Clone specific chemical defense responses in Norway spruce to infestations by two pathogenic fungi.

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Abstract

Chemical defense responses against the two pathogenic fungi *Endoconidiophora polonica* (Ep) and *Heterobasidion parviporum* (Hp) were investigated using four clones of Norway spruce (*Picea abies*) with different susceptibility to *Heterobasidion* sp. Eight year old trees were inoculated with Ep and Hp to minimize the variation due to environment. After three weeks the bark tissue at the upper border of the inoculation hole were extracted with hexane and analyzed by GC-MS. Both treatment and clonal differences were found based on induced mono-, sesqui- and diterpenes. In addition, the Hp produced toxin, fomanoxin, was identified in lowest amount in the most Hp susceptible clone. The clonal trees seem to use different defense strategies towards the two fungi. One of the clones was able to induce strong chemical defense against both fungi, one clone induced chemical defense only against Ep and the most susceptible clone exhibited the least capacity to produce an effective defense against Ep and Hp. Two diterpenes were found to be distinctly different between clones with different susceptibilities, which can be used as chemical indication of Norway spruce resistance against fungi

Key words: *Endoconidiophora polonica, Ceratocystis, Heterobasidion parviporum, Picea abies*, clone, induced response, terpenes

Introduction

Conifers are longlived trees facing a number of treats during their lifetime. The ability to react on various stressors by producing defense chemicals is most probably the reason for survival (Keefover-Ring et al 2015). Many organisms that utilize conifers as hosts such as bark beetles and fungi have adjusted their metabolism to cope with the more or less toxic chemicals. The bark beetle transfer fungi including *Endoconidiophora polonica* (Ep) (=*Ceratocystis polonica*), to the trees to facilitate its tree colonization by which start terpenes and thus reduce the terpene content and probably release aggregation pheromone to facilitate for the mass attack of beetles (Zhao et. al **2016**) and survival of the larvae. *Heterobasidion parviporum* (Hp) which is the most economical important pest on spruce produce toxins that kill the cells following degradation of the terpenes (Kusumoto et al 2014). However studies have shown that Norway spruce trees have individually different resistance against spruce bark beetle and root rot fungi. There exist trees that are much less or not attacked by bark beetles while many of them were killed by mass attacks (Schiebe et. al 2012; Zhao et. al 2011a). Some genotypes are less infested by root rot fungus Hp than other genotypes (Swedjemark and Karlsson 2004; Swedjemark et. al 1998). These result indicated tree defenses is an important regulator of bark beetle population densities and root rot diseases which can can be exploited to reduce tree-killing by bark beetles and pathogens. The observed chemical difference between resistant and susceptible trees opens for an investigation to identify resistant markers and to study individual performance during introduction of various stressors to the trees.

Experiments with Norway spruce clones have shown that tree resistance is partly determined by genetically controlled host tree characteristics. Identifying resistant trees and selecting genotypes that are more resistant to multiple pests are of great importance for future forestry in Sweden. Norway spruce resistance to root rot fungi (*Heterobasidion* spp.), the most serious pathogens of the tree species in Sweden, has been determined by observing natural infestations and performing artificial inoculation studies in clonal trees. A deeper understanding of conifer defense mechanism and conifer - bark beetle - fungus interactions are urgently needed to breed Norway spruce genotypes that are more resistant to bark beetles and fungi.

By introducing the two above mentioned fungi on the same specimens of spruce clonal materials from Skogforsk clone archive with known different susceptibility to Hp and chemically analyze the infested tissue by GC-MS the produced defense compounds and fungi detoxification compounds, we will be able to study the variation in defense among individual spruce trees.

Results of the study shows that spruce specimens have different ability to produce defense chemicals towards the same or different infestation, thus, such a multifaceted defense gain the forest community survival facing various threats over time.

Materials and methods

The experiments were carried out on potted plants at Skogforsk at Ekebo 2013. The material (Table 1, Fig 1) is present in the nursery at Skogforsk, Ekebo. It was cut in 2007. The material was tested for resistance by inoculation as 2 year-old plants in 1998. Originally there were 150 clones from 15 families. They were included in the RESROBS project and were tested in Greece, Italy and Norway. Selected clones are outplanted on a heavily *H. annosum* infected site in Denmark, where we continuously check for disease. They were also outplanted on a site in Tönnersjö in southern Sweden (1998). On that site we have done twig inoculations 2010. In addition the clones are also outplanted on 3 more sites in southern Sweden.

Four clones of Norway spruce were chosen based on their ability to resist *H. parviporum* infection. This was evaluated by measuring the lesion length. Clone 132 and 329 had both long lesion lengths and assumed to have a lower ability to resist Hp growth whereas clone nr 393 and 457 had short lesion

lengths and assumed to be less susceptible to Hp (Table 1). The grafts of these clones were potted in 2008

Fungi materials

The fungi strains used were: *H. parviporum* Sä 159-5 and *E. polonica* NFLI *1993-208/115-2*, chosen based on known pathogenicity towards *P. abies*. The Hp inoculate was used by Lind et al. (2005) and the Ep-strain was collected from a Norway spruce log infested with the bark beetle *Polygraphus poligraphus* L. which transferred the fungi to this tree (Krokene and Solheim, 1996*).* This strain has been used in several inoculation studies by the Norwegian group (Zhao et. al 2010; Zhao et. al 2011b)

Inoculation procedure

Inoculations were made in July 2014. To minimize the possible differences between speciments and inoculation procedure, six trees per clone were inoculated with Hp on one side and Ep on the other side with a distance of ca 30 cm in between (Figure 1). Inoculations of the trees were performed using two sterilized 4 mm cork borers, one for Ep and another for Hp to avoid possible contamination. Inoculum consisted of mycelium that had been growing on malt agar (2% malt, 1.5% agar) for 1 week. The fungal plug with agar inoculum was inserted in the hole and parafilm was wired around the stem and closed the wound (Fig 2). 3 weeks after the inoculations the healing process were inspected, and two bark plugs were taken around each inoculation holes to characterize tree induced response as well the metabolites produced by fungi in different clones. To know the constitutive terpene level in different Norway spruce clones, an extra sample were taken from the opposite site of Hp inoculation at the same height of the Hp inoculation but 30 cm below the Ep inoculation, where has been proved no terpene induction in our previous study .

Sampling for chemical analyses

Bark samples for chemical analyses were taken from the experimental trees 3 weeks after inoculation using a 5 mm cork borer. Samples consisted of two bark plugs taken close to the upper and lower border of each inoculation hole.

The outer cork bark was removed from the bark plugs and the plugs were marked and wrapped in aluminum foil and frozen in liquid nitrogen. After transport to KTH the plugs were chopped in 1mm^2 pieces and extracted in 0.5ml n-hexane (Lancaster) containing (0.158 mg/mL) pentadecane (Lancaster synthesis, England) as an internal standard and (0.12 mg/mL) 3-tert-butyl-4-hydroxy-anisol (Fluka Switzerland) as an antioxidant. The samples were cut into ca 2x2 cm small pieces and extracted with 0.5 ml hexane at room temperature for 48h before the extract was transferred to new vials and kept at -25 °C until GC-MS analyses. The bark plugs were dried at 80 °C for 6h, and then weighted on a Sartorius electronic balance for calculation of absolute amounts.

GC-MS analyses

The hexane extracts were analyzed using a Varian 3400 GC connected to a Finnigan SSQ 7000 MS to separate, identify and quantify the volatile constituents. A DB-wax column (J&W USA, 30 m, 0.25 mm i.d., 0.25 µl film thickness) was used, and the temperature program was set as 40 °C for 1 min , followed by an increase to 235 °C with 4 °C min^{-1}, and hold time 29 minutes. 1µl extract were injected to a split/splitless injector with a 30 s splitless injection at 230 °C. Aux 240. Helium was used as the carrier gas at a constant flow of 1 ml min^{-1}, the temperature at the ion source was 150 °C, the mass detector was operated with a mass range of 30-350, and the electron impact ionization was 70 eV. One microliter of hexane extract of each sample was injected into the GC-MS with a CTC autosampler using a (5 µl) syringe. The terpene hydrocarbons were identified by comparing retention times and mass spectra with available authentic standards, and by comparing retention indexes (RIs) and mass spectra with Massfinder 3 (Hochmuth Scientific Consulting, Germany) and the reference libraries of NIST (National

Institute of Standards and Technology). The absolute amounts of terpenes were calculated relative to the internal standards and expressed as mg (or μg) g^{-1} dry wt, and expressed as pentadecane equivalents. The relative amounts of terpenes were calculated as the ratio of the area of each peak to the sum of all the areas of terpene hydrocarbons in a defined GC fraction, and expressed as percentages.

Statistical analyses

The absolute amounts were calculated relative to internal standard and dry weight as described in an earlier article and relative proportions (normalized to 100%). The absolute amounts where then normalized with square root transformation and the relative amounts where arc sin transformed.

In cases when the means of specific substances were not obviously different between the treatments and clones e.g. when the compound was below the detection limit in one group and abundant in the other group, we tested for statistical differences. First Levene's test was used to assess the equality of variance between the means of absolute amounts of specific compounds for both between the inoculation treatments or between the clones. Means of equal variance were subjected to One-Way ANOVA and as a post hoc we used Tukey's HSD. $P<0.05$. When Levene's test showed a significant difference in variance the non-parametric Kruskal-Wallis test was used. Levene's test, ANOVA and the non-parametric ANOVA were performed in SAS University Edition.

Results

Clonal differences to inoculations of Hp and Ep: The four clones induced terpene biosynthesis differently (Fig 3). Clone 457 responded the strongest of all four clones to inoculation by Hp and Ep with mono and diterpene production. The induced amounts of was at similar level, which was in contrast to the other clones, especially 329 which was strongly induced by Hp but only to a minor extent to Ep (Fig 3 and 5). Sesquiterpenes were also induced showing a different pattern for each clone.

Induction of monoterpenes: The clones had different monoterpene hydrocarbon profiles, 132 had high amounts of β-phellandrene, 329 and 457 high amounts of limonene, and 393 high 3-carene level. Interestingly, 393 producing 3-carene was the most resistant to Hp. The monoterpene GC-MS profiles of control and inoculated tissues were similar in relative amounts.

Oxygenated monoterpenes: Several oxygenated monoterpenes was present in the tissues close to Hp and Ep inoculations (Fig 4). Generally, Hp infested tissues contained larger number of oxygenated monoterpenes than did Ep tissues regardless of clones. Individual clones were slightly different in the composition of oxygenated monoterpenes. The clone 457 responded most strongly to infestation forming highest number of oxygenated monoterpenes. α-Terpineol was the main oxygenated monoterpene present in both the Hp and Ep infested phloems of almost all specimens. Others, like pinocarveol, terpinen-4-ol, cis-verbenol and myrtenol were also abundant. Interestingly, the absolute amounts of total oxygenated monoterpenes were similar in Hp infested tissues (Fig 4).

Diterpenes: Large differences in the diterpene content were found between clones, especially between geranyllinalool (GL) and thunbergol (TH), both highly inducible. Clone 132 did not produce TH in contrast to 457 that produced mainly TH in high quantity. The proportion of thunbergol and geranyllinalool TH/GL is higher in the two least susceptible clones (393 and 457) and the lowest with the two most susceptible clones (132 and 329, Fig 5)

Clonal differences in fomanoxin content: There were differences in the presence of *Heterobasidion* produced toxin fomanoxin in the infested phloem of the clones, with the lowest amount in clone 132 and the highest in clone 457 (Fig 6).

Discussion

The ability to react against inoculation of either of the two fungi seems to be very different among the clones. The chemical response of one of the clones (457) was strongly induced by both Hp and Ep whereas another clone (329) only responded to inoculation by Hp, the more aggressive fungus. The chemical response of clone 132 was weak and this was the most susceptible clone of the four tested (Table 1).

Hp produces a complex mixture of toxic compounds where fomanoxin (F) is the most volatile constituent. Toxin production by Hp in the phloem varied among the clones (Fig 6) and was present in highest amounts in the less susceptible clone 457 whereas only trace amounts were found in clone 132, the most susceptible clone. Earlier, it was found that the production of F was lower when Hp was growing in a medium with high concentration of oxygenated monoterpenes (Kusumoto et al 2015). (+)-α-Pinene, α-terpineol, bornyl acetate, abietic and dehydroabietic acid showed inhibiting effect on the Hp mycelial growth. Our findings are seemingly in contradiction to Kusumotos findings but in this study we have inoculated the mycelium in living trees which most probably change the conditions for the production of the toxin.

Large quantities of diterpenoids were induced in the least susceptible clones 457 and 393. This is in accordance with earlier findings (Xosé, Personal communication) Except for certain diterpenes in common; the diterpene profile was similar within a clone but different among clones.

Earlier we have identified TH as a resistant marker when mature spruces were inoculated with Ep (Zhao et al 2010). In this study, we found that TH amount was much higher in two resistant clones than the two susceptible ones, either after Ep or Hp inoculation. It also is worth noticing that the production of TH is absent in clone 132, possibly due to lack of the cyclisation synthase forming TH from GL. Differently, GL amount was significantly higher in two susceptible clones than the two resistant clones, after Hp inoculation. These two diterpenes can be used as indicator of Norway spruce resistance and susceptibility respectively.

In this study we have confirmed earlier observations that clones respond differently against a fungus and also different against different fungi. Tree chemical responses against the two fungi Hp and Ep were also different within the same specimen. Thus, the chemical defense seems to be local and exhibit a variety of chemical compounds with various effects on the stressors, so far only partly known.

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Fig 1 The spruce clones at Ekebo 2013

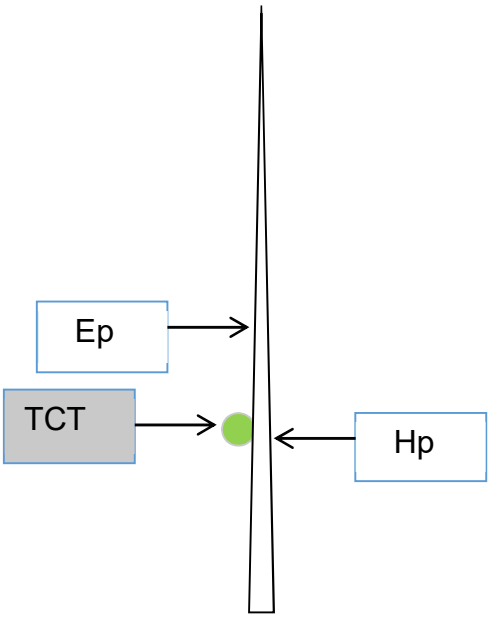

Fig. 2 Inoculation points and sample points. Distance between the inoculations is 30 cm

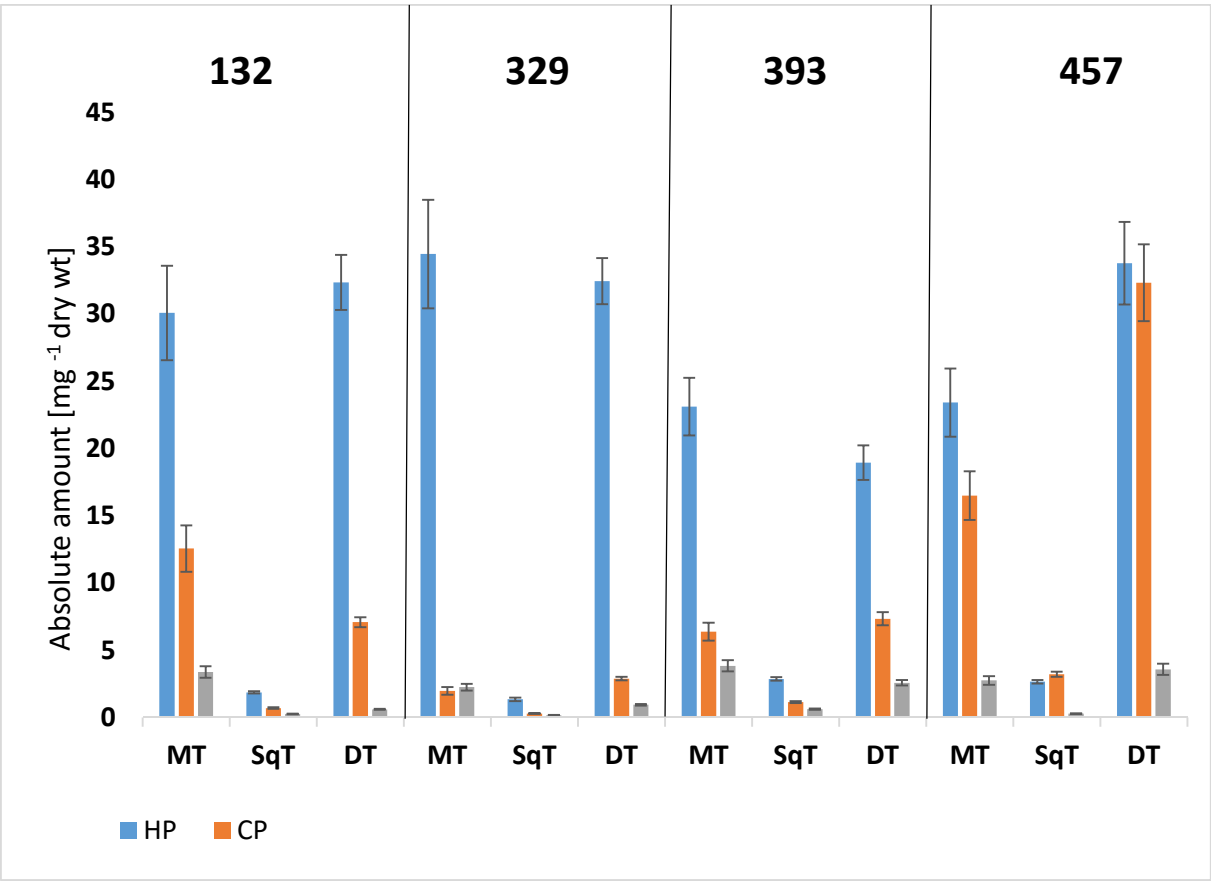

Figure 3. Mean values representing absolute amounts of monoterpenes (MT), sesquiterpenes (SqT) and diterpenes (DT) in Norway spruce 8 year old trees after induction of *Heterobasidion parviporum* (Hp) and *Endoconidiophora polonica* (Cp) in four clones (132,329,393 and 457). Error bars denote standard deviation. Decrease in susceptibility to Hp: clone numbers 132>329>457>393

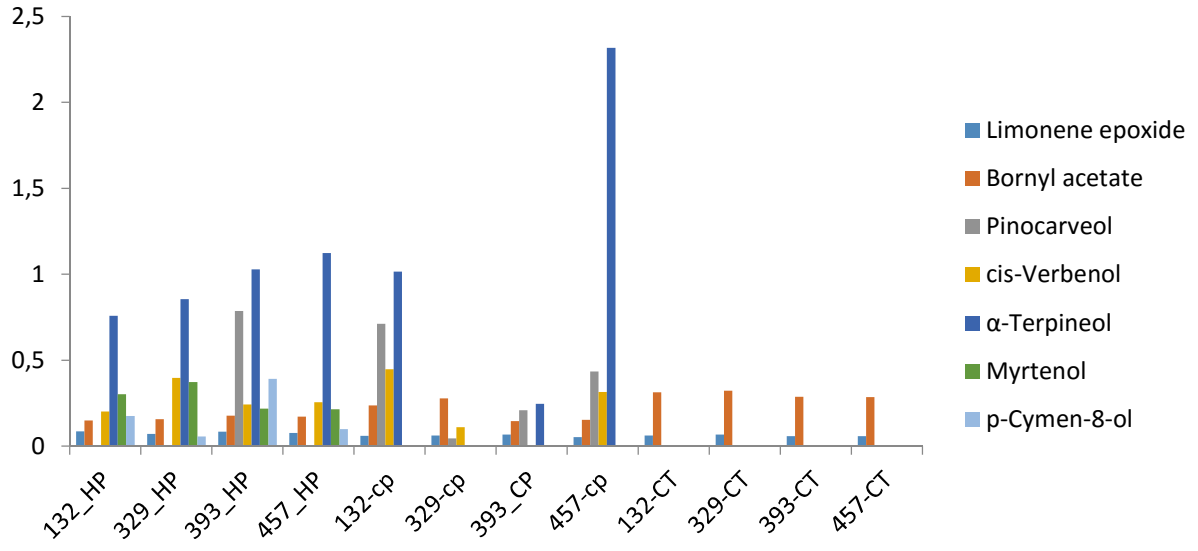

Fig 4. Mean absolute amounts of oxygenated monoterpenes in infested phloem for each clone

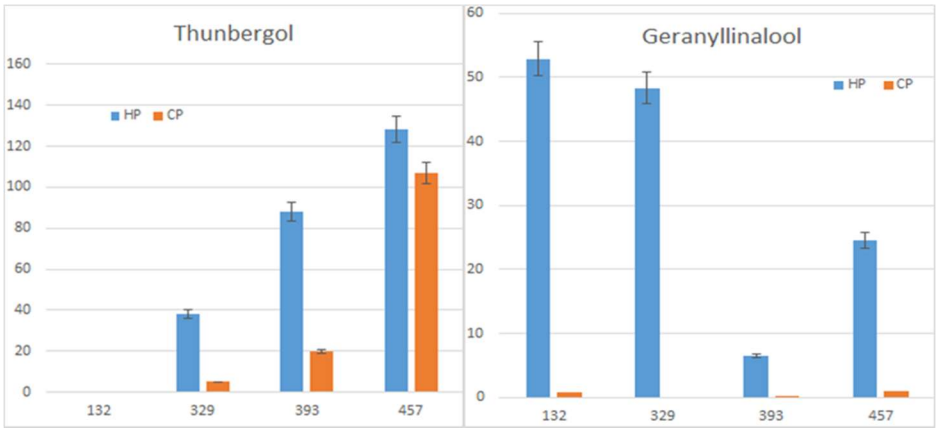

Fig 5 Induced responses of *H. parviporum* and *E.polonica*
in four spruce clones of the diterpene alcohols thunbergol and geranyllinalool.

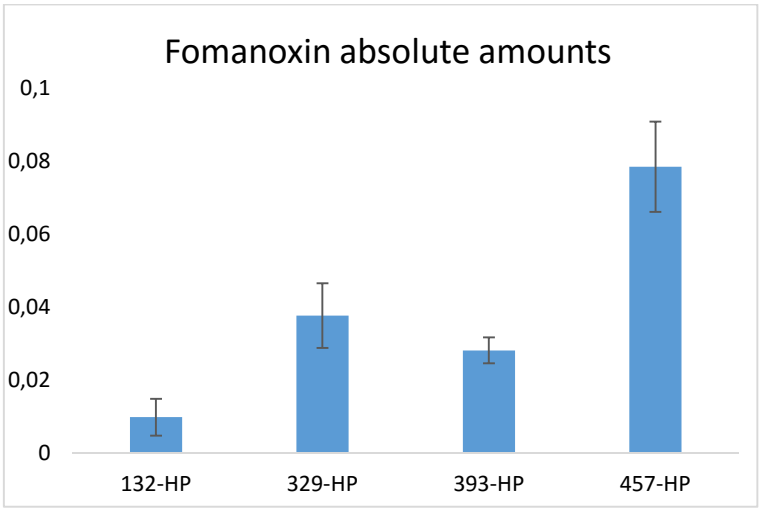

Figure 6 Absolute amounts (in C15 equivalents) of fomanoxin present in the infested phloem of the clones.

Table 1. Clones tested for resistance 1998 and included in this study.

Spruce clones	**Infestation success rate %**
S21K9220132	86.6
S21K9220329	70.7
S21K9220393	38
S21K9220457	44.7