentials of -1.03 MPa) in a 30-year-old forest stand showed comparably small reaction zones in response to *L. yunnanensis* inoculation pointing to enhanced defence capability (Sallé et al. 2008).

Predisposition to biotic infestation is to a large degree influenced by variations in individual stress levels of trees over time (Führer et al. 1997; Netherer et al. 2015). However, the scope of this experiment did not include examination of physiological levels of drought stress in the test trees, nor specific analyses regarding available carbon resources and contents/composition of terpenes in the phloem affected by the fungus. Although reduced effectiveness in stopping the invasion of *E. polonica* was associated with limited constitutive resin reservoirs in fully covered trees, we overall observed positive relationships between lesion size and resin flow rates after inoculation. Small mechanical wounds and pre-treatment with fungal inoculations can increase the resistance of Norway spruce trees to further infections (Krokene et al. 1999). In response to low-density fungal inoculation, trees with longer necrotic lesions in the phloem showed reduced secondary wound reaction after subsequent mass inoculation with *E. polonica* four weeks later, indicating a stronger stimulation of defence capacity (Krokene et al. 2001).

These earlier results are in agreement with the positive correlation of September 2014 resin flow and area of necrosis due to *E. polonica* observed in the present study. However, such relationship was clear only for the trees fully deprived of water. Semi-cover and control trees did not significantly increase resin production in response to inoculation and wounding, which seems to be inconsistent with the major hypotheses of plant defence expression (Lombardero et al. 2000; Rosner & Hannrup 2004). The comparably higher rates of resin flow in September 2014, as medium-term response to fungal inoculation at trees subject to drought suggest that mobilisation of resources for secondary metabolism was not yet impaired in these trees but activated by the need to stop the fungus. A similar pronounced increase in resin flow rates and monoterpene contents after fungal inoculation was observed in pure Norway spruce stands of moderate growth as opposed to vitally growing spruce trees in mixed stands (Baier et al. 2002). As long as (drought) stress is not too severe, resistance of Norway spruce can be enhanced (Christiansen & Glosli 1996; Christiansen & Bakke 1997). Within a few days, changes in bark anatomy involving the induction of traumatic resin ducts and swelling of polyphenolic parenchyma cells are initiated and display highest effectiveness three to nine weeks after low-density inoculation with *E. polonica* (Krokene et al. 2003). We assume that the increased resin flow in September 2014 observed in fully covered spruce trees of the *Rosalia* roof experiment was also due to the formation of traumatic resin ducts, stimulated by wounding and necrosis. Strong stimulation of tree defences can prevent trees from being successfully mass attacked by bark beetles (Zhao et al. 2011), but might also speed up the exhaustion of carbon resources, as argued by Lieutier et al. (2009).

5. Conclusion

The preliminary results gained in this experiment encourage the examination of medium to long-term drought effects on Norway spruce resistance following an integrative approach as suggested by Ryan et al. (2015). There are still a lot of unresolved questions: To what extent does constitutive resin flow of Norway spruce trees change over a drought manipulation period of 3–6 years? In response to inoculation of the blue-stain fungus *E. polonica*, what about the relationship between resistance parameters such as size of hypersensitive wound reaction, extent of induced resin flow, monoterpene contents/composition and stress intensity as indicated by tree physiological characteristics? What is the role of particular phenols in tree resistance and to what extent does phenol composition indicate tree resistance? Are adaptations of bark anatomical characteristics, e.g. size and number of radial resin canals, to the changed conditions of water supply visible after several consecutive years of drought stress (see Ferrenberg et al. 2015)? There are still inconsistencies about the influence of growth conditions, stress status and stress history on attractiveness of Norway spruce for attacks by *I. typographus* and its resistance to invaders. Given that low-density attacks activate defence capability of trees, is tree disposition to further attacks sustainably decreased and at which expenses? Investigation of a broad array of resis-

tance parameters will lead to a better understanding of how the investment of non-structural carbohydrate reserves into secondary metabolism changes under drought stress, when depletion of resources eventually occurs, and at which point trees are most susceptible to bark beetle attack.

Acknowledgements

The authors thank Pavel Mezei from the Slovak Academy of Sciences for valuable support in fieldwork. We highly appreciate the constructive feedback from an anonymous reviewer. This study was supported by the Austrian Science Fund (FWF; TRP194-B16) as part of the project “Modelling Dynamic Predisposition to *Ips typographus*” and the trust “120 Jahre Bodenkultur”.

References

- Anderegg, W. R. L., Hicke, J. A., Fisher, R. A., Allen, C. D., Aukema, J., Bentz, B. et al., 2015: Tree mortality from drought, insects, and their interactions in a changing climate. *New Phytologist*, 208:674–683.
- Baier, P., 1996: Defence reactions of Norway spruce (*Picea abies* Karst.) to controlled attacks of *Ips typographus* (L.) (Col., Scolytidae) in relation to tree parameters. *Journal of Applied Entomology*, 120:587–593.
- Baier, P., Führer, E., Kirisits, T., Rosner, S., 2002: Defence reactions of Norway spruce against bark beetles and the associated fungus *Ceratocystis polonica* in secondary pure and mixed species stands. *Forest Ecology and Management*, 159:73–86.
- Blackwell, E., 2011: Risk assessment of bark beetle outbreaks after an avalanche occurrence in the Dürrenstein Wilderness Area. Master thesis at the University of Natural Resources and Life Sciences, Vienna (BOKU), Austria.
- Boone, C. K., Aukema, B. H., Bohlmann, J., Carroll, A. L., Raffa, K. F., 2011: Efficacy of tree defence physiology varies with bark beetle population density: a basis for positive feedback in eruptive species. *Canadian Journal of Forest Research*, 41: 1174–1188.
- Brignolas, F., Lacroix, B., Lieutier, F., Sauvard, D., Drouet, A., Claudot, A.-C. et al., 1995: Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark beetle-associated fungus. *Plant Physiology*, 109:821–827.
- Brignolas, F., Lieutier, F., Sauvard, D., Christiansen, E., Berryman, A. A., 1998: Phenolic predictors for Norway spruce resistance to the bark beetle *Ips typographus* (Coleoptera: Scolytidae) and an associated fungus, *Ceratocystis polonica*. *Canadian Journal of Forest Research*, 28:720–728.
- Bryant, J. P., Chapin, III F. S., Klein, D. R., 1983: Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory (hare *Lepus capensis*). *Oikos*, 40:357–368.
- Christiansen, E., Waring, R. H., Berryman, A. A., 1987: Resistance of conifers to bark beetle attack: searching for general relationships. *Forest Ecology and Management*, 22:89–106.
- Christiansen, E., Glosli, A. M., 1996: Mild Drought enhances the Resistance of Norway spruce to a Bark Beetle transmitted Blue-stain Fungus. In: Mattson, W. J., Niemelä, P., Rousi M. (eds.): Dynamics of forest herbivory: quest for pattern and principle. USDA Forest Service General Technical Report NC-183, N.C. Forest Experimental Station, St. Paul NM, 55108, p. 192–199.
- Christiansen, E., Bakke, A., 1997: Does Drought really enhance *Ips typographus* Epidemics? – A Scandinavian Perspective. In: Grégoire, J. C., Liebhold, A. M., Stephen, F. M., Day, K. R., Salom, S. M., (eds.): Proceedings of the IUFRO working parties S7.03.03, S7.03.05, S7.03.07 joint meeting: Integrating cultural tactics into the management of bark beetle and reforestation pests. 1–3 September 1996, Vallombrosa, Italy. General Technical Report NE-236. Radnor, PA. Northeastern Forest Experiment Station.
- Christiansen, E., Krokene, P., Berryman, A. A., Franceschi, V. R., Krekling, T., Lieutier, F. et al., 1999: Mechanical injury and fungal infection induce acquired resistance in Norway spruce. *Tree Physiology*, 19:399–403.
- Croisé, L., Lieutier, F., Cochard, H., Dreyer, E., 2001: Effects of drought stress and high density stem inoculations with *Lep-tographium wingfieldii* on hydraulic properties of young Scots pine trees. *Tree Physiology*, 21:427–436.
- De Beer, Z. W., Duong, T. A., Barnes, I., Wingfield, B. D., Wingfield, M. J., 2014: Redefining *Ceratocystis* and allied genera. *Studies in Mycology*, 79:187–219.
- Dunn, J. P., Lorio, P. L., Jr., 1993: Modified water regimes after photosynthesis, xylem water potential, cambial growth, and resistance of juvenile *Pinus taeda* L. to *Dendroctonus frontalis* (Coleoptera: Scolytidae). *Physiological and Chemical Ecology*, 22:948–957.
- Dreyer, E., Guérard, N., Lieutier, F., Pasquier-Barré, F., Lung, B., Piou, D., 2002: Interactions between nutrients and water supply to potted *Pinus sylvestris* trees and their susceptibility to several pests and pathogens. In: Lieutier, F. (ed.): Effects of Water Stress on Pine Susceptibility to various Pest and Disease Guilds. Final Scientific Report of the EU Project FAIR 3 CT96-1854, 172 p.
- Eyles, A., Bonello, P., Ganley, R., Mohammed, C., 2010: Induced resistance to pests and pathogens in trees. *New Phytologist*, 185:893–908.
- Ferrenberg, S., Kane, J. M., Langenhan, J. M., 2015: To grow or defend? Pine seedlings grow less but induce more defences when a key resource is limited. *Tree Physiology*, 35:107–111.
- Fossdal, C. G., Yaqoob, N., Krokene, P., Kvaalen, H., Solheim, H., Yakovlev, I. A., 2012: Local and systemic changes in expression of resistance genes, nb-lrr genes and their putative microRNAs in Norway spruce after wounding and inoculation with the pathogen *Ceratocystis polonica*. *BMC Plant Biology*, 12:105.
- Franceschi, V. R., Krokene, P., Christiansen, E., Krekling, T., 2005: Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist*, 167:353–376.
- Führer, E., Lindenthal, J., Baier, P., 1997: Baummortalität bei Fichte: Zusammenhänge zwischen prämortaler Vitalitätsdynamik und dem Befall durch rindenbrütende Insekten. *Mitteilungen Deutsche Gesellschaft für allgemeine und angewandte Entomologie*, 11:645–648.
- Gasch, J., 1985: Standortserkundung und Standortskartierung mit allgemeiner Waldbauplanung im Lehrforst Ofenbach der Universität für Bodenkultur. Diploma thesis, University of Natural Resources and Life Sciences, Vienna (BOKU), Austria.
- Gaylord, M. L., Kolb, T. E., Pockman, W. T., Plaut, J. A., Yezzer, E. A., Macalady, A. K. et al., 2013: Drought predisposes pinyon-juniper woodlands to insect attacks and mortality. *New Phytologist*, 198:567–578.
- Gaylord, M. L., Kolb, T. E., McDowell, N. G., 2015: Mechanisms of piñon pine mortality after severe drought: a retrospective study of mature trees. *Tree Physiology*, 35:806–816.

- Grubelnik, R., 1998: Untersuchungen über die Zusammensetzung der Mycoflora von *Ips typographus* L. auf ausgewählten Wald-Standorten unter besonderen Berücksichtigung der pathogenen Art *Ceratocystis polonica*. Diploma thesis at the University of Natural Resources and Life Sciences, Vienna (BOKU), Austria.
- Herms, D. A., Mattson, W. J., 1992: The dilemma of plants: to grow or defend. *The Quarterly Review of Biology*, 67:283–335.
- Hulcr, J., Atkinson, T. H., Cognato, A. I., Jordal, B. H., McKenna, D. D., 2015: Morphology, Taxonomy, and Phylogenetics of Bark Beetles. In: Vega, F. E., Hofstetter, R. W. (eds.): *Bark Beetles: Biology and Ecology of Native and Invasive Species*. Elsevier Inc., San Diego, p. 41–84.
- Kirisits, T., 2004: Fungal Associates of European Bark Beetles with special Emphasis on the Ophiostomatoid Fungi. In: Lieutier, F., Day, K. R., Battisti, A., Grégoire, J.-C. (eds.): *Bark and wood boring insects in living trees in Europe, a synthesis*. Kluwer Academic Publishers. Dordrecht, Netherlands, p. 181–235.
- Kirisits, T., Dämpfle, L., Kräutler, K., 2013: *Hymenoscyphus albidus* is not associated with an anamorphic stage and displays slower growth than *Hymenoscyphus pseudoalbidus* on agar media. *Forest Pathology* 43, p. 386–389.
- Knebel, L., Robison, D. J., Wentworth, T. R., Klepzig, K. D., 2008: Resin flow responses to fertilization, wounding and fungal inoculation in loblolly pine (*Pinus taeda*) in North Carolina. *Tree Physiology*, 28:847–853.
- Krokene, P., Christiansen, E., Solheim, H., Franceschi, V. R., Berryman, A. A., 1999: Induced resistance to pathogenic fungi in Norway spruce. *Plant Physiology*, 121:565–569.
- Krokene, P., Solheim, H., Christiansen, E., 2001: Induction of disease resistance in Norway spruce (*Picea abies*) by necrotizing fungi. *Plant Pathology*, 50:230–233.
- Krokene, P., Solheim, H., Krekling, T., Christiansen, E., 2003: Inducible anatomical defence responses in Norway spruce stems and their possible role in induced resistance. *Tree Physiology*, 23:191–197.
- Krokene, P., Lahr, E., Dalena, L. S., Skrøppa, T., Solheim, H., 2012: Effect of phenology on susceptibility of Norway spruce (*Picea abies*) to fungal pathogens. *Plant Pathology*, 61:57–62.
- Lieutier, F., Sauvard, D., Brignolas, F., Picron, V., Yart, A., Bastien, C., Jay-Allemand, C., 1996: Changes in phenolic metabolites of scots-pine phloem induced by *Ophiostoma brunneo-ciliatum*, a bark-beetle-associated fungus. *European Journal of Forest Pathology*, 26:145–158.
- Lieutier, F., Brignolas, F., Sauvard, D., Yart, A., Galet, C., Brunet et al., 2003: Intra- and inter-provenance variability in phloem phenols of *Picea abies* and relationship to a bark beetle-associated fungus. *Tree Physiology*, 23:247–256.
- Lieutier, F., 2004: Host Resistance to Bark Beetles and its Variations. In: Lieutier, F., Day, K. R., Battisti, A., Gregoire, J. C., Evans, H. F. (eds.): *Bark and wood boring insects in living trees in Europe, a synthesis*. Kluwer Academic Publishers. Dordrecht, Netherlands, p. 135–180.
- Lieutier, F., Yart, A., Salle, A., 2009: Stimulation of tree defences by Ophiostomatoid fungi can explain attack success of bark beetles on conifers. *Annals of Forest Science*, 66:801.
- Lombardero, M. J., Ayres, M. P., Lorio, P. L., Ruel, J. J., 2000: Environmental effects on constitutive and inducible defences of *Pinus taeda*. *Ecology Letters*, 3:329–339.
- Lorio, P. L., Jr., Stephen, F. M., Paine, T. D., 1995: Environment and ontogeny modify loblolly pine response to induced acute water deficits and bark beetle attacks. *Forest Ecology and Management*, 73:97–110.
- Marini, M., Ayres, M. P., Battisti, A., Faccoli, M., 2012: Climate affects severity and altitudinal distribution of outbreaks in an eruptive bark beetle. *Climatic Change*, 115:327–341.
- Martikainen, P., Siitonen, J., Kaila, L., Punttila, P., Rauh, J., 1999: Bark beetles (Coleoptera, Scolytidae) and associated beetle species in mature managed and old growth boreal forests in southern Finland. *Forest Ecology and Management*, 116:233–245.
- Mason, P. A., Singer, M. S., 2015: Defensive mixology: Combining acquired chemicals towards defence. *Functional Ecology*, 29:441–450.
- Netherer, S., Matthews, B., Katzensteiner, K., Blackwell, E., Henschke, P., Hietz, P. et al., 2015: Do water limiting conditions predispose Norway spruce to bark beetle attack? *New Phytologist*, 205:1128–1141.
- Niinemets, Ü., 2010: Responses of forest trees to single and multiple environmental stresses from seedlings to mature plants: Past stress history, stress interactions, tolerance and acclimation. *Forest Ecology and Management*, 260:1623–1639.
- Novak, M., Krajnc, A. U., Lah, L., Zupanec, N., Krasevec, N., Krizman, M. et al., 2014: Low-density *Ceratocystis polonica* inoculation of Norway spruce (*Picea abies*) triggers accumulation of monoterpenes with antifungal properties. *European Journal of Forest Research*, 133:573–583.
- Rosner, S., Hannrup, B., 2004: Resin canal traits relevant for constitutive resistance of Norway spruce against bark beetles: environmental and genetic variability. *Forest Ecology and Management*, 200:77–87.
- Ryan, M. G., Sapes, G., Sala, A., Hood, S. M., 2015: Tree physiology and bark beetles. *New Phytologist*, 205:955–957.
- Sallé, A., Hui, Y., Yart, A., Lieutier, F., 2008: Seasonal water stress and the resistance of *Pinus yunnanensis* to a bark-beetle-associated fungus. *Tree Physiology*, 28:679–687.
- Seidl, R., Müller, J., Hothorn, T., Bäessler, C., Heurich, M., Kautz, M., 2015: Small beetle, large-scale drivers: how regional and landscape factors affect outbreaks of the European spruce bark beetle. *Journal of Applied Ecology*, 9:1365–2664.
- Skrøppa, T., Solheim, H., Hietala, A., 2015: Variation in phloem resistance of Norway spruce clones and families to *Heterobasidion parviporum* and *Ceratocystis polonica* and its relationship to phenology and growth traits. *Scandinavian Journal of Forest Research*, 30:103–111.
- Winter, M. B., Baier, R., Ammer, C., 2015: Regeneration dynamics and resilience of unmanaged mountain forests in the Northern Limestone Alps following bark beetle induced spruce dieback. *European Journal of Forest Research*, 134:949–968.
- Zhao, T., Krokene, P., Hu, J., Christiansen, E., Björklund, N., Långström, B., Solheim, H. et al., 2011: Induced terpene accumulation in Norway spruce inhibits bark beetle colonization in a dose-dependent manner. *PLoS ONE*, 6: e26649.