

Breeding for Resistance in Norway Spruce to the Root and Butt Rot Fungi *Heterobasidion* spp.

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Abstract

Results from previous studies of resistance in Norway spruce (*Picea abies* (L.) Karst.) to the pathogens *Heterobasidion* spp. show significant genotypic variation in fungal growth and spore susceptibility among Norway spruce clones. The genetic variation and the heritability are large enough for practical breeding purposes and there is no adverse correlation between *Heterobasidion* infection and growth traits. There is an economic incentive to use plant materials which are more resistant than those used in reforestation today. Recent research on developing the methods for testing resistance in plant material have demonstrated that we need to identify new markers and early traits for screening young material before we can include resistance to the pathogen in existing breeding programs. In an ongoing project we are analyzing the differences in the chemical defense and the transcriptome of *Heterobasidion* spp.-infected *P. abies* clones with different susceptibility to *Heterobasidion* spp. infection. Preliminary results show that the quantity of certain substances involved in defense mechanisms is specific for resistant clones. For example, astringin may be a constitutive chemical marker for resistance.

Key words: *Heterobasidion* spp., Norway spruce, *Picea abies*, resistance breeding

Introduction

The root pathogens *Heterobasidion* spp. are able to infect living conifer roots of all ages (Asiegbu et al. 2005). The *Heterobasidion* species complex consists of five different species, of which two are common in Scandinavia: *Heterobasidion annosum* and *Heterobasidion parviporum* (Dahlman 2010). The complex causes serious damage to coniferous forests throughout the Northern Hemisphere and, under optimal conditions, can rapidly destroy a stand. The fungus spreads both by spores and by vegetative growth: spores land on recently exposed wood, such as freshly cut stumps, germinate, and form a mycelium, which colonizes the stump and its roots. It then infects healthy trees by growing via root contacts and grafts between stumps and trees and then extends through the roots and up into the stem.

Results from our previous studies (inoculation of differently aged clonal material and screening of naturally infected clone stands) of resistance in Norway spruce (*Picea abies* (L.) Karst.) to *Heterobasidion* spp. are promising. There is significant genetic variation in resistance to fungal growth and in spore susceptibility in Norway spruce. The results show the same pattern in inoculation experiments and in naturally-infected Norway spruce clone stands (Karlsson and Swedjemark 2006, Swedjemark 1995). The genetic variation and the heritability are large enough for practical breeding purposes and there is no adverse correlation between *Heterobasidion* infection and growth traits. The economic calculations show that losses due to root rot are significant; there is an economic incentive to use plant materials which are more resistant than those used today (Karlsson and Swedjemark 2006).

Genetic variation in Norway spruce has been detected for several traits such as growth capacity and wood density (Karlsson and Högberg 1998). Economic gain from using improved plant material could be even greater if resistance factors were included in selection programs. Previous results from *Heterobasidion* spp. inoculation experiments and inventories in field trials have shown that genetic variation in fungal extension and infection frequency is of the same magnitude as in other traits used in applied tree breeding. The genetic improvement was 15 percent less than the average

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infection frequency when selecting the 10 percent top clones (Karlsson and Swedjemark 2006, Swedjemark 1995).

Inoculation experiments on twigs showed that the infection pattern is the same in twigs as in cuttings, which makes it possible to perform non-destructive inoculation experiments in field trials. In inoculation experiments on cuttings, a systemic induced resistance reaction was found up to 18 months after the first treatment with the pathogen (Swedjemark and Karlsson 2006, Swedjemark et al. 2007).

Being long-lived organisms, Norway spruce trees rely on both induced and constitutive defenses to restrict the spread of invading fungi and insects. The first line of defense in Norway spruce trees is the bark. The combination of the physical properties of lignified and suberized walls and the chemical properties of phenolics and terpenes makes bark a very efficient barrier against infection. Once an injury or an infection is recognized by the tree, induced defenses are activated, including cell wall re-enforcements, production of lytic enzymes, and secondary metabolites such as phenols, stilbenes, lignans, flavonoids, and terpenes (Keeling and Bohlmann 2006, Witzell and Martin 2008)

One generally debated hypothesis claims that the production of defense compounds depend on abiotic factors, e.g., the amount of nutrition available (Herms and Mattson 1992). In one of our studies on spruce, we have partly confirmed this hypothesis, as the induced amounts of monoterpenes were less in trees that obtained an excess nutrition. We also found that the absolute amounts of terpenes were positively correlated to the inoculated fungal performance (Danielsson et al. 2011).

The terpene chemistry in healthy *P. abies* is well documented, both within and between grafts, and in natural stands (Borg-Karlson et al. 1993, Persson et al. 1996). Large variations between and within trees are found for the monoterpenes, and there also exists “chemo types” characterized by the occurrence of 3-carene. We have shown that only a few monoterpene enantiomers, including (+)-3-carene, are induced by fungi (Zhao et al. 2010) representing specific terpene synthases. With our technique, we can analyze the effects of stress elicitors in more detail, and we aim to show that individual changes in terpenes are highly dynamic and inducible.

The phenols stilbene, (*E*)-astringin and corresponding astringin dimers (piceasides), and a number of unknown phenolics, seem to be of major importance as resistant markers (Danielsson et al. 2011) and will be the focus of our further investigations. We have recently identified clones that produce enhanced amounts of astringin which is of interest in our search of resistant markers.

The aim of our research is to find the best constitutive or induced markers for resistance to be able to select parent clones to be included in existing breeding programs for Norway spruce. Knowledge of reliable resistance markers is essential for the production of future plant materials intended for selection of parent clones for the breeding population, and thus for commercial reforestation material.

Material and Methods

Plant Material

Since 1994, we have had access to Norway spruce plant and tree material included in the Swedish regional clonal forestry program at The Forest Research Institute in Sweden (Karlsson and Högberg 1998). During the years, we have tested ca 1,000 spruce clones for resistance to *Heterobasidion* spp., using artificial inoculation of plants, trees, twigs, stumps, and somatic embryos. In addition we have screened naturally infested clonal stands by felling trees, using a borer, or a root rot detector (Rotfinder[®]). Grafts of selected clones have been prepared and we have made controlled crossings between parents with known levels of resistance.

Methods for Artificial Inoculation

- Cuttings, roots, and twigs. Wounds are made aseptically in the bark to reach the xylem surface. Mycelium is attached to the wound. Incubation period is ca 3 weeks. At sampling, the stem is consecutively cut into 5 mm thick disks. The disks from each

sample are incubated under humid conditions and are then checked for conidia of the fungi. The fungal extension is estimated for each sample.

- Artificial inoculation on stumps. The trees are felled, leaving stumps ca 50 cm high. Immediately after felling, the stumps are covered with a suspension of *Heterobasidion* spp. conidiospores (suspended in sterile water). After incubation (ca 3 months), the stumps are sliced into 1 cm thick discs, numbered, and stored in a plastic bag under humid conditions. After ca 10 days, the discs are checked for conidia development on the mycelia and the extension of fungal growth is measured.

Quantitative RT-PCR Analysis of PAL-Transcripts

The enzyme PAL (Phenylalanin Ammonia-Lyase) is central in the phenylpropanoid pathway and lignin biosynthesis, but also in production of stilbenes and flavonoids. Using quantitative real-time polymerase chain reaction (Quantitative RT-PCR) analysis of PAL transcript levels, the level of activation is measured in the tissues around the fungal growth. Small samples close to the fungi and at certain distances from the fungi are collected and frozen in -80 °C and processed according to Hietala et al. (2003).

Methods for Terpene and Phenol Analysis

Samples extracted in hexane, methanol, or by solid phase micro extraction (SPME) or thermal adsorption/desorption methods, are taken in order to measure the “metabolomics.” We have well-established protocols for the extraction procedures and the separation of constituents in the complex mixtures is reliable. Sampling by solvent extraction gives information about the constitutive and induced resins, and the hexane extracts is analyzed by the two-dimensional GC-MS to separate the enantiomers of the chiral terpenes (Persson et al 1993). The dynamics of selected chiral monoterpenes α -pinene, β -pinene, and limonene and sesquiterpenes is analyzed in detail by SPME-GC-MS technique adsorption solvent or thermal desorption in 2D-GC-MS. Volatiles released by healthy and infested trees are collected and we localize the front of the mycelium by measuring the emission of specific de novo-produced stress compounds (methyl salicylate and α - and β -farnesenes).

The methanol/water extract rich in phenolic compounds is analyzed by using reversed phase-HPLC-MSⁿ. Efforts to identify the unknown compounds is made by HPLC-prep-microprobe-NMR in cooperation with the NMR group at the Max Planck Institute of Chemical Ecology in Jena, Germany, a group already focusing in this area.

Recent Results and Discussion

A significant problem when studying resistance in forest ecosystems is the relevance of results from artificial trials in young material versus the natural situation. Are we really measuring actual resistance to the pathogen, and what will happen during a rotation period? The correlation between inoculation results is poor when comparing inoculation in the same clones from different propagation batches (Karlsson et al. 2008). Recent results indicate that it may be an environmental disturbance (stress) of the cuttings which triggers the induced resistance system, which may influence results from inoculation experiments. Induced resistance has showed to be a clone-dependent trait. A systemic-induced resistance reaction was found 18 months after the first treatment with *Heterobasidion* (Swedjemark et al. 2007). Inoculated twigs heavily attacked by *Sacchiphantes abietis* were more efficient in excluding the pathogen from the wood, indicating an induced resistance reaction (Swedjemark and Karlsson 2006). Plants infested with mites were tested for levels of α - and β -farnesenes (indicating stress); clones exposed to mite infection had high levels of volatiles and had a shorter extension of fungal growth when inoculated compared to unaffected control plants, indicating that the induced resistance reaction influenced the result. (Borg-Karlson and Swedjemark, unpublished data). Recent research has shown that induced stress affects tree resistance, where the least visually stressed trees were the most infested by blue stain fungi (Zhao et al. 2010). However, recent results show that the transcriptional regulation always shows the same pattern in the same spruce clones of different ages when inoculated with *Heterobasidion*.

(Danielsson et al. 2011) These findings make it possible to rely on inoculation studies if supplemented with analysis of transcriptional regulation.

Inoculations of *H. annosum* in roots of clones with high and low susceptibility show that the initial phenol composition differed between highly and less susceptible clones. Less susceptible clones were characterized by higher constitutive concentrations of the sesquiterpene α -longipinene, the stilbene (*E*)-astringin and astringin dimers (piceasides), and a number of unknown phenolics, possibly indicating further biochemical reactions downstream.

The levels of the flavonoid (+)-catechin showed a temporal variation; it accumulated between 5 and 15 days after inoculation (dpi) in response to *H. annosum* infection in the less susceptible genotypes. The transcriptome data suggested that the accumulation of free (+)-catechin was preceded by an induction of genes in the flavonoid and proanthocyanidin biosynthesis pathway such as leucoanthocyanidin reductase. Quantitative PCR analyses verified the induction of genes in the phenylpropanoid and flavonoid pathway. The qPCR data also highlighted genotype-dependent differences in the transcriptional regulation of these pathways.

It is obvious from our results that the transcriptional changes in responses to *H. annosum* or wounding treatments are substantially different between the two less susceptible genotypes. The pattern of extractable (+)-catechin in bark differ between the clones. The qPCR data also indicate differences between the clones; the PAL genes are down-regulated already at 15 dpi in wounded susceptible clones while they remain slightly up-regulated at 28 dpi irrespective of treatment in more resistant clones. The result indicates that the genotypes may perhaps depend on different successful defense strategies in the interaction with *Heterobasidion*. Clearly, an extended comparison between the chemistry and the transcriptional responses in the interaction with *Heterobasidion* between several independent genotypes is needed to catalogue the mechanisms of successful host defense strategies (Danielsson et al. 2011).

Conclusions

- There is significant genetic variation in resistance to fungal growth and in spore susceptibility in Norway spruce. Genetic variation and the heritability are large enough for practical breeding purposes and there is no adverse correlation between *Heterobasidion* infection and growth traits.
- Concentration of secondary metabolites in the tree differs between trees more or less susceptible to the pathogen: astringin could be a candidate as a constitutive marker for resistance.
- Concentration of specific phenols differs between trees more or less susceptible to the pathogen.
- Constitutive concentrations of terpenes do not seem to be specific for trees more or less resistant to *Heterobasidion*.
- The transcriptome always shows the same pattern in the same spruce clones when inoculated with *Heterobasidion*.
- Transcriptome data correlates with the chemical profile.

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