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Variation in secondary chemistry within a natural population of birch: Effects of genotype, environment and ontogeny.

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Keywords: *Betula pendula*, clonal repeatability, condensed tannins, flavonoids, hydrolysable tannins, intraspecific variation, population, resistance, secondary chemistry, triterpenoids.

The aim of this thesis was to study the foliar and shoot secondary chemical variation due to genotype, environment and ontogeny within a natural population of European white birch (*Betula pendula*) by analysing phenolics and triterpenoids of 30 naturally regenerated 20-year-old parental trees and clonal plantlets originating from those same trees.

The differences between parental trees accounted for most of the variation, both in foliar and shoot phenolics, whereas a high variation in shoot triterpenoids was found both between parental trees and within a tree. The effect of genotype, especially in the quality of secondary chemistry, appeared to be strong. Both the yearly conditions and the developmental stage of bud and leaf greatly affected the secondary chemistry of mature trees.

Studies using clonal plantlets further confirmed the strong genetic control over secondary chemistry in European white birch. The high variability in clonal repeatability values indicated that genetic determination differed, depending on the studied compound or compound group. The effect of environment was also highly dependent on the studied chemical trait; i.e., the accumulation of some compounds was more sensitive to different environmental conditions than others. Furthermore, the responses of individual genotypes to the environment differed from each other. This could be seen as a significant genotype by environment interaction, which indicates phenotypic plasticity in the accumulation of birch secondary chemicals. A comparison between ontogenic stages of European white birch revealed the significant role of ontogeny on some studied compounds; e.g., triterpenoids, which were found to act as anti-feeding components during the juvenile stage of *B. pendula*.

These studies indicate a high temporal and spatial variation in secondary chemistry within a natural population of *B. pendula*, which creates an unpredictable and multidimensional structure of plant quality at the population level; e.g., for herbivores, thus enabling birch populations to adapt to different abiotic and biotic stress factors in highly variable environment.

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ABBREVIATIONS

API	atmospheric pressure ionisation
DAD	diode array detection
DHPPG	3,4'-dihydroxypropiophenone-3- β -D-glucopyranoside
DW	dry weight
ES	electrospray
G x E	genotype by environment interaction
HPLC	high pressure liquid chromatography
LMWP	low-molecular-weight phenolics
MS	mass spectrometry
SIM	selected ion monitoring

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles and previously unpublished results. The articles are referred to in the text by their Roman numerals I-IV.

- I Laitinen, M.-L., Julkunen-Tiitto, R. and Rousi, M. 2000. Variation in phenolic compounds within a birch (*Betula pendula*) population. *Journal of Chemical Ecology* 26: 1609-1621.
- II Laitinen, M.-L., Julkunen-Tiitto, R. and Rousi, M. 2002. Foliar phenolic composition of European white birch during bud unfolding and leaf development. *Physiologia Plantarum* 114: 450-460.
- III Laitinen, M.-L., Julkunen-Tiitto, R., Yamaji, K., Heinonen, J. and Rousi, M. 2003. Variation in birch bark secondary chemistry between clones and within a clone: implications for herbivory by hares. *Oikos* 103: 000-000.
- IV Laitinen, M.-L., Julkunen-Tiitto, R., Tahvanainen, J., Heinonen, J. and Rousi, M. Variation in birch (*Betula pendula*) bark secondary chemistry due to genotype, environment and ontogeny. Submitted for publication.

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1. INTRODUCTION

Plants are known to produce a large number of low molecular weight compounds. While the presence and structures of these chemicals has been recognised only relatively recently, humans have exploited them throughout history as an aid in food catering (e.g., fish and arrow poisons), and for economic purposes such as dyes, perfumes and medicines (e.g. Mann 1987, Waterman and Mole 1994). Due to the remarkable development of analytical methodology; e.g., in gas chromatography (GC) and high-pressure liquid chromatography (HPLC) coupled to mass spectroscopy (MS), it has been possible to characterise even minor components in plants (Rhodes 1994).

Previously, most of these compounds were classified as secondary metabolites because they did not seem to have any clear function in the organisms that produced them. However, more recently, such compounds have been shown to exhibit a diverse spectrum of biological functions (e.g., Waterman 1992, Koes et al. 1994, Strack 1997). Many secondary compounds are now known to be of ecological importance, not only due to their role as protectants from harmful effects of UV-light and other abiotic factors (e.g., Waterman 1992, Koostira 1994, Dixon and Paiva 1995, Bharti and Khurana 1997), but also as a defence against herbivores and pathogens (e.g., Malhotra et al. 1996, Hartley and Jones 1997), as aid to pollination (e.g., Wogt et al. 1994, Taylor and Hepler 1997, Xu et al. 1997) or as signaling molecules (Kerr and McElroy 1993, Klessig and Malamy 1994, Mackerness 2000). Thus many secondary metabolites are known to enhance the prospects of plant survival through an interaction with environment (Waterman 1992).

In contrast to primary metabolites, secondary compounds vary widely in their distribution among plant species (Rhodes 1994). In addition, the intraspecific variation in plant secondary metabolite

composition is considerable (e.g., Harborne and Turner 1984, Bohm 1987). In plants, the synthesis and accumulation of individual secondary compounds reflect different evolutionary stages. Some of these compounds occur only sporadically, whereas others are distributed widely throughout the plant kingdom (e.g., Harborne 1980, Rhodes 1994). Individual secondary compounds can be specific to orders, families, species, and sometimes even intraspecific taxa (e.g., Harborne 1980, Wollenweber and Dietz 1981). Such variation may also occur at the level of an individual plant; i.e., secondary products are not found uniformly throughout the plant but are often limited to particular organs, and even to particular cells and tissues within that organ (e.g., Wiermann 1981, Rhodes 1994).

Two widely distributed groups of secondary compounds that are present in all plants are phenolics and terpenoids, whereas only one third of known plant species contain nitrogen-based metabolites, such as alkaloids, cyanogens or glucosinolates (Harborne 1997).

A phenol is a chemical compound with at least one aromatic ring bearing one or more hydroxyl groups. Many of these compounds occur as different derivatives formed by condensation or addition reactions, thus making a wide variety of chemical compounds found in plants (e.g., Harborne 1980, Strack 1997). Phenolics are important to plants, because they give mechanical support, contribute to flower and fruit colouring, protect against pathogens and herbivores and they are effective in protecting tissues from damaging UV-light (e.g., Strack 1997).

The terpenoids are the largest family of natural plant compounds (Connolly and Hill 1991, Harborne 1997). The alternate name for terpenoids used in the literature is isoprenoids. These compounds are built up of C₅ isoprene units, and the nomenclature of terpenoids reflects the number of isoprene units present (Bramley 1997). Terpenoids have diverse functional roles in plants; e.g., as hormones, photosynthetic

pigments, electron carriers and components of membranes (e.g., Mc Garvey and Croteau, 1995). Moreover, some terpenoids are known to act as plant defence, as attractants for pollinators and as seed dispersers (Harborne 1991).

Previously, several secondary compounds from both of these groups; i.e., phenolics and terpenoids, have been identified and found in pollen, buds, leaves and bark or twigs in birch species (*Betula* sp.) (e.g., Wollenweber and Dietz 1981, Meurer et al. 1988, Taipale and Lapinjoki 1991, Vainiotalo et al. 1991, Meurer-Grimes 1995, Šmite et al. 1995, Julkunen-Tiitto et al. 1996, Ossipov et al. 1997, Keinänen and Julkunen-Tiitto 1998, Keinänen et al. 1999a,b, Salminen et al. 1999, Valkama et al. 2003). In addition, birch species are known to have a characteristic phenolic pattern (Hegnauer 1989), and this interspecific variation in birch secondary chemistry has been used in several chemotaxonomic studies (e.g., Meurer-Grimes 1995, Julkunen-Tiitto et al. 1996, Keinänen et al. 1999b). Even though the production of secondary compounds in plants is influenced by many environmental factors (Waterman and Mole 1989, Waterman 1992, Dixon and Paiva 1995), and by the age of the individual plant; i.e., by ontogeny (Bohm 1987, Bryant and Julkunen-Tiitto 1995, Julkunen-Tiitto et al. 1996), the qualitative and quantitative variation in birch secondary chemistry has been found to be taxonomically significant as a useful tool for species recognition (Julkunen-Tiitto et al. 1996). Due to hybridisation in birches, the secondary chemistry has also been used to find a reliable and quick method for distinguishing different species; e.g., *B. pendula* and *B. pubescens* in older mixed stands (Santamour and Lundgren 1996).

The intraspecific variation in secondary chemistry, both qualitative and quantitative, has been found to be considerable among seedlings and even among genotypes of European white birch (Tahvanainen et al. 1991, Keinänen et al. 1999a). This might be due to differences in

abiotic and biotic factors; e.g., fertilization, defoliation, elevated CO₂, ozone, UV-light, temperature and SO₂-pollution are all known to affect the chemical responses of individual trees, seedlings or clones within the same birch species (Lavola and Julkunen-Tiitto 1994, Lavola et al. 1994, Keinänen et al. 1999a, Lavola, 1998a,b, Saleem et al. 2001, Kuokkanen et al. 2001, 2003, Yamaji et al. 2003). However, it has been proposed that the chemical content of birch seedlings or clonal plantlets is mainly genetically determined (Tahvanainen et al. 1991, Keinänen et al. 1999a).

In the northern boreal zone, shoots and twigs of deciduous trees and shrubs are the primary source of food for hares (*Lepus* sp.) in the wintertime (e.g., Bryant and Kuropat 1980). In Scandinavia, the birch plantations in reforestation areas are under frequent vole and hare feeding (Rousi et al. 1990, Rousi 1990). In addition, birches are food for several insect herbivores (Hanhimäki et al. 1995, Rousi et al. 1997, Tikkanen et al. 2003). For example, herbivory by mammals can alter growth, reproduction, survival, and other fitness components in birch forests (e.g., Bryant et al. 1983 and references therein). Thus, several previous studies have concentrated on the variation and determinants of resistance in birch (e.g., Tahvanainen et al. 1991, Rousi et al. 1997, Laitinen et al. 2002, Pusenius et al. 2002). In addition to the importance of secondary chemistry in birch herbivory resistance, pharmacological effects have also been of recent interest; e.g. as antioxidants, and antimicrobial effects of birch phenolics (Kähkönen et al. 1999, Rauha et al. 2000).

Even though many studies have concentrated on birch secondary chemistry, the knowledge of the variation in secondary chemistry in a natural population is scanty. Many of the studies have been carried out on plants grown under optimal greenhouse conditions or in field-grown plants that were well protected from stress (e.g., by fertilization and irrigation). Due to practical constraints in many studies, the amount of studied trees or clones has also been quite

low, or the age of experimental trees is usually only one or a few years (e.g., Keinänen et al. 1999a, Lavola and Julkunen-Tiitto, 1994, Lavola 1998b, Tegelberg et al. 2001, 2002). In addition, in some studies different seedlings had to be used for subsequent sampling times or as a control treatment, due to destructive methods; i.e., different genotypes were compared. Just what is the effect of intraspecific variation on those results is not clearly known. In addition, there have been only a few studies where both the effect of genotype and environment, and their interaction has been studied (Hakulinen et al. 1995, Keinänen et al. 1999a, Veteli et al. 2002, Orians et al. 2003, Yamaji et al. 2003) in order to find out the possible differences in responses of individual genotypes to environment. Thus the objectives of this thesis were:

- (1) to study the secondary chemical variation in a naturally regenerated, 20-year-old *Betula pendula* Roth population by analysing the variation within a tree (II, IV), between individual trees (I, IV), between different developmental stages during the growing season (II) and between years (I).
- (2) to study the effect of genotype (III, IV), environment and genotype by environment interaction (IV) on chemical variation by using micropropagated plantlets from the parental trees of a natural birch population.
- (3) to study the effect of ontogeny on the secondary chemistry of European white birch by comparing mature trees and young micropropagated plantlets from the same trees (IV and unpublished data).
- (4) to test the variation among clones in bark secondary chemistry in relation to herbivory by hares (III).

2. MATERIALS AND METHODS

2.1. Plant material

European white birch (*B. pendula*) plants were used in all of our studies (I-IV). This species is widely distributed throughout the Northern hemisphere (Ashburner, 1993). In Finland, European white birch is common with the exception of the northernmost regions of Lapland (Nylén 1995, Hämet-Ahti et al. 1998). European white birch has two types of shoots; short and long. Short shoots usually have two or three leaves, which burst fairly simultaneously in the spring, whereas in long shoots new leaves are produced throughout the growing season (Maillette 1982). Differences in the age of short and long shoot leaves may affect their chemical composition. To avoid this variation only short shoot leaves were used as leaf samples.

The forest stand selected for our studies (I, II) and subsequent material for micro-propagation, was a naturally regenerated, mixed *B. pendula* and *B. pubescens* forest situated in Punkaharju, southeastern Finland (61°48' N, 29°18' E). The area was logged in 1979 and seeded from naturally regenerated forests. In the spring of 1997, thirty parental trees were randomly selected from this stand. Trees were ca. 20 years old and, on average, 12 m tall. Micropropagation of parental trees began during the summer of 1997. The aim was to propagate 300 plantlets from each parental tree. An amount of over 150 plantlets was achieved for 19 trees. Prior to our studies, all plantlets had the same growth environment; i.e., plantlets were placed randomly in a greenhouse and in field conditions, and they had the same amount of fertilisation, etc. The plant material used in studies I-IV, and for unpublished results can be seen in Table 1.

Table 1. Plant material used in different studies.

PLANT MATERIAL	STUDIES				
	I	II	III	IV	unpublished
Parental trees	all 30 trees, full-grown leaves, air-dried	10 randomly selected trees, buds and leaves, freeze-dried		all 30 trees, current years growth, twigs, air-dried	
Micropropagated plantlets			1 year old plantlets, 19 clones, winter dormant twigs, fresh frozen	3 year old plantlets, current year's growth, 14 clones, twigs, air-dried	2 year old plantlets, full-grown leaves, 2 clones, air-dried

2.2. Field experiments

2.2.1. Study on effects of genotype, environment and ontogeny

A field experiment was established in June 1999 to study the effect of genotype, environment and genotype by environment interaction on the variation in birch shoot secondary chemistry. Micropropagated plantlets were planted on four study-areas in Punkaharju and Parikkala, southeastern Finland. For each study-area, plantlets from 14 clones were planted in 6 to 9 blocks, four plantlets / clone / block. Each clone was randomly situated within a block. One twig of the current year's growth per clone from six randomly selected blocks per study area was collected in June 2001. At the same time, samples from parental trees were collected to compare the micro-propagated material to parental trees (IV).

2.2.2. Hare feeding experiment

An open-field feeding experiment was conducted in Punkaharju, south-eastern Finland, in ten forest-stands, in order to get

information about the palatability of our study clones (III). For each forest stand a feeding station was established by pseudo planting one-year-old micropropagated plantlets in the snow from nineteen clones in January 1999. The feeding activity by hares (*Lepus timidus*) was monitored for 2.5 months and results were expressed as number of eaten twigs per clone (III).

2.3. Chemical analysis

Plant material, a composite sample of thirty leaves (I), individual buds or leaves (II), removed bark (III) and stem pieces (IV) were extracted with 100% methanol for chemical analysis of phenolics. For the analysis of triterpenoids, diethyl ether was used for whole extraction (III), or after methanol extraction (IV). Depending on the sample sizes, large volume (10 ml for I, III and unpublished data; 15 ml for IV) or small volume (0.5 ml) extractions (II) were made by using a clipping homogenizer. The extracts were evaporated to dryness in a vacuum evaporator (I, III, IV) or under nitrogen (II) and stored at -20°C .

The low molecular weight phenolics

(LMWP) were analysed by high-pressure liquid chromatography (HPLC) with gradient elution, using diode array detection (DAD) (I-IV and unpublished results). The identification of compounds was based on comparisons of the retention times and the spectral characteristics, as previously described (Julkunen-Tiitto et al. 1996, Keinänen and Julkunen-Tiitto 1998). Further identification of the compounds was made by high-pressure liquid chromatography - mass spectrometry (HPLC-MS) analysis (II), as described in Julkunen-Tiitto and Sorsa (2001). For analysing individual triterpenoids from birch bark extracts a modified method from Taipale et al. (1993) was used in paper III, and in study IV a new HPLC-API-ES-method was used (Julkunen-Tiitto et al. in preparation). The identification of triterpenoid components in samples was based on mass spectra (m/z), and selected ion monitoring (SIM) quantification was based on a purified papyriferic acid. The amount of condensed tannins was determined from the extracts (I, III, IV and unpublished data) and from the residue (I, unpublished data) by a butanol-HCl test, as described in Porter et al. 1986 and Hagerman 1995.

3. RESULTS AND DISCUSSION

3.1. Secondary chemistry of birch leaves and bark

The leaves and bark of *Betula* sp. are known to contain various secondary compounds, such as, phenolic glycosides, phenolic aglycones, tannins and triterpenoid compounds (Reichardt 1981, Wollenweber and Dietz 1981, Taipale and Lapinjoki 1991, Vainiotalo et al. 1991, Taipale et al. 1993, 1994, Julkunen-Tiitto et al. 1996, Ossipov et al. 1997, Keinänen and Julkunen-Tiitto 1998, Lavola 1998a,b, Keinänen et al. 1999b, Riipi et al. 2002, Šmite et al. 1995, Salminen et al. 2002). The compositions of the phenolic (Fig. 1) and triterpenoid compounds (Fig. 2) found

in our studies were consistent with earlier studies on *B. pendula* (e.g., Vainiotalo et al. 1991, Julkunen-Tiitto et al. 1996, Keinänen et al. 1999a). The main phenolic compounds present in full-grown leaves were myricetin-3-galactoside and quercetin-3-galactoside and the main phenolic compound group was condensed tannins (I). In European white birch bark the main phenolic compounds were (+)-catechin, chlorogenic acid and quercetin-3-galactoside, and the main phenolic compound group was condensed tannins (III, IV) (Fig. 1). The main triterpenoids were papyriferic acid and pendulic acid (III, IV) (Fig. 2).

3.2. Variation in foliar chemistry

3.2.1. Qualitative variation due to genotype, environment and ontogeny

Genotype, environment and ontogeny, are known to have an effect on plant secondary chemistry (e.g. Coleman and Jones 1991). A previous study where the effects of defoliation, fertilization and genotype on foliar chemistry of *B. pendula* clones were studied suggested that the chemistry of the leaves was mainly controlled by genotype; i.e., clones could be grouped by their phenolic profiles (Keinänen et al. 1999a). The high variation in quality of foliar phenolics between parental trees in our natural study population (I, II), and the similarity between chemical profiles of full-grown leaves between years (I) and also between full-grown leaves and leaves collected on mid-May (I, II) supports the importance of a genetic effect.

The chemical profile may also change due to plant ontogeny or phenology (Bohm 1987, Bryant and Julkunen-Tiitto 1995). In *Alnus glutinosa* leaves the flavonoid aglycone pattern remained unchanged over the growing season, and even over successive years (Danier et al. 1991). Conversely, for mountain birch, the developmental stage of the leaf greatly affected the chemical profile (Nurmi et al. 1996, Riipi et al. 2002), which was

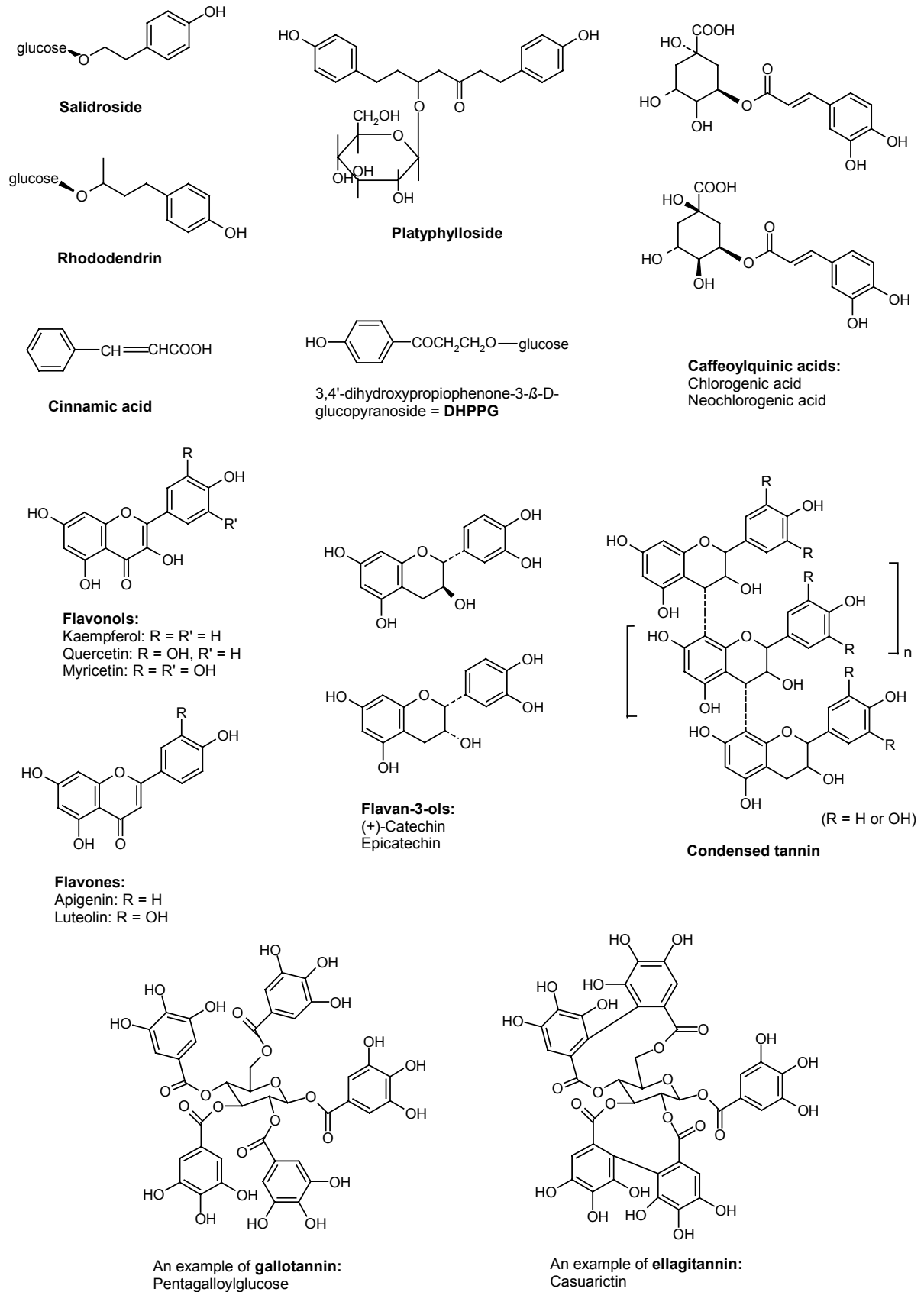


Figure 1. The basic structures of phenolics detected in European white birch (*Betula pendula*) leaves and bark.

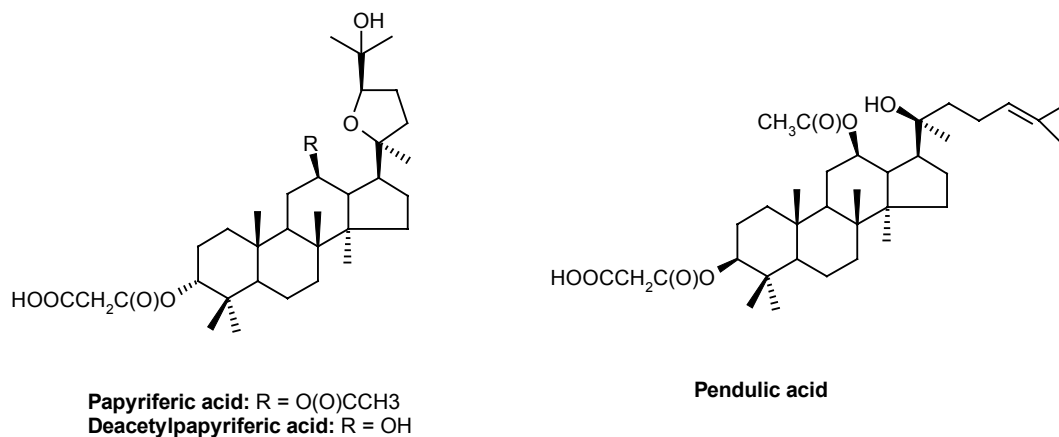


Figure 2. The structures of triterpenoids detected in European white birch (*Betula pendula*) bark.

also the case for European white birch buds and leaves (II). Both in mountain birch and European white birch, early developmental stages contain many individual hydrolysable tannin and flavonoid aglycone compounds whereas, older leaves contain mainly myricetin and quercetin derivatives (Riipi et al. 2002, I, II) and condensed

tannins (Riipi et al. 2002).

In contrast to phenological differences ontogeny does not seem to have a strong effect on the quality of *B. pendula* leaves; i.e., there were strong similarities in the chemical profiles between parental trees and micropropagated plantlets (Fig. 3, unpublished data), which was also in the

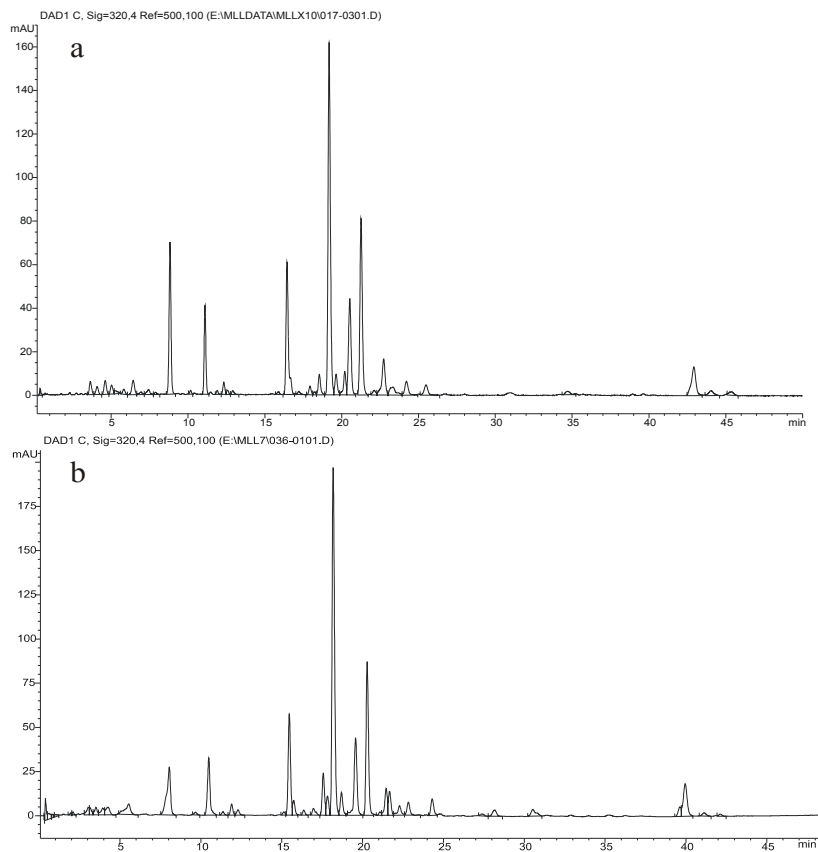


Figure 3. HPLC-grams for a) leaves from parental tree number 16 in 1998 and b) clonal plantlet (clone 16) leaves in 2000.

study of *Alnus glutinosa* leaves (Daniere et al. 1991). Thus, it seems that the stability in foliar phenolic profiles of individual trees can be used as a good tool for chemotaxonomic recognition (I). However, the dependence of foliar phenolic quality on the developmental stage (II) must be taken into account when comparing individual trees.

3.2.2. Quantitative variation within a population: annual and seasonal effects

The variation found in the concentrations of foliar phenolics of birch (*Betula* sp.) species, both in space and time, may be attributed not only to the individual genotypes, but also to the differences in the amount of leaf damage, seasonal change and available nutrients (Suomela et al. 1995, Nurmi et al. 1996, Kause et al. 1999, Keinänen et al. 1999a). In our studies, a high variation in the concentrations of individual secondary compounds in buds and leaves was found between individual trees within a birch population (I, II), whereas the intra-tree variation was small compared to variation between trees (II). Thus, the chemical composition between leaves from the same level of tree canopy is quite homogenous.

A clear annual difference was found for all studied phenolic compounds and compound groups (I). In addition, the individual trees responded differently to yearly conditions, suggesting that the resistance of an individual tree is dependent on the specific environmental conditions (I). Both biotic and abiotic factors are known to affect the foliar chemistry in birch (e.g. Lavola et al. 1994, Keinänen et al. 1999a, Tegelberg et al. 2001, 2002, Saleem et al. 2001, Kuokkanen et al. 2001, 2003, Yamaji et al. 2003) and individual genotypes may have different thresholds for induced responses (e.g., Coleman and Jones 1991). Thus, the annual differences in foliar secondary chemistry in our study may be mostly due to the differences in weather conditions between the study years or different degrees of herbivory.

The chemical quantity of phenolics in birch species also varies significantly during the seasonal development of leaves (Baldwin and MacLean 1987, Nurmi et al. 1996, Riipi et al. 2002, II). In our study, clear main trends in phenolic concentrations were found during bud unfolding and leaf development (II). It has been proposed that the young leaves are of greater value to plants (Krischik and Denno 1983, Harper 1989) and offer greater nutritional quality to herbivores than older leaves (Ayers and Mac Lean 1987, Stamp and Bowers 1990). Thus, it would be an effective strategy for a plant to produce phenolics for defence against herbivores in young leaves. However, in *B. pendula* the youngest leaves and buds did not always contain higher concentrations of individual phenolic compounds compared to mature leaves (II), which is consistent with suggestions that the direction of seasonal trends also depends on individual phenolic compound or compound groups (Loponen et al. 1997, Ossipov et al. 1997, Estiarte et al. 1999, Riipi et al. 2002). In our study, the concentrations of hydrolysable tannins, flavonoid aglycones and catechin derivatives declined during bud unfolding and leaf development, whereas the concentrations of DHPPG, phenolic acids and flavonol glycosides showed increasing trends (II). The rapid changes in the early developmental stages; e.g., for gallotannins and the increase and subsequent slight decrease in flavonoid glycosides in our study (II), supports the results of Riipi et al. (2002).

The production of individual phenolic compounds at different developmental stages of plant growth has been predicted by recent theories; e.g., the amounts of compounds are predicted by their biosynthetic origin (Haukioja et al. 1998), by competition between protein and phenolic synthesis due to common precursor (Jones and Hartley 1999) or changes in defence compounds are explained by adaptive responses to insect feeding behaviour (Haukioja 2003). The use of concentrations of defensive

compounds in allocation studies has recently been argued (Koricheva 1999). The chemical concentrations are thought to be useful when studying the herbivory, because concentrations are relevant estimates of plant quality for herbivores. In allocation studies, however, the use of absolute content should also accompany the use of concentrations (Koricheva 1999). In a leaf developmental study with mountain birch, Riipi et al. (2002) used graphical vector analysis (GVA) (e.g., Timmer and Stone 1978, Haase and Rose 1995, Koricheva 1999, Fig 4.) to obtain further information on the allocation during seasonal changes. The GVA for results from the bud and leaf development study (II) showed that a decrease in total gallotannins during the growing season was due to both a decrease in biosynthesis and dilution effect (Fig. 5, GVA for three study trees). In addition, a decrease in the amounts per leaf suggests that gallotannins were either catabolized or otherwise transformed (Fig. 5), which also happened in the leaves of mountain birch (Riipi et al. 2002). In addition, the observed trend; i.e., an increase and subsequent slight decrease during leaf development for quercetin derivatives was due to their increased biosynthesis at the beginning of

development, and due to a dilution effect at the later developmental stages (Fig. 5, GVA for three study trees).

Trends in both hydrolysable tannins and flavonol glycosides followed predictions derived from the hypothesis using biosynthetic origin; i.e., during rapid growth, plants are supposed to use compounds which do not compete directly with protein synthesis, such as hydrolysable tannins (II). On the contrary, catechin derivatives and flavonoid aglycones, for example, had an opposite trend from the predictions. Thus, these results do not totally support the idea of competition between protein and phenolic syntheses at the level of individual compounds or compound groups (II). When explaining the results of secondary compound accumulation and allocation by different theories, it must be remembered that secondary compounds may undergo turnover. Thus, their accumulation is affected not only by synthesis, dilution or competition with protein synthesis but also by turnover (Rhodes 1994). In addition, at a given developmental stage of an organ, a large increase in the accumulation rate does not necessarily indicate synthesis of a product *de novo*, but could be the result of translocation of a compound (Wiermann

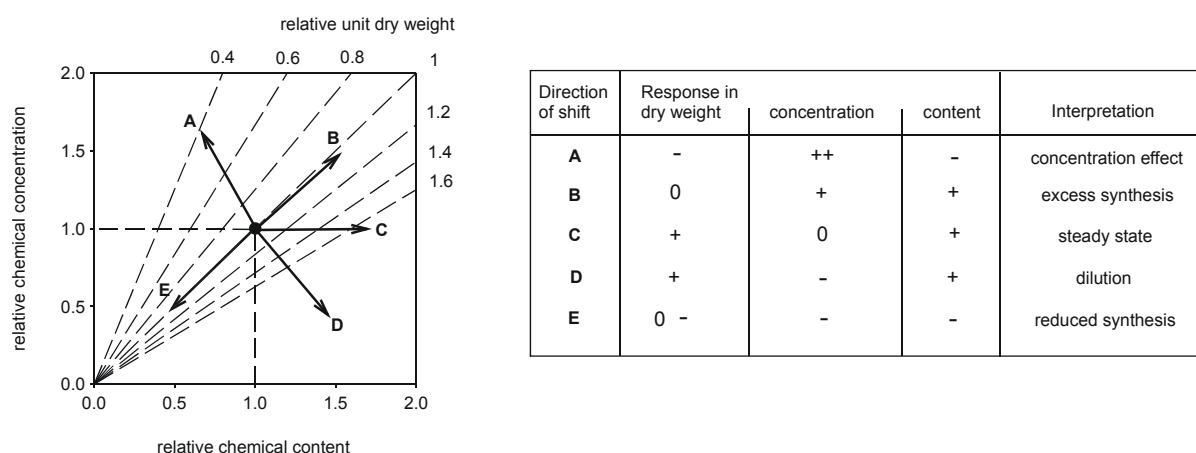


Figure 4. Interpretation of directional shifts in chemical concentration, chemical content and dry weight. Modified from Timmer and Stone (1978), Haase and Rose (1995) and Koricheva (1999). Diagonal lines indicate relative plant unit biomass; the reference point coordinate has a value of (1,1,1).

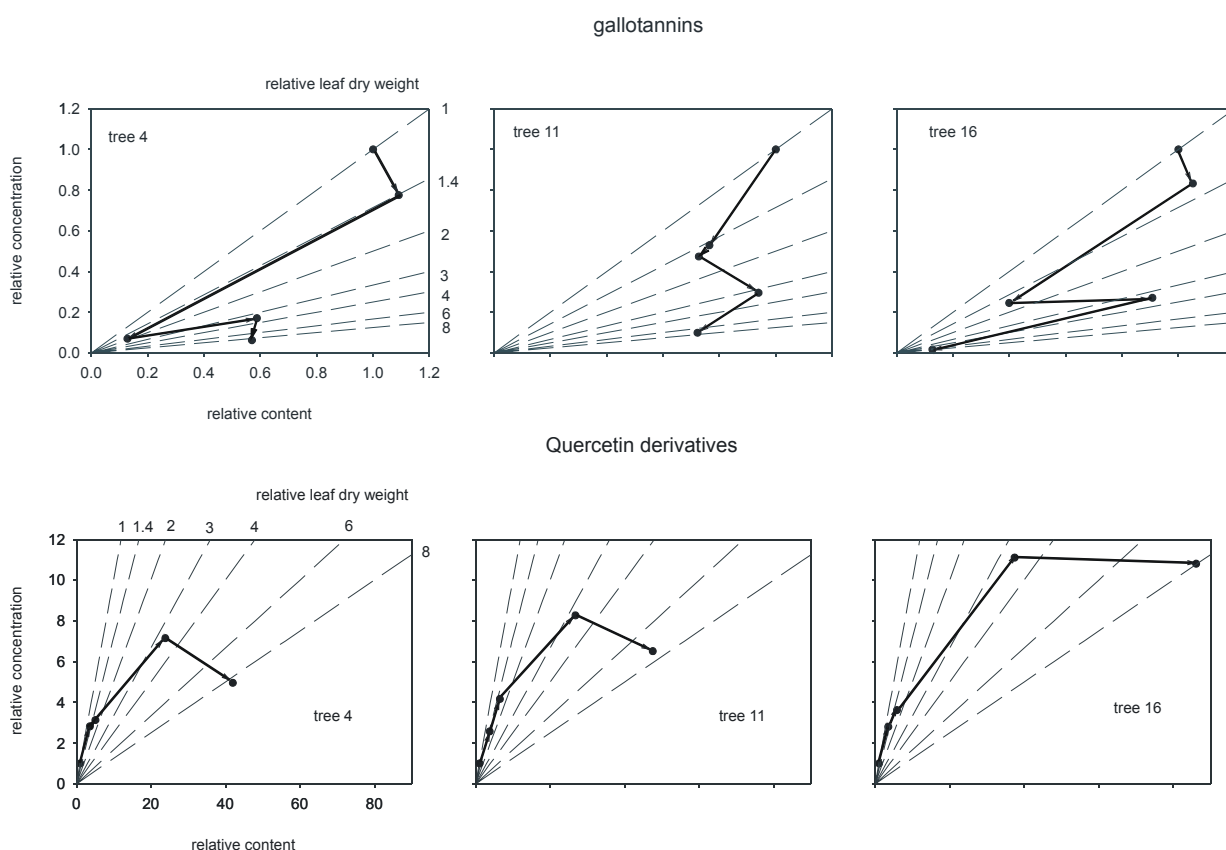


Figure 5. Effects of the developmental stage on the concentration and content of gallotannins and quercetin derivatives. The first developmental stage was used as a reference point (1,1,1).

1981). Furthermore, especially when energy costs and defence are thought different models lack e.g. the costs of enzymes needed for synthesis and storage, the easiness or toughness of metabolism and turnover of compound (Gershenson 1994, Berenbaum 1995).

3.2.3. Ontogeny and secondary chemical concentrations

One reason for intraspecific variation is that the secondary chemistry can change considerably during plant ontogenic development. Especially juvenile stages are thought to be better defended than mature stage of the same species (Bryant and Julkunen-Tiitto 1995). When the concentrations of foliar secondary chemistry between parental trees (I) and

micropropagated plantlets (unpublished data) was compared (Table 2), a clear difference was found in the amount of condensed tannins; i.e., young plantlets had two times more condensed tannins in their full-grown leaves than mature trees. Also a slight difference between ontogenic stages was seen for myricetin-3-glucoside+myricetin-3-glucuronide and quercetin derivatives (Table 2).

The age of the micropropagated plantlets was 2 years, thus weak differences between plantlets and parental trees may be due to the fact that 3-year-old saplings, trees of 1.5-3 m in height and 20-year-old trees seem to be quite similar in their chemical compositions (I). However, when parental trees and clonal plantlets are compared, caution is needed due to different growing conditions.

Table 2. An example of the variation in leaf phenolic compounds. Results are for genotypes 15 and 16 from study I. Also some unpublished (unpub) data is included. DHPPG = 3,4'-dihydroxypropiophenone-3- β -D-glucopyranoside; nchla = neochlorogenic acid; Mgal = myricetin-3-galactoside; Mglu = myricetin-3-glucoside + myricetin-3-glucuronide; Qgal = quercetin-3-galactoside; Qglu = quercetin-3-glucoside + quercetin-3-glucuronide; Cond.tannins = condensed tannins analysed from both the extract and residue. Results are expressed as mg per g (DW). The concentration of composition sample from 30 leaves (parental trees) or an average \pm SE (clonal plantlets) is shown.

Tree /clone	Sample / article	year	Plant age	DHPPG	nchla	Mgal	Mglu	Qgal	Qglu	Cond. tannins
T15	full-grown leaf / I	1997	20	3.43	1.47	5.15	1.77	9.06	3.15	40.22
T15	full-grown leaf / I	1998	20	4.43	1.86	6.65	2.49	13.32	5.15	44.43
C15	full-grown leaf / unpub	2000	2	4.47 \pm 0.31	1.00 \pm 0.04	6.63 \pm 1.04	3.57 \pm 0.48	13.95 \pm 0.49	6.31 \pm 0.02	104.6 \pm 4.5
T16	full-grown leaf / I	1997	20	0.59	0.00	2.63	0.36	8.00	0.52	47.78
T16	full-grown leaf / I	1998	20	1.99	0.00	6.32	0.62	10.58	0.74	81.65
C16	full-grown leaf / unpub	2000	2	2.34 \pm 0.05	0.12 \pm 0.02	5.73 \pm 0.38	0.87 \pm 0.00	15.69 \pm 0.66	1.13 \pm 0.01	137.4 \pm 3.3

3.3. Variation in the secondary chemistry of birch bark

3.3.1. Variation within a natural population, mature trees

In previous studies, a high variation in birch bark secondary chemistry was found between different species (e.g., Taipale et al. 1994, Julkunen-Tiitto et al. 1996) and among seedlings of *B. pendula* (Tahvanainen et al. 1991), but information on the intraspecific variation within a natural population has been lacking. A high variation between parental trees was found for bark secondary chemistry within our study population (IV and Table 3). On the contrary, variation within an individual tree was quite low for all of the studied compounds, except for pendulic acid derivatives and total terpenoids. The between tree variation accounted less than

30% of the total variation in terpenoids (IV and Table 3).

These results are in accordance with our leaf chemistry study (II), in which most of the phenolic variation was explained by between tree differences. The yearly conditions, nutrients, UV-radiation, temperature, and developmental stage are all known to affect the chemical variation in European white birch (Keinänen et al. 1999a, Tegelberg et al. 2001, 2002, Kuokkanen et al. 2001, I, II). In addition, variation in secondary chemistry occurs at the individual plant level; i.e., the compounds differ depending on plant organ (e.g., Rhodes 1994, Keski-Saari and Julkunen-Tiitto 2003). In spite of the many sources of variation differing between these two studies, the results of foliar chemistry (II) fit well to confidence limits of shoot chemistry results (IV).

3.3.2. Effect of genotype and clonal repeatability

The bark secondary chemistry among European white birch seedlings is claimed to be mainly genetically controlled (Tahvanainen et al. 1991). In our studies, there were significant differences between studied clones concerning bark chemistry (III, IV), which suggests that the effect of genotype is strong. However, the within a clone variation was also high; e.g., for triterpenoids, even though the plantlets were grown under greenhouse conditions to minimize the effect of environmental variation (III). In spite of the different amounts of clones used, different growing conditions and different developmental

stages, the results from one-year-old (III) and three-year-old plantlets (IV) are similar, especially for chemical traits with high clonal repeatability (Table 3); e.g., for quercetin - 3 - galactoside, quercetin - 3 - glucoside + quercetin - 3 - glucuronide, chlorogenic acid, caffeoyl quinic acids and cinnamic acids. These results reinforce the strong genetic basis for the accumulation of birch shoot secondary compounds.

When the clonal repeatability (heritability in broad sense) was calculated, there was a high variation that depended on analysed compound and compound group (Table 3 and IV). The differences in heritability between individual secondary compounds are thought to be due to different origins and different bioactivities

Table 3. Variations between clones are presented as a proportion of total variation and variations between trees are presented as a proportion of the total variation with 95% confidence limits (Searle, 1997) and clonal repeatability = $V_G / V_P \pm SE$ (Falconer 1989, Dickerson 1969) for clonal plantlets. Results are from studies III and IV. † = DHPPG was found only in tree 17.

Compound / Compound group	variation between clones (III)	variation between parental trees (IV)	95% confidence limits (IV)	clonal repeatability (IV) \pm SE (IV)
(+)-catechin	0.56	0.73	0.60, 0.84	0.12 0.07
Quercetin-3-galactoside	0.49	0.65	0.51, 0.79	0.50 0.21
Quercetin-3-glucoside, Quercetin-3-glucuronide	0.73	0.84	0.75, 0.91	0.75 0.30
Quercetin-3-arabinoside	0.80	0.66	0.52, 0.80	0.33 0.14
Chlorogenic acid	0.89	0.88	0.80, 0.93	0.60 0.25
Rhododendrin	0.70	0.47	0.30, 0.65	0.35 0.16
Platyphylloside	0.67	0.71	0.57, 0.83	0.19 0.09
Salidroside	0.47	0.76	0.64, 0.86	0.24 0.12
DHPPG	0.62	†	†	0.74 0.30
Papyriferic acid	0.35	0.68	0.54, 0.81	0.48 0.20
Pendulic acid	0.50	0.27	0.12, 0.47	0.36 0.17
Catechin derivatives	0.37	0.73	0.60, 0.84	0.13 0.07
Quercetin derivatives	0.60	0.67	0.53, 0.80	0.32 0.14
Caffeoyl quinic acids	0.88	0.86	0.78, 0.92	0.60 0.25
Cinnamic acid	0.71	0.78	0.67, 0.87	0.72 0.29
Flavonoid aglycones	0.63	0.62	0.47, 0.77	0.25 0.11
LMWP	0.30	0.75	0.63, 0.85	0.12 0.06
Condensed tannins	0.79	0.60	0.45, 0.75	0.25 0.11
Total triterpenoids	0.38	0.29	0.13, 0.48	0.44 0.20

of compounds, and/or the intensity of selection and length of selection time (Falconer 1989, Orians et al. 1996). In other words, a long and intense selection is thought to lead to low heritability values over time (Falconer 1989), and life history traits often show low heritability (Mosseau and Roff 1987, Falconer 1989). Thus, the rather low clonal repeatability found for condensed tannins (IV), for example, may be due to the high bioactivity (e.g., Waterman 1988, Hagerman et al. 1998, Kraus et al. 2003) and ancient origin (e.g., Seigler 1998). Even though the clonal repeatability (i.e., the broad sense heritability), indicated that a genetic basis for bark chemistry was especially strong for many compounds (IV), one must bear in mind that clonal repeatability is the upper limit of heritability, because with clonal material the additive and non-additive components of genotypic variance (V_G) cannot be separated (e.g., Falconer 1989). The additive genetic variation can provide a measure of breeding value of individuals. Thus, to determine possibilities for selection to seed production for forestry purposes, the additive genetic variation and genetic correlations for these traits should be studied among other things (e.g., Falconer 1989, Talbert 1992).

3.3.3. Effect of environment and environmental sensitivity

Many environmental factors are known to affect the secondary metabolism of deciduous trees (e.g., Hakulinen et al. 1995, Keinänen et al. 1999a, Saleem et al. 2001, Tegelberg et al. 2001, Keski-Saari and Julkunen-Tiitto 2003, Yamaji et al. 2003). The effect of growing environment in our studies could be seen as differences between greenhouse grown plantlets of the same clone (III) and as the differences between plantlets of the same clone, both within and between growing sites (IV). For some phenolic compounds; e.g., (+)-catechin and chlorogenic acid, there was a significant effect of growing site (IV), but no genotype by environment interaction

(G x E), whereas, a significant G x E was found for several studied traits, especially for triterpenoids, indicating the specialization of studied genotypes (IV). These results support previous studies where significant genotype-fertilization, genotype-defoliation and genotype-ozone interactions have been reported for some of the foliar phenolic compounds in *B. pendula* (Lavola et al. 1994, Keinänen et al. 1999a, Yamaji et al. 2003). Similar differences in the specialization of genotypes, depending on the studied chemical trait, have also been reported for phenolic accumulation in willows (Hakulinen et al. 1995, Veteli et al. 2002, Orians et al. 2003).

In forestry, superior trees have been selected for both seed and vegetative production over a long time. The difficulty in decision-making is linked to the desired objective; e.g., whether we want to find trees that perform well on a specific site or on a wider range of environments (Talbert 1992). Equivalent responses of genotypes to changes in the environment suggest that different clones are not specialised to certain growing conditions (Via 1984, van Buijtenen 1992) and, thus, a selection for genotypes that perform well on a broad range of environments can be made for these traits. On the other hand, a superior genotype in one environment may not always retain its relative advance in other environments (e.g., Via 1984 and references therein). This sort of genetic variation in phenotypic plasticity can be seen as a significant G x E (Via and Lande 1985, Falconer 1989), which was also observed for triterpenoids in *B. pendula* shoots (IV). In spite of the statistically significant G x E found in our study (IV), the genotypes seem to be relatively stable in their performance at various sites; e.g., in spite of small changes in the rank order, clones 19 and 20 had relatively high concentrations of triterpenoids in all sites (IV). Thus, a selection of some superior clones; e.g., for high papyriferic acid accumulation, seems to be possible.

3.3.4. Effect of ontogeny: parental trees vs. micropropagated plantlets

When the two ontogenic stages of *B. pendula* were compared, the phenolic quality seems to be independent of the ontogenic stage; i.e., the phenolic profile of mature trees and plantlets is similar (IV), as was also the case for the foliar phenolic profile (see section 3.2.1). On the contrary, the triterpenoid pattern of young trees differed from the pattern of parental trees (IV).

There was also a clear effect of ontogeny when the quantity of different compounds between parental trees and plantlets were compared (Table 4, IV). The great ontogenic difference was found especially for triterpenoid concentrations (Table 4, IV), which is in accordance with a previous study on Alaska paper birch, where a 25 fold greater amount of triterpenoid papyriferic acid was found in juvenile stems than in stems collected from mature trees (Reichardt et al. 1984). The triterpenoid result also supports a hypothesis that claims juvenile stages to be more defended than mature stages (Bryant and Julkunen-Tiitto 1995). However, this was not the case for all studied compounds, or compound groups; e.g., parental trees contained more (+)-catechin, salidroside and condensed tannins than tree-year-old plantlets (Table 4, IV). This may be due to different roles of these compounds in plants. For example, catechins are known to have a role as structural elements of condensed tannins (Strack 1997, Seigler 1998), whereas triterpenoids are known to be active anti-feeding compounds in juvenile stages (e.g. Reichardt et al. 1984, III).

When results from one-year-old plantlets (III) were compared with the results in study IV (Table 4), the effect of ontogeny seems to be more complicated, which may be due to different growing conditions and different developmental stages in these two studies. Regardless of these differences, the effect of ontogeny seems to be strong for chlorogenic acid,

DHPPG, triterpenoids and condensed tannins (marked bold in Table 4). The increasing trend due to ontogeny in condensed tannins (Table 4, IV) supports the earlier results (Julkunen-Tiitto et al. 1996).

The amount of terpenoids was lower in one-year old seedlings of *B. pendula* than in one-year-old plantlets (Julkunen-Tiitto et al. 1996 vs. III), whereas the chemistry of one-year-old plantlets and the 3-year-old *B. pendula* seedlings was similar (Tegelberg et al. 2002, III). Furthermore, the secondary compound concentrations differed between 3-year-old seedlings and 3-year-old plantlets; i.e., the concentrations were higher in seedlings than in plantlets, especially for (+)-catechin, rhododendrin and platyphylloside (Tegelberg et al. 2002, IV). This may be due to the fact that plantlets in study III and the seedlings in the study of Tegelberg et al. (2002) were both fertilised and samples were taken at the winter dormant stage, whereas in study IV the plantlets were grown under natural conditions and samples were taken at the end of June. Another explanation for the similarity between 3-year-old seedlings and one-year-old plantlets may be in the effect of micropropagation. Micro-propagation-induced rejuvenation is suggested not to affect similarly all features of micropropagated *B. pendula* trees (Jones et al. 1996), which may also be the case for bark secondary chemistry.

3.4. Secondary chemistry, plant resistance and adaptation

3.4.1. Bark chemistry and herbivory by hares

Different birch species, families and genotypes vary in their resistance to browsers (Rousi et al. 1993, 1997, Bryant and Julkunen-Tiitto 1995, Mutikainen et al. 2000, Laitinen et al. 2002, Pusenius et al. 2002, III). The resistance to browsing is supposed to rely largely on the resin droplets found on the stem surface of *B. pendula* and other birch species, especially

Table 4. Mean concentrations in mg g⁻¹ (DW) for samples taken from parental trees or plantlets of different ages. Minimum and maximum values are also shown. † = DHPPG was found only in tree 17. The effect of different chemotypes between studies was eliminated by using only the results of 14 parental trees or clones, which were the same for all studies. The compounds marked in bold had a clear ontogenic effect when comparing the results of all three age groups.

Compound / Compound group	1-year-old (III)			3-year-old (IV)			20-year-old (IV)		
	mean	min	max	mean	min	max	mean	min	max
(+)-catechin	12.33	6.58	16.45	1.92	0.55	4.32	4.78	1.93	7.64
Quercetin-3-galactoside	3.42	2.08	5.25	1.94	0.93	3.32	2.93	1.69	5.27
Quercetin-3-glucoside + Quercetin-3-glucuronide	0.85	0.21	1.74	0.65	0.09	1.13	0.96	0.19	1.81
Quercetin-3-arabinoside	1.55	0.71	3.37	0.36	0.20	0.64	0.78	0.52	1.40
Neochlorogenic acid	2.32	0.21	4.65	0.18	0	0.62	0.44	0	1.25
Chlorogenic acid	0.77	0.11	2.82	0.99	0	2.64	3.08	0.40	5.50
Rhododendrin	11.41	3.14	16.05	1.11	0	2.43	1.41	0	2.00
Platyphylloside	24.54	15.5	32.50	0.61	0.07	1.69	0.40	0	1.19
Salidroside	2.02	1.03	2.76	0.62	0.32	1.15	2.33	1.12	4.56
DHPPG	0.19	0	0.56	0.70	0	2.14	0.02†	0	0.22
Papyriferic acids	21.94	7.64	42.75	16.59	4.60	30.74	0.14	0.05	0.21
Pendulic acids	9.39	1.88	22.80	13.35	3.88	34.81	0.11	0.03	0.37
Catechin derivatives	19.89	13.45	23.37	2.86	1.18	5.92	7.18	3.89	9.99
Quercetin derivatives	3.29	1.75	5.76	2.81	2.25	5.40	5.93	3.58	9.53
Caffeoyl quinic acids	3.55	0.77	8.21	1.52	0.20	3.19	5.12	1.03	7.99
Cinnamic acid derivatives	0.28	0	0.62	0.41	0	1.45	0.57	0	1.72
Flavonoid aglycones	0.68	0.20	1.22	1.84	0.82	3.09	1.35	0.70	2.45
Condensed tannins	28.12	13.93	43.66	27.72	15.22	41.03	108.26	88.33	126.92
Total triterpenoids	31.53	10.68	64.02	29.32	8.51	64.60	0.25	0.11	0.56

in the juvenile stage (Reichardt et al. 1984, Rousi et al. 1991, Laitinen et al. 2002). The bark and resin are known to contain different phenolics and triterpenoids (e.g. Reichardt 1981, Taipale and Lapinjoki 1991, Vainiotalo et al. 1991, Julkunen-Tiitto et al. 1996), and some are thought to regulate browsing; e.g., papyriferic acid, platyphylloside and condensed tannins are thought to act as feeding deterrents (Reichardt et al. 1984, Sunnerheim et al. 1988, Waterman 1988, Sunnerheim-Sjöberg and Knutsson 1995, Ayers et al. 1997).

In our feeding experiments, hare feeding showed a negative correlation with total triterpenoids and flavonoid-aglycones (III). The main anti-feeding component

found in Alaskan paper birch was a triterpenoid, papyriferic acid (Reichardt 1984). In study III the main triterpenoid compounds were papyriferic acid and pendulic acid. Thus, our results indicate that different triterpenoids may act together as anti-feeding components in juvenile *B. pendula* stems (III). The effect of flavonoid-aglycones as anti-feeding components has not been previously reported, although they have been reported to act as anti-microbial agents (e.g. Malhotra et al. 1996). Flavonoid aglycones have been found in the secretions of birch buds and leaves (Valkama et al. 2003, II), where they are secreted with terpenoid-rich material by trichomes (Wollenweber and Dietz 1981, Valkama et al. 2003). The

secretory glands covering birch shoots are thought to be of the same origin as leaf trichomes (Lapinjoki et al. 1991) and, thus, the result of flavonoid aglycones may be due to the simultaneous secretion of terpenoids and flavonoid-aglycones. The presence of anti-microbial flavonoid-aglycones on the bark surface may also be needed to prevent further damage by pathogens when stems are either damaged or cut. However, more detailed studies are needed to clarify the role of flavonoid-aglycones in birch resistance.

3.4.2. Chemical variation and plant resistance

The long life of a tree increases the likelihood of successful reproduction, thus increasing its fitness. The potential life span of tree is limited by the activities of pathogens, pests and the frequency and intensity of fires, etc. (Lanner 2002). The long life and increase in the size of the plant also increases the complexity of the tree-herbivore system. Because trees have to defend themselves against a wide range of different herbivores, it has been suggested that there cannot be a single general resistance mechanism (e.g., Tikkanen et al. 2003). Birch species are attacked by many different mammalian and insect herbivores (e.g., Rousi et al. 1993, 1997, Hanhimäki et al. 1995) and there are differences between genotypes in their resistance to herbivory (e.g., Rousi et al. 1997, Laitinen et al. 2002, Pusenius et al. 2002, Tikkanen et al. 2003, III). Different responses of herbivore species to changes in host plant quality (van der Meijden 1996, Glynn et al. 2003) further demonstrate the importance of multiple resistance mechanisms needed for plant adaptation.

Even though plants resistance to herbivores is thought to result from different genetically based chemical and structural traits, the phenotypic variation in herbivore resistance is supposed to result primarily from variation in plant nutrient and secondary metabolite concentrations (Herms and Mattson 1992). Unlike primary

metabolites, secondary compounds are generally idiosyncratic in their distribution, both between and within plant species (e.g., Berenbaum 1995). The complexity in plant chemistry within a population could provide additional protection against herbivores, as high variation in chemical composition may decrease the likelihood that herbivores will evolve resistance to certain defence compounds (Schultz 1983, Whitham 1983). In addition to quantitative variation, the qualitative variation may represent an adaptive alternative to increase defence (Pearson 1989, Jones and Firn 1991). If an herbivore is capable of detoxifying a particular secondary metabolite, then increases in secondary metabolism will not necessarily equate to increases in defence (Herms and Mattson 1992). On the contrary, small amounts of biosynthetically different chemicals may enhance the benefit of a plant defence by synergism (e.g., Berenbaum et al. 1991).

The European white birch secondary chemistry varies significantly between individuals within a natural population, between different genotypes, between different years, between different developmental stages and between different growing sites (I, II, III, IV). Also, different plant parts; e.g., leaves and stem, differ both in quality and quantity of secondary chemicals (I, II vs. III, IV). All this qualitative and quantitative variation in secondary chemistry provides potential for simultaneous resistance to a wide variety of herbivores and other environmental factors. In addition, differing responses of *B. pendula* genotypes to environment (i.e., phenotypic plasticity of secondary compound accumulation; Lavola et al. 1994, Keinänen et al. 1999a, Yamaji et al. 2003, IV) is important in the adaptation of birch populations to variable abiotic and biotic environmental conditions, and it also enables birch trees to have the most adaptive phenotype in a particular environment and, thus, survive under highly variable and changing environmental conditions.

1. CONCLUDING REMARKS

1. There is an enormous temporal and spatial variation in secondary chemistry in a natural population of *B. pendula*, due to differences among plant organs, developmental stages, ontogeny and edaphic and climatic effects.
2. Due to high intraspecific variation in birch secondary chemistry, experiments should use plant material from a wide genetic background to gain population or species level generalizations.
3. There is a strong effect of genotype on secondary chemical profile. Thus, the chemical quality can be used as a chemotaxonomic tool if the developmental stage of the plant part and the ontogenic stage of the study plants are considered.
4. The sensitivity of birch secondary compound accumulation to environmental effect is highly dependent on the studied compound and plant genotype.
5. The significant genotype by environment interaction for some anti-feeding compounds indicates a high phenotypic plasticity, thus enabling the *B. pendula* population to survive under various environmental conditions.
6. The effect of ontogeny is strong, especially for some chemical traits.

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