Stilbenes as Constitutive and Induced Protection Compounds in Scots Pine (*Pinus sylvestris* L.)

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Abstract

The goals of our studies are to describe the natural variation in the concentration of constitutive heartwood extractives; estimate the genetic parameters related to heartwood characteristics; determine whether there is a genetic connection between constitutive and inducible production of stilbenes; and, together with technical experts, to develop fast and reliable techniques to quantify stilbenes from wood samples and determine whether there are rapid ways to utilize the existing variation in silviculture. We are also collaborating with molecular geneticists to find the markers for pine stilbene biosynthesis that could be used in early selection.

The study material includes three generations of Scots pine (*Pinus sylvestris* L.): the first generation consists of grafted clones in seed orchards; their half-sib progenies growing in about 40-year-old progeny trials are the second generation; and the third generation includes seedlings in a nursery. The first and the second generations provide heartwood samples for stilbene analysis and the third generation has been used to study mechanical induction of stilbenes. Moreover, after heartwood samples from a large natural stand have been surveyed for stilbenes and total phenolics (end of 2012), we will be ready to make experiments on their seedling progenies and their biotic challengers.

We hypothesize that trees having strong inducible defense ability against biotic and abiotic stresses in their living tissues may also have high concentration of stilbenes in their heartwood when they mature. Such trees would be optimal to cultivate as timber with natural stilbene impregnation. One vision with practical importance is the exploitation of the genetic variation in stilbene production for the improvement of Scots pine regeneration material. For the evaluation of the possibilities and strategies to improve Scots pine trees to produce more durable heartwood, we need to estimate the interaction between genotype and environment as well as the genetic correlation between quality and growth traits.

Key words: *Pinus sylvestris*, stilbenes, protection compounds

Introduction

Because of its natural characteristics, Scots pine (*Pinus sylvestris* L.) heartwood is an important timber resource in Nordic countries, the potential of which is currently not fully utilized. Due to its high content of durability providing extractives, Scots pine heartwood timber has been used in construction where high decay resistance was required. Today, after more than 100 years of using super effective pressure treated wood, the interest on the natural durability of Scots pine (*Pinus Sylvestris* L.) heartwood has increased due to the legal restrictions on the use of wood preservation chemicals. Natural decay resistance is a special and important wood quality trait of Scots pine heartwood; however, it is very laborious to measure and study.

Heartwood is dead tissue, where extractives such as stilbenes have accumulated constitutively under developmental inevitability. It has an important role in living trees (Taylor et al. 2002), and its features continue to be important when heartwood serves as construction material. One of the most

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important goals of our studies has been to find indirect ways to grade heartwood material according to its natural decay resistance. Based on previous studies, it is evident that the concentration of extractives is an important factor in giving natural decay resistance to heartwood (Scheffer and Cowling 1966). Besides resin acids (Harju et al. 2002), stilbenes pinosylvin (PS) and its monomethyl ether (PSM) are the most important extractives in Scots pine heartwood (Venäläinen et al. 2004). Laboratory decay tests with the brown-rot fungus *Coniophora puteana* (strain BAM Ebw. 15) of heartwood material have shown a strong negative correlation between the concentration of stilbenes and total phenolics for mass loss (Harju and Venäläinen 2006, Heijari et al. 2005, Leinonen et al. 2008). The result suggests that quantifying stilbenes or total phenolics might be used as an indirect measure of natural decay resistance.

The living tissues in the trunk of Scots pine—namely the sapwood, phloem, and the cambium located between them—are constitutively protected on the outside by the bark and on the inside by the heartwood (Franceschi et al. 2005). However, attacks by damaging agents such as insects and their fungal associates trigger additional defensive responses in the form of induced production of secondary compounds and changes in wood anatomy (Lieutier et al. 1989, Nagy et al. 2005, Raffa et al. 2005).

Scots pine seedlings have been shown to produce stilbenes in various types of tissue in response to different kinds of stress factors, such as ultraviolet light (Schoeppner and Kindl 1979), ozone (Rosemann et al. 1991), and infection by fungi (Bonello et al. 1993, Gehlert et al. 1990). In intact sapwood, stilbenes do not occur in detectable amounts. However, even the mere mechanical wounding of Scots pine sapwood (Nilsson et al. 2002) induces the production of chemical defenses. The discolored sapwood induced by mechanical wounding has a similar high-performance liquid chromatography (HPLC) chromatogram as natural heartwood (Nilsson et al. 2002).

In this paper we describe the natural variation in the concentration of constitutive heartwood extractives and present estimates for the genetic parameters related to heartwood characteristics in Scots pine. We have used mechanical wounding to induce production of secondary compounds in the sapwood of 3-year-old seedlings and we present genetic parameters for some chemical and growth traits from that experiment.

Methods and Materials

Heartwood Studies

Study material consisted of two progeny trials with identical half-sib progenies, 53 of which were included in this study (10 trees/progeny). The first 5.4 ha progeny trial area is in Leppävirta (62°25'N, 27°45'E) and the second 5.5 ha area in Savonranta (62°15'N, 29°00'E), both in eastern Finland. The trees were 43 and 45 years old, respectively, at the time of the increment core sampling of heartwood The original design for both trials was 12 randomized blocks, with three rows of three trees per plot, at a spacing of 2×2 m (equivalent to 2,500 trees/ha).

The diameter at breast height (DBH) was measured, and 5 mm increment cores were drilled radially through the stem at about breast height in Savonranta, and in Leppävirta at about 90 cm. The pith-including cores were placed in plastic tubes and stored in darkness at -20 °C. The boundary between the sapwood and heartwood was marked on the increment cores under UV light, and the radius of the heartwood was measured from both sides of the pith. From each increment core, sections covering annual rings five to eight counted from the pith were separated from both sides of the pith. The aim was to obtain heartwood annual rings with the same cambial age. One section was used for the Folin–Ciocalteu (FC) assay to measure the concentration of total phenolics, and the other section was used for gas chromatography – mass spectrometry (GC–MS) analysis of stilbenes. The heartwood samples for the chemical analyses were oven dried at 60 °C for 24 h, weighed, and stored in a desiccator prior to grinding with an analytical mill (Kinematica). The milled samples were stored in darkness in sealed test tubes and glass bottles at -20 °C.

Mechanical Wounding of the Seedlings

The seeds were collected in October 2000 from 18 unrelated trees from a progeny trial in Mäkrä (Kerimäki, Mäkrä, 61°50'N, 29°23'E), the heartwood of which had earlier been studied (Harju et al. 2003, Tiitta et al. 2003, Venäläinen et al. 2004). Seeds from each known mother tree comprised a half-sib family, where the fathers originated from an unknown stochastic pollen pool. The seedlings were grown for 3 years in a greenhouse at the Punkaharju Research Unit (61°48'N, 29°19'E) of the Finnish Forest Research Institute.

A total of 10 seedlings from each half-sib family were wounded on April 2004, when elongation of the buds of the seedlings had just begun. Ten seedlings of each family were left unwounded as controls. When wounding, about 10 holes were drilled through the stem with a 2.5 mm drill at intervals of about 2 cm along the 2002 internode (2-year-old segment of the stem). After about 3 months of growth, the wounded internodes were cut apart, the bark and phloem removed, and the internodes frozen at -20°C. Similar sections were cut from the control seedlings. A longitudinal 5 mm section from above and below each wound hole was sampled (fig. 1). The samples were prepared for chemical analysis using the same methods as used for the increment cores. The analysis of stilbenes, resin acids, and lignans in the seedlings was carried out in accordance with the procedure for stilbenes described in Karppanen et al. (2007). More details of the experiment are found in Harju et al. (2009).

Figure 1—(left) Wounding of a 3-year-old Scots pine seedling by drilling holes through the stem, and (right) longitudinal section of the wounded stem under UV light (wavelength 313 nm). The brightly fluorescent areas of the stem were sampled for chemical analysis.

Analysis of Stilbenes and Total Phenolics

The gas chromatography-mass spectrometry (GC–MS) analysis of stilbenes was carried out as described by Karppanen et al. (2007). However, slight changes were made to the procedure. For a detailed description of the accelerated GC–MS analysis used in the study, please contact Tarja Tapanila (tarja.tapanila@metla.fi). The Folin–Ciocalteu assay for measuring total phenolics is described in Harju and Venäläinen (2006). The absorptivity was measured after 20 to 90 min at 735 nm against a mixture containing all reagents except the sample. Tannic acid (Merck; 1.59446.0010) was used as a standard. Therefore, the results are expressed as milligram tannic acid equivalents (TAE) per gram of dry mass of wood.

Estimation of Genetic Parameters

Heritabilities were estimated using the formula $\hat{h}^2 = 4\hat{\sigma}_f^2 / (\hat{\sigma}_f^2 + \hat{\sigma}_e^2)$. True half-sibs and unrelated parents were assumed. Variance components were estimated using a model, where block effects were regarded as fixed and family effects as random effects. The model did not include interaction effect. A mixed procedure of Statistical Package for Social Sciences (SPSS) system with restricted maximum likelihood (REML) technique (SPSS Inc. 2006) was used to estimate the variance components. Genetic correlation between the traits in the same environment (Leppävirta) was estimated as $r_A = \text{cov}_{xy} / (\text{Var}_x \, \text{Var}_y)^{0.5}$ and the genetic correlation between the two environments was estimated applying the formula 22.8 in Lynch and Walsh (1998).

Results and Discussion

Heartwood Studies

For the grading of existing timber or for tree improvement it is important to know the distribution of the stilbene concentration and the concentration of total phenolics in the heartwood among the trees within a single stand. In all the materials studied, the distribution was slightly skewed to the right, which is typical for many biological distributions.

We estimated heritability for some chemical and growth variables (table 1). Quantification of stilbenes is continuing for the progeny trial Savonranta. To date, the results show that for heartwood stilbenes and total phenolics, the prospects of breeding are really promising compared to other traits studied. The heritabilities were quite similar, but the coefficients of additive genetic variation were about half of the ones estimated by Fries et al. (2000) from a full-sib Scots pine progeny trial of about the same age as the trials in our study.

Table 1—Estimates for heritability in narrow sense and the coefficient of additive genetic variation (CV$_A$) for the chemical and growth characteristics of Scots pine

	Heritability (CV$_A$%)		
	Leppävirta		**Savonranta**
	2003	**2009**	**2010**
PS[a]		0.67 (35)[c]	
PSM		0.41 (33)[c]	
PS + PSM		0.56 (33)[c]	
Total phenolics	0.71 (33)[b]	0.52 (26)[c]	0.54 (31)
Density	0.60 (6)	0.58 (7)[c]	0.26 (6)
DBH	0.15 (6)	0.28 (8)[c]	0.12 (5)
Heartwood proportion	0.23 (16)	0.16 (13)[c]	0.16 (11)

[a] PS = pinosylvin, PSM = pinosylvin monomethyl ether.
[b] Harju and Venäläinen (2006).
[c] Partanen et al. (2011).

Genetic correlation between the two environments (Leppävirta and Savonranta), which are located about 100 km from each other, were ≥ 0.80 for the concentration of total phenolics, density, DBH, and the proportion of heartwood. There were evident rank changes between the sites among the families, but there were many families that held their rank position in both environments.

For the progeny trial in Leppävirta, it was possible to estimate the genetic correlation between the measured traits. For the extractives mentioned in table 1, the genetic correlations were ≥ 0.75. Genetic correlation between the concentration of extractives and the proportion of heartwood was around 0.20. Heartwood density and DBH had a negative genetic correlation of -0.35, and they both had unfavorable negative genetic correlation with the concentration of extractives varying from -0.06 to -0.33. However these negative genetic correlations could be handled during the thinning of the actual production stands by cutting the largest trees, which produce the poorest quality of wood.

Induced Stilbenes in Seedlings

In our study, besides the increased amount of resin acids (from the average of 3.4 mg/g in control seedlings to the average of 71.9 mg/g in wounded seedlings), typical heartwood compounds PS and PSM were found (fig. 2) from the brightly fluorescing areas of the mechanically wounded seedlings (Harju et al. 2009). Two lignans, nortrachelogenin (NTG) and matairesinol (MR), typical for Scots pine knot wood (Holmbom et al. 2003), were found as well (fig. 2).
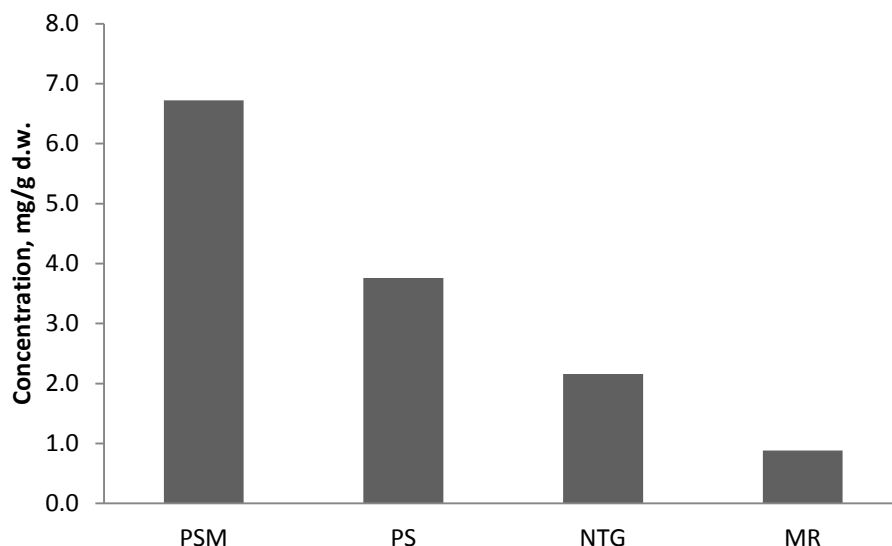
Figure 2—Average concentration of pinosylvin , pinosylvin monomethyl ether , nortrachelogenin , and matairesinol found from the brightly fluorescing areas of the mechanically wounded seedlings.

We were especially interested in quantifying the variation among the seedlings and in the proportion of the heritable variation in response to the wounding. Except for the concentration of PSM and resin acids, the heritabilities in the narrow sense were surprisingly high for all the traits that were measured (table 2). The coefficient of additive genetic variation was higher for the chemical than for the growth traits.

Table 2—Estimates for the heritability in narrow sense ($\hat{h}^2$) and the coefficient of additive genetic variation (CV$_A$) for growth and chemical traits induced by mechanical wounding of 3-year-old seedlings (Harju et al. 2009)

Variable	n	Heritability in narrow sense, $\hat{h}^2$ (SE)	Coefficient of additive genetic variation, CV$_A$, %
PS[a]	175	0.71 (0.36)	29
PSM	176	0.35 (0.25)	15
NTG	175	0.73 (0.37)	37
MR	171	1.03 (0.46)	52
Sum of resin acids	176	0.31 (0.24)	21
Height	180	0.84 (0.40)	10
Diameter of the sampled section[b], mm	180	0.64 (0.34)	8

[a] PS = pinosylvin; PSM = pinosylvin monomethyl ether; NTG = nortrachelogenin; MR = matairesinol.
n = the number of seedlings. Number of half-sib families was 18.
[b] Measured from the middle of the section.

In this material, there was no phenotypic correlation between the growth traits and the concentration of extractives. Because of the small number of studied families, it was not possible to estimate genetic correlation. However, correlation of family means was estimated. Except for resin acids, the family mean correlation between chemical and growth traits was negative, varying from -0.22 to -0.39 and did not differ statistically significantly from zero (n = 18). For resin acids, the correlation was positive, but did not differ statistically significantly from zero (Harju et al. 2009).

If mechanical wounding of the seedlings induces a reaction related to the extractive composition of their mother's heartwood, then wounding could be utilized in developing an early testing method in the breeding of durable Scots pine heartwood. Thus, we also estimated heritability from the regression of the offspring mean values based on their mothers, i.e., regression of the induced concentration of extractives in the xylem of the seedlings on the concentration of extractives in the

heartwood of their mothers. The heritability value for PS was 0.31, which suggests that there might be a positive connection between the constitutive and inducible synthesis of PS (Harju et al. 2009).

We are now interested in the role of induced production of stilbenes for tree survival and we are looking for proper research tools to approach the topic. In terms of tree breeding, it would be profitable if the constitutive and induced resistance could be selected for simultaneously.

Acknowledgments

Literature Cited

Bonello, P.; Heller, W.; Sandermann, H. Jr. 1993. Ozone effects on root-disease susceptibility and defence responses in mycorrhizal and non-mycorrhizal seedlings of Scots pine (*Pinus sylvestris* L.). New Phytologist. 124: 653–663.

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.

Fries, A.; Ericsson, T.; Gref, R. 2000. High heritability of wood extractives in *Pinus sylvestris* progeny tests. Canadian Journal of Forest Research. 30: 1707–1713.

Gehlert, R.; Schöppner, A.; Kindl, H. 1990. Stilbene synthase from seedlings of *Pinus sylvestris*: purification and induction in response to fungal infection. Molecular Plant-Microbe Interactions. 3: 444–449.

Harju, A.M.; Anttonen, S.; Viitanen, H.; Kainulainen, P.; Saranpää, P.; Vapaavuori, E. 2003. Chemical factors affecting brown-rot decay resistance of Scots pine heartwood. Trees. 17: 263–268.

Harju, A.M.; Kainulainen, P.; Venäläinen, M.; Tiitta, M.; Viitanen, H. 2002. Differences in resin acid concentration between brown-rot resistant and susceptible Scots pine heartwood. Holzforschung. 56:.479–486.

Harju, A.M.; Venäläinen, M. 2006. Measuring the decay resistance of Scots pine heartwood indirectly by the Folin-Ciocalteu assay. Canadian Journal of Forest Research. 36: 1797–1804.

Harju, A.M.; Venäläinen, M.; Saranpää, P.; Laakso, T. 2009. Mechanical wounding of the Scots pine seedlings results in stilbene and lignan biosynthesis. Tree Physiology. 29(1): 19–25.

Heijari, J.; Nerg, A.M.; Kaakinen, S.; Vapaavuori; E.; Raitio, H.; Levula, T.; Viitanen, H.; Holopainen, J.K.; Kainulainen, P. 2005. Resistance of Scots pine wood to brown-rot fungi after long-term forest fertilization. Trees. 19: 728–734.

Holmbom, B.; Eckerman, C.; Eklund, P.; Hemming, J.; Nisula, L.; Reunanen, M.; Sjöholm, R.; Sundberg, A.; Sundberg, K.; Willför, S. 2003. Knots in trees - a new rich source of lignans. Phytochemistry Reviews. 2: 331–340.

Karppanen, O.; Venäläinen, M.; Harju, A.M.; Willför, S.; Pietarinen, S.; Laakso, T.; Kainulainen, P. 2007. Knotwood as a window to the indirect measurement of the decay resistance of Scots pine heartwood. Holzforschung. 61: 600–604.

Leinonen, A.; Harju, A.M.; Venäläinen, M.; Saranpää, P.; Laakso, T. 2008. FT-NIR spectroscopy in predicting the decay resistance related characteristics of solid Scots pine (*Pinus sylvestris* L.) heartwood. Holzforschung. 62: 284–288.

Lieutier, F.; Cheniclet, C.; Garcia, J. 1989. Comparison of the defense reactions of *Pinus pinaster* and *Pinus sylvestris* to attacks by two bark beetles (Coleoptera: Scolytidae) and their associated fungi. Environmental Entomology. 18: 228–234.

Lynch, M.; Walsh, B. 1998. Genetics and analysis of quantitative traits. Sunderland, MA: Sinauer Associates, Inc.

Nagy, N.E.; Krokene, P.; Solheim, H. 2005. Anatomical-based defense responses of Scots pine (*Pinus sylvestris* L.) stems to two fungal pathogens. Tree Physiology. 26: 159–167.

Nilsson, M.; Wikman, S.; Eklund, L. 2002. Induction of discolored wood in Scots pine (*Pinus sylvestris*). Tree Physiology. 22: 331–338.

Partanen, J.; Harju, A.M.; Venäläinen, M.; Kärkkäinen, K. 2011. Highly heritable heartwood properties of Scots pine: possibilities for selective seed harvest in seed orchards. Canadian Journal of Forest Research. 41(10): 1993–2000.

Raffa, K.F.; Aukema, B.H.; Erbilgin, N.; Klepzig, K.D.; Wallin, K.F. 2005. Interactions among conifer terpenoids and bark beetles across multiple levels of scale: an attempt to understand links between population patterns and physiological processes. In: Romero, J., ed. Chemical ecology and phytochemistry of forest ecosystems. Proceedings of the Phytochemical Society of North America. Recent Advances in Phytochemistry. 39: 79–118.

Rosemann, D.; Heller, W.; Sandermann, H., Jr. 1991. Biochemical plant responses to ozone II. Induction of stilbene biosynthesis in Scots pine (*Pinus sylvestris* L.) seedlings. Plant Physiology. 97: 1280–1286.

Scheffer, T.C.; Cowling, E. 1966. Natural resistance of wood to microbial deterioration. Annual Review of Phytopathology. 4: 147–170.

Schoeppner, A.; Kindl, H. 1979. Stilbene synthase (pinosylvine synthase) and its induction by ultraviolet light. FEBS Letters. 108: 349–352.

SPSS Inc. 2006. SPSS 14.0.2. for Windows. Chicago: SPSS Inc.

Taylor, A.M.; Gartner, B.L.; Morrell, J.J. 2002. Heartwood formation and natural durability – a review. Wood and Fiber Science. 34(4): 587–611.

Tiitta, M.; Kainulainen, P.; Harju, A.M.; Venäläinen, M.; Manninen, A.M.; Vuorinen, M.; Viitanen, H. 2003. Comparing the effect of chemical and physical properties on complex electrical impedance of Scots pine wood. Holzforschung. 57: 433-–439.

Venäläinen, M.; Harju, A.M.; Saranpää, P.; Kainulainen, P.; Tiitta, M.; Velling, P. 2004. The concentration of phenolics in brown-rot decay resistant and susceptible Scots pine heartwood. Wood Science and Technology. 38: 109–118.