University of Joensuu, PhD Dissertations in Biology

No: 52

Susanne Heiska

The yield and cultivation reliability of herbal willow

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Biosciences of the University of Joensuu, for public criticism in the Auditorium H1 of the University, Yliopistokatu 4, on 30th November, 2007, at 12 noon

Pre-examiners
Professor Heikki Hokkanen
Department of Applied Biology,
University of Helsinki, Finland

Professor Jarmo Holopainen
Department of Environmental Science,
University of Kuopio, Finland

Examiner
Professor Pirjo Mäkelä
Department of Applied Biology,
University of Helsinki, Finland

Supervisors
Professor Riitta Julkunen-Tiitto
Faculty of Biosciences,
University of Joensuu, Finland;
Docent Matti Rousi
Finnish Forest Research Institute,
Punkaharju Research Station, Punkaharju, Finland

University of Joensuu
Joensuu 2007

Julkaisija Joensuun yliopisto, Biotieteiden tiedekunta
PL 111, 80101 Joensuu
Publisher University of Joensuu, Faculty of Biosciences
P.O.Box 111, FI-80101 Joensuu, Finland

Toimittaja FT
Editor Dr Heikki Simola

Jakelu Joensuun yliopiston kirjasto / Julkaisujen myynti
PL 107, 80101 Joensuu
puh. 013-251 2652, fax 013-251 2691
email: *joepub@joensuu.fi*
Distribution Joensuu University Library / Sales of publications
P.O.Box 107, FI-80101 Joensuu, Finland
tel. +358-13-251 2652, fax +358-13-251 2691
email: *joepub@joensuu.fi*

Verkkojulkaisu *http://joypub.joensuu.fi/joypub/faculties.php?selF=11*
väitöskirjan yhteenveto-osa; toim. Markku A. Huttunen
and Tomi Rosti
ISBN 978-952-219-035-2 (PDF)
Internet version *http://joypub.joensuu.fi/joypub/faculties.php?selF=11*
summary of the dissertation; ed. by Markku A. Huttunen
and Tomi Rosti
ISBN 978-952-219-035-2 (PDF)

Sarjan edeltäjä Joensuun yliopiston Luonnontieteellisiä julkaisuja (vuoteen 1999)
Predecessor Univ. Joensuu, Publications in Sciences (discontinued 1999)

ISSN 1795-7257 (printed); ISSN 1457-2486 (PDF)
ISBN 978-952-219-034-5 (printed)

Joensuun Yliopistopaino
2007

Heiska, Susanne

The yield and cultivation reliability of herbal willow.
- University of Joensuu, 2007, 88 pp.
University of Joensuu, PhD Dissertations in Biology, No: 52. ISSN 1795-7257

ISBN 978-952-219-034-5

Keywords: herbal willow cultivation, *Salix myrsinifolia*, plastic mulch, fertilisation, clonal variation, total salicylate yield, growth, resistance, phenolic chemistry

Eight clones of dark-leaved willow (*Salix myrsinifolia* Salisb.) and two *S. myrsinifolia* x *phylicifolia* hybrids were cultivated for two years (2001-2003) in Kaavi and Punkaharju, Eastern Finland by different cultivation methods with the aim of comparing the effects of cultivation method and clone on plant growth, total salicylate yield and willow resistance for herbivores and plant pathogens. The cultivation methods included different combinations of soil management practices, black plastic mulch and fertilisation. Willow growth was measured in Kaavi and in Punkaharju six times during the growing seasons 2001-2002, and the above-ground biomass of the willows was measured at the end of the growing season 2002. The severity of *Melampsora*-rust was calculated from leaf samples collected at the end of the growing seasons 2001 and 2002. The feeding damage caused by insects was measured in the field during the growing seasons and winter browsing by voles was studied in the field and in the laboratory feeding experiment. Concentrations of salicylates and other phenolic glucosides and condensed tannins were analysed from the leaves and bark of the willows grown in Kaavi. The yield of total salicylates after two-year cultivation was also measured. The use of plastic mulch doubled willow growth and total salicylate yield compared to the control treatment in unmulched soil. Rust severity and insect feeding seemed, however, to be more dependent on willow clone. Vole browsing in the field and in the laboratory was higher amongst the plants grown in unmulched soil compared to those grown in plastic mulch. Feeding was controlled by the diameter of the twig and the concentrations of salicylates and condensed tannins in the bark. Generally, willow chemistry was highly dependent on the clone, and the effects of cultivation methods on plant chemistry also differed amongst the clones. These results indicate that for reliable cultivation, it is important to match the cultivation method with the selected plant material.

Susanne Heiska, Natural Product Research Laboratories, Faculty of Biosciences, University of Joensuu, P.O. Box 111, FI-80101 Joensuu, Finland

ABBREVIATIONS

ANOVA	analysis of variance
dw.	dry weight
HPLC	high performance liquid chromatography
N	nitrogen
RH	relative humidity
SE.	standard error

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I. Heiska, S., Rousi, M., Turtola, S., Meier, B., Tirkkonen., V. and Julkunen-Tiitto, R. 2005. The effect of genotype and cultivation method on the total salicylate yield of dark-leaved willows (*Salix myrsinifolia*) Planta Medica 71:1134-1139.

II. Heiska, S., Tikkanen, O-P., Rousi, M., Turtola, S., Tirkkonen, V., Meier, B. and Julkunen-Tiitto, R. 2007. The susceptibility of herbal willow to *Melampsora* rust and herbivores. European Journal of Plant Pathology 118:275-285.

III. Heiska, S., Tikkanen, O-P., Rousi, M. and Julkunen-Tiitto, R. Bark salicylates and condensed tannins reduce vole browsing amongst cultivated dark-leaved willows (*Salix myrsinifolia*). Chemoecology. Published electronically DOI: 10.1007/s00049-007-0358-9.

IV. Heiska, S. Paunonen, R., Turtola, S., Tirkkonen, V., Meier, B., Rousi, M. and Julkunen-Tiitto, R. Foliar phenolic composition of dark-leaved willows (*Salix myrsinifolia* Salisb.) cultivated by different methods. Environmental and Experimental Botany. Submitted.

The original publications are referred in the text by their roman numerals. In addition to the original publications, this dissertation includes unpublished material.

1. INTRODUCTION

1.1. The use of willow salicylates and their synthetic derivatives

The properties of willow bark in treating fever and alleviating headache and rheumatic pain have been known since the ancient Chinese, Egyptian, Greek, Indian, and Roman civilizations. One of the earliest record of willow use in folk medicine dates back more than 3500 years; instructions for making willow extracts and decoctions were described in the famous Egyptian treatise called Ebers´ Papyrus (Pierpoint 1994; Braña et al. 2005; Setty et al. 2005). However, the use of willow drugs has also been in more recent focus (Schmid et al. 2001ab).

Willow bark is regarded as probably the most famous example of a modern drug developed from a herbal remedy. The active compound in willow bark and leaves is salicin, a low molecular weight phenolic glucoside (Schmid et al. 2001b). High concentration of salicin and other salicylates that can be easily hydrolysed into salicin are characteristic of willows (Julkunen-Tiitto 1989). However, in 1826 salicin was isolated for the first time from the leaves of *Filipendula ulmaria* by an Italian chemist **Ludovico Brunatelli** (Braña et al. 2005). Three years later a French scientist **Henri Leroux** isolated salicin from willow bark (Braña et al. 2005; Pierpoint 1994).

The costs of salicin purification from plant material led to a search for synthetic derivatives of salicin and finally, the discovery of salicylic acid in the mid-1800s. The use of salicylic acid was limited by the severe gastric irritation it caused. By 1853 **Charles von Gerhardt**, a French chemist, acetylated salicylic acid in order to make it better tolerated by patients. Gerhard had no interest in marketing, and his discovery was abandoned for a few decades. Acetylic salicylic acid was rediscovered at the end of the 19th century in collaboration between **Arthur Eichengrün** and his assistant **Felix Hoffman**, both working for Bayer. By 1899, acetylsalicylic acid was marketed under the trade name of Aspirin (Pierpoint 1994; Hedner and Everts 1998; Braña et al. 2005; Setty et al. 2005).

Today Aspirin is widely used but despite the use of the acetylated form of salicylic acid in the drug, recent clinical studies show that herbal drugs derived from willow bark are highly effective and better tolerated by patients than the synthetic derivative (Chrubasic et al. 2000; Schmid et al. 2001a). The tolerability of willow drugs is based on the absorption of salicin from the stomach and oxidation into an acid form after the absorption (Schmid et al. 2001b). Though salicin is thought to be the main analgesic in willow bark, other components such as tannins, flavonoids, and salicin esters may contribute to its overall effect, making herbal drugs more effective than the synthetic derivatives (Schmid et al. 2001a). Thus, there is a resurgence of interest in herbal remedies made of willow as a treatment for chronic pain syndromes (Setty and Sigal 2005).

White willow (*Salix alba*) is probably the willow species most commonly used for medicinal purposes due to the high concentration of salicin in its bark, but crack willow (*S. fragilis*), purple willow (*S. purpurea*) and violet willow (*S. daphnoides*) may also be sold under the label of willow bark (Setty and Sigal 2005). Dried stem and leaves especially of northern dark-leaved willow (*S. myrsinifolia*), one of the more than 20 willow species that are native to Finland,

has been reported as containing salicin concentrations of 1-3 mg/g and 2-7 mg/g, respectively and salicortin concentrations of 10-50 mg/g and 50-80 mg/g, respectively (Julkunen-Tiitto and Meier 1992). Due to the rapid growth and high concentration of salicylates, *S. myrsinifolia* is considered as being suitable for cultivation as a 'herbal willow', a willow that can be used as raw material for herbal production. Currently, bark and leaves collected from naturally grown Finnish willows are used as minor components in herbal products. Still no methods for the cultivation of herbal willows have been developed (Raipala-Cormier; Frantsilan yrttitila, personal communication).

1.2. From the collection of herbs to the cultivation of special crops

Traditionally herbs and medicinal plants used in folk medicine have been collected from the naturally grown plants, and already the Vikings were reported to trade in medicinal plants. The collecting and systematic cultivation of medicinal plants was, however, common in the monasteries at the end of 17th century. In the 'Era of Liberty' of the Swedish-Finnish Kingdom, starting in the early 18th century, cultivation of medicinal plants was politically exhorted, and apothecaries and doctors were considered responsible for their cultivation. At that time research on medicinal plants and on their cultivation was started at the Academy of Turku. During Russian Rule in the 19th century, the importation of herbs and drugs from the east was encouraged by low customs, and thus the cultivation of medicinal plants decreased dramatically in Finland. The importation of drugs became more difficult during World War I and World War II, and interest in cultivation, drying and trade in medicinal plants increased again. The enthusiasm was, however, short-lived; the deficiency in food supplies during the rebuilding period after the wars made it necessary to focus on food production. (Peldán 1967).

Herbs and medicinal plants were not been widely cultivated and collected in Finland after World War II, probably because of the high costs of labour-intensive production and a climate with short growing seasons and cold winters, which restrict the crop selection. Currently, the diversity of cultivated plants is quite narrow; roughly 80% of the 2.3 million ha of utilised agricultural area in Finland is used for growing grass and cereals, mainly a few cultivars of barley (*Hordeum vulgare*) and oats (*Avena sativa*). The cultivation of special crops, such as sugar beet (*Beta vulgaris*) and turnip rape (*Brassica rapa*) is centred in the southern and central parts of Finland. However, EU-funded programs for developing the rural areas since the 1990s have aroused interest in the cultivation of special crops, and during the last few years, cultivation of special crops including herbs and medicinal plants has been increasing rapidly. In 2006 special crops were cultivated in an area of 228 100 ha, while the area under organic herb cultivation was 16 000 ha. These areas are high compared to the other Nordic countries, but the crop selection is narrow; nearly 90% of the area in organic herb cultivation was used for growing caraway (*Carum carvi*). (Anon. 2006; Galambosi 2006).

Effective crop rotation and diversified use of pest control methods is hard to maintain in a one-sided cultivation system based on only a few crops, which may lead to an increased risk of herbivore generalisation in the cultivated area and soil contamination by weeds and pathogens (Altieri 1999).

Thus the introduction of new crops that can be cultivated in the northern climate would surely enrich the diversity of the cultivated areas. As roughly one tenth of the utilised agricultural area in Finland is annually in set-aside (Anon. 2006), the cultivation area of herbs and medicinal plants could be increased without risk to food production. Especially perennial crops have a positive effect on reducing soil erosion and nutrient leaching by keeping the soil covered during the winter-time (e.g. Morgan 2005). The over-wintering of some insects and migration of small mammals may also benefit from the cultivation of perennials (Tolbert and Schiller 1996; Pywell 2005).

1.3. Cultivated willow

Herbal willow could be a promising alternative to the cultivation of special crops. Cultivated in a short rotation system with a two-year harvesting-cycle (Julkunen-Tiitto and Meier 1992) and with a frequency of plantation renewal in 10-20 years periods, the cultivation of herbal willow might be conducted with a relatively low work input. In addition, the economic value of the yield, especially of northern willows might be high, due to the high content of salicylates and thus high yield quality. Developing cultivation techniques is, however, essential for large-scale cultivation.

Most of the willows can be easily planted using stem cuttings. The competition ability of the cuttings during the stand establishment is, however, poor, and weed interference during that time might be critical (Labrecque 1994; Sage 1998; Abrahamson et al. 2002). Chemical pesticides cannot be recommended for herbal willow because of the risk of residues in the end product (Zuin and Wilegas 2000). On the other hand, effective mechanical control of weeds probably would require several repeated controlling treatments leading to increased production costs and risk of erosion and soil compaction (Kouwenhoven 1997; Vangessel et al. 1998). Different mulch materials are widely used in nurseries and horticulture (Robinson 1988; Green 2003). Of these materials, black plastic seems to be the most effective solution to the weed problem (Davies 1988; Houle and Babeux 1994) and thus could be used in the cultivation of herbal willow.

Willow growth can be increased by fertilisation (e.g. Hansson et al. 1999; Hytönen and Kaunisto 1999; Weih and Nordh 2002), but on the other hand growing conditions favouring resource allocation to growth may reduce the production of salicylates and other phenolic glucosides (Bryant et al. 1983; Coley et al. 1985; Herms and Mattson 1992; Hakulinen et al. 1995; but see also Hamilton et al. 2001). Cultivation methods regulating growth may also have an effect on biomass allocation between the stem and leaves (Fang et al. 1999; Bullard et al. 2002; Proe et al. 2002) and thus modify salicylate yield. Willow responses to environmental factors such as nutrients and soil moisture are known to vary amongst different genotypes (Weih and Nordh 2002; Turtola et al. 2005), and thus high salicylate yields in herbal willow cultivation might be achieved only by fitting the cultivation methods to the clones.

As a widespread taxon, willows play an important role in multi-trophic interaction between plants and herbivores and their enemies by serving as food and shelter (e.g. Keith 1983; Sipura 1999). During the growing seasons, willows are often attacked by leaf beetles (*Chrysomelidae*) and during the winter,

willow bark serves as an important source of food for small mammals such as voles (*Microtus* spp.) and hares (*Lepus* spp.). The feeding of small mammals may cause considerable damage, especially when the herbivores are abundant (Gill 1992; Abrahamson et al. 2002; Hiltunen 2002). Of willow diseases only *Melampsora* rust has caused economically significant injury in willow plantations (Dawson and McCracken 1994; Ramstedt 1999; McCracken and Dawson 2001). As pesticide use should be avoided in herbal willow, the role of cultivation method and selection of resistant clones is important for keeping the extent of damage to a minimum.

Willow phenolic compounds are generally considered to be important in willow defence against herbivores and pathogens. The ecological significance of willow leaf chemistry is, however, many-sided. As an example, some willow-feeding leaf beetles, such as *Lochmaea capreae* and *Galerucella lineola,* avoid willows with high concentration of salicylates or chlorogenic acid (Tahvanainen et al. 1985; Ikonen et al. 2001). On the other hand, willow leaves rich in salicylates are preferred by *Phratora vitellinae*, which can use the ingested salicin in their defence against generalist predators (Pasteels et al. 1983; Rowell-Rahier 1984; Rank 1992; Rank et al. 1998). Especially in monoculture, long-time cultivation may change the balance between plants, herbivores and their enemies, and cultivated willows may show increased susceptibility to herbivores and pathogens (McCracken 1994; Gruppe et al. 1999). In order to improve cultivation reliability, the effect of cultivation methods on willow chemistry and herbivore abundance in the cultivated area should be evaluated.

1.4. Aims of this study

Throughout history, rises and falls in interest in the cultivation of herbs and medicinal plants have been influenced by various national crises, and cultivation has been manipulated by politicies and various subsidies (Peldàn 1967). Successful introduction of a new crop requires good timing and a niche in the market. One of the most important reasons for failing with a new crop, however, seems to be the lack of well developed cultivation methods (Galambosi 2005). In this study, the possibilities for introducing herbal willow for cultivation were evaluated in field and laboratory experiments with a view to developing reliable cultivation methods.

The most important aim of these experiments was to study, how the use of plastic mulch and fertilisation affects the growth (I, II) and chemistry (III, IV) of different willow clones, and how the salicylate yield relates to growth (I, II). Herbivores and pathogens were also observed in the field with the purpose of evaluating the effect of different cultivation methods on cultivation reliability (II). Vole browsing amongst cultivated willows was examined in the laboratory feeding trials in order to study the effect of cultivation method and bark chemistry on browsing (III). Ten willow clones were included in this study to show whether the yield and cultivation reliability can be influenced more by clone selection or by developing cultivation methods, and whether the effects of cultivation methods vary depending on the clone (I, II, III, IV).

2. MATERIAL AND METHODS

2.1. Plant material and field experiments

Eight clones of dark-leaved willow and two natural hybrids (*S. myrsinifolia* x *phylicifolia*) were selected for their vigorous phenotypic growth and used in this study. The origin of the plants was in the areas of Kaavi and Joensuu, Eastern Finland. All the plant material used in this study was established from cuttings made of second year shoots and cultivated in the field during 2001-2003.

All the willow clones were cultivated in two locations in South Eastern Finland, in Luikonlahti, **Kaavi** (I, II, III, IV) and in **Punkaharju** (I, II), using different cultivation methods combining **soil tillage** (I, II), **mulching** (I, II, III, IV) and **fertilising** (I, II, IV). In Kaavi, the willows were grown in ploughed and harrowed soil using six combinations of mulch (black plastic and bare soil) and fertiliser (unfertilised, low and high levels). In Punkaharju, the plants were grown in uncultivated soil that was fertilised as in Kaavi. The cultivation methods are explained in detail in I and II.

The experimental design in Kaavi was a split-plot design with eight complete blocks (see Annex 1 for the experimental design). The cultivation methods were randomised in the main plots, and clones were randomly placed in the sub-plots. Sub-plot size was 1.25 m^2 and contained 12 plant individuals, planted in three rows with a cutting density of 9 cuttings per 1 m^2. The experimental design in Punkaharju was a split-plot design with five complete blocks, with fertilisation in the main plot factor and clones in the sub-plot factor (see Annex 2 for the experimental design). Plot size and cutting density were the same as in the Kaavi experiment.

2.2. Observations in the field

2.2.1. Willow growth and survival

Willow growth and survival were measured in Kaavi and in Punkaharju during growing seasons 2001 and 2002 (I, II). In Kaavi, four of the twelve plants in each sub plot were randomly selected for growth measurements. To monitor the development of the willow stand, the height of the highest shoot of these selected plants, hereafter called '**main shoot height**', was measured six times during the growing seasons 2001 and 2002 (II). The **shoot number** of the plants was calculated at the end of the growing seasons 2001 and 2002 (I, II). The height and **diameter** of all shoots of the selected plants were also measured at the end of the growing season 2002 (I, II). The **biomass** of the plants was measured at the end of the growing season 2002 (I). Dry masses of the stems and leaves were measured separately. Willow growth and biomass were measured in Punkaharju in the same way as those in Kaavi (I, II).

Some of the willows died during the experiment and willow **survival** was measured in Kaavi and in Punkaharju by counting the number of all living and dead plants at the end of the growing season 2001, and at the beginning and end of the growing season 2002 (I, II).

2.2.2. Herbivores and pathogens

For evaluating the cultivation reliability of herbal willows, the abundance of willow leaf and stem eating herbivores was observed in Kaavi and Punkaharju during the growing seasons 2001 and 2002 (II). As the number of leaf-eating herbivores was high in both experiments at the beginning of August 2001, six of

the twelve plants in each plot in both experiments were selected for an estimation of **leaf damage,** mainly caused by leaf beetles (*Phratora vitellinae* and *Galerucella lineola*) (Chrysomelidae, Coleoptera) and moth larvae (Noctuidae, Lepidoptera). The damage caused by insects was estimated visually as described in II.

Aphids (Aphididae, Homoptera) were observed as dense colonies at the beginning of July 2002 in Punkaharju. Three plants in each plot were systematically chosen for a visual estimation of **aphid abundance**, described in more detail in II.

Voles browsed the willows in Kaavi during the winters 2001-2002 (II) and 2002-2003 (III). **Vole browsing** was measured in each plot by counting the total number of plants that showed any signs of vole feeding in spring 2002 (II) and spring 2003 (III).

Melampsora rust infected the willows in Kaavi and Punkaharju and orange pustules were seen on willow leaves during the growing seasons 2001 and 2002 (II). For the analysis of natural **rust severity**, the youngest fully expanded leaf of the same plants that were used for the measurements of main shoot height was collected at the end of August and the beginning of September in 2001 and 2002. The leaves were dried in a drying room as described in II. The severity of the pustules on the lower surface of each leaf was observed from the dry leaves with a microscope (II).

2.3. Multi-choice feeding experiment with voles

Voles clearly selected willows in Kaavi experiment (II). To study the selection criteria affecting vole browsing, the palatability of the field-grown willows was also tested in a laboratory feeding trial with voles (*Microtus agrestis*). The experiment was conducted at the University of Joensuu during the winter 2002-2003.

Plant material for the trial was collected in the Kaavi experimental area in December 2002. Because of the limited number of voles available for the experiment, only eight clones, 1, 2, 3, 4, 6, 7, 8 and 10, were used for the experiment. Clone 5 was not used because of high mortality (II) and clone 9 was not used as preliminary chemical analyses showed it to be a hybrid with chemical components resembling those of *S. phylicifolia*. Winter dormant shoots of the plants were collected from amongst the unfertilised plants as described in III. A 17 cm piece of each shoot, measured from the base, was cut for the feeding experiment.

The feeding experiment was conducted with 16 voles, which were trapped in the wild in Punkaharju and kept in separate cages during the experiment. During the experiment, each vole received a random combination of 10 different twigs, as described in III. The duration of the experiment was 10 h, and the whole procedure was replicated in three days with the same voles.

The experiment was planned with a structure of balanced incomplete blocks with vole-by-day as a block (48 levels) and combinations of mulching treatments and clones (16 levels) as experimental units. Within one day, each experimental unit was introduced to 7 voles, so that each of the 120 possible pair combinations was offered to 4 different voles (see Annex 3 for the experimental design).

Prior to the initiation of the trial, the **diameters** of the twigs were measured with a slide calliper. **Bark thickness** was measured with a microscope as explained in III. After the termination of the trial, twigs were removed from the cage and

the **removed bark area** was scanned as described in III. An estimate for the **dry mass** of the bark area removed by voles was calculated as described in III.

2.4. Chemical analysis

2.4.1. Total salicylates

To estimate the quality of willow raw material and the yield of salicylates, the above-ground parts of one randomly sampled plant in each plot in the Kaavi experiment were harvested in August 2002 (I). The harvested plants were chopped with a garden shedder in the field and dried in a drying room at a relative humidity (RH) of 10%. The dried material was then milled to a powder. Salicylates were extracted from the powder and hydrolysed into salicin according to Meier et al. (1985).

Salicin, hereafter called '**total salicylate concentration**' was analysed from the hydrolysed samples using high performance liquid chromatography (HPLC). The system is described in more detail in I. HPLC runs were monitored at a wavelength of 220 nm and salicin was quantified against a commercial standard (Sigma).

Total salicylate yield was estimated by multiplying the total salicylate concentration by the hectare biomass (I).

2.4.2. Leaf phenolics

The effect of clone and cultivation method on leaf phenolic chemistry was tested on the plants grown in the Kaavi experiment (IV). Because of poor growth (I, II) and survival (II), clones 3 and 5 were not used in the chemical analysis.

One of the twelve plants in each plot was randomly chosen for the analysis. The youngest fully expanded leaf of each of these plants was collected between the end of August and the beginning of the September 2001 and at the beginning of September 2002 (IV). The collected leaves were dried in a drying room (at RH 10%).

5-10 mg samples were taken from the dried leaves, and phenolic compounds of the samples were extracted in methanol as described in IV. The phenolic compounds were analysed by HPLC as described in I, and runs were monitored at wavelengths of 220, 270, 320 and 360 nm.

Concentrations of salicylates, including diglycoside of salicyl alcohol, salicin and salicortin; **flavonoids**, including hyperin and luteolin-7-glucoside, **and chlorogenic acid** were quantified against commercial or purified standards as described in IV. The molecular structures of the analysed compounds are presented in Annex 4.

The total amount of the methanol-soluble **condensed tannins** was analysed from HPLC samples by the acid-butanol test (Hagerman 1995) and using tannins purified from *Betula nana* leaves (IV).

Salicylate potential, an estimate was calculated for the maximum yield of total salicylates. The concentrations of leaf diglycoside of salicyl alcohol, salicin, salicortin and tremulacin in 2002 (IV, Table 1) were divided by their molecular masses and multiplied by the molecular mass of salicin to estimate the total salicylate concentration in the leaves. Concentrations were summarised and multiplied by the leaf biomass measured from the plants grown in the same plot (I).

2.4.3. Bark phenolics and nitrogen

To study the effects of bark chemical components on vole feeding, bark phenolic compounds and total nitrogen were analysed from the plant material collected for vole feeding trials (III). For the analysis, a 4 cm piece of the shoot was cut above the part that was used in

the feeding trial. The bark was removed from the wood part and chopped as described in III.

Phenolic compounds were extracted from 100-200 mg samples of fresh bark with methanol as described in III and analysed by HPLC (I, III). HPLC runs were monitored at wavelengths of 220, 270 and 320 nm.

Concentrations of the salicylates, including salicin, salicortin and HCH-salicortin; **other phenolic glucosides**, including picein, triandrin and triandrin derivative, **catechins**, including gallocatechin and (+)-catechin, and **flavonoids,** including luteolin-7-glucoside and hyperin, were quantified against commercial or purified standards as described in III.

Concentrations of methanol-soluble **condensed tannins** were analysed from the bark methanol extract used for HPLC analysis, and insoluble tannins were analysed from extraction residues after drying. The acid-butanol test (Hagerman 1995) was used for the analysis (III).

Concentrations of **total nitrogen** were analysed from 10-20 mg samples of dried bark by LECO analysator (model FP-528) as described in III.

3. RESULTS AND DISCUSSION

3.1. Willow main shoot height and biomass – doubled growth with plastic mulch

Weed competition is the most common reason of failure in willow cultivation (Abrahamson et al. 2002). The 'critical weedless period' of willows, the period following the planting and during which weed competition is critical for establishment (e.g. Weaver 1984), seems to be surprisingly long; willows have been reported to compete effectively with weeds starting at the second growing season following the year of establishment (Sage 1998; Abrahamson et al. 2002). Careful site preparation prior to planting is therefore recommended in willow production (Danfors et al. 1997; Abrahamson et al. 2002).

In this study, willow **main shoot height** and **biomass** were more than two-fold and nearly ten-fold amongst the plants grown in ploughed and harrowed soil in Kaavi compared to those in uncultivated soil in Punkaharju (I, II). A high cutting density of 9 cuttings per 1 m^2 was used in order to enhance willow competition by rapid canopy closure. However, the canopy remained open in Punkaharju during the cultivation period 2001-2002, but in Kaavi, the canopy was closed in July 2002. The different soil management practices and the different weed flora (II) were probably the main reasons for the differences in growth between these two experimental sites.

Plastic mulch is widely used in horticulture and in short rotation coppicing systems for suppressing weed competition and evening up soil temperature and water relations (e.g. Clarkson 1960; Robinson 1988; Streck et al. 1994; Green et al. 2003). In Kaavi, plastic mulch enhanced the early development of herbal willows (II) and doubled the biomass measured at the end of a 2-year harvesting cycle (I). A similar increase in growth has also been reported in earlier studies with other willow species and poplar (Labrecque et al. 1993; Green et al. 2003, but see Houle and Babeux 1994). The effect of mulch was probably for the most part based on its effect on controlling weeds; in earlier studies, weed competition has been shown to reduce willow growth more than water or nutrient stress (Sage 1999).

The effect of the clone on growth was pronounced during the whole cultivation period, the growth responses of the plants to mulch varying depending on the clone

(I, II). This may be an indication of different competitive abilities of the clones. A properly established willow stand with a closed canopy is considered to compete effectively with weeds (Abrahamson et al. 2002). Thus, the effect of mulch on growth may decline, and clone responses to mulch may change during the forthcoming harvesting cycles. More long-time experiments are therefore needed to show the suitability of the clones for cultivation.

Willow growth was not affected by fertilising in 2001, but the fertiliser effect became more pronounced during 2002 (II). To prevent nutrient leaching from the soil, only low rates of fertiliser were used in 2001. In energy willows, fertilising is not recommended during stand establishment, but during the following growing seasons, 75 kg/ha N can be used to enhance the growth of stem biomass without a risk of increased leaching (Mortensen et al. 1998; Adgbidi et al. 2001). As the herbal willows in this study were growing in a dense stand and the leaves were also harvested, a high rate of 150 kg/ha N of fertiliser was used. Regardless of the high fertiliser rate, growth was more affected by the mulch than by fertiliser (I, II, but see Houle and Babeux 1994). The pH value of the soil was quite low in both experiments (I), and some of the added nutrients probably were not available to the plants. Soil liming could have considerably increased the fertiliser effect and willow growth. In nature, however, willows grow in environments with rather low soil pH, and thus, lime was not used in this study.

3.2. Melampsora rust – reducing risks in cultivation by clone selection

Willow leaf rust, typically caused by complex mixtures of pathotypes belonging to the genus *Melampsora*, is reported to be the most important single factor limiting willow cultivation (e.g. Dawson and McCracken 1994; Ramstedt 1999; McCracken and Dawson 2001; Niemi et al. 2006). Rust pathogenicity is known to vary depending on the pathotype structure, but the resistance of the willows also varies depending on the clone (Pei et al. 1996; McCracken et al. 2000). In this study, **rust severity** varied highly between the years and experiments and amongst the clones, but cultivation method had only a weak effect on rust severity (II). The ranking order of the clones, however, seemed to be quite similar throughout the years and experimental sites, indicating that the pathogen structure was probably quite similar in Kaavi and in Punkaharju.

Rust infection can interfere with willow growth by accelerating leaf abscission (Dawson and McCracken 1994; Abrahamson et al. 2002) or delaying bud burst in the following growing season (Steenackers et al. 1996). Winter hardiness may also be lowered by heavy rust infection (Verwijst 1996). In this study, rust severity was low in the fast growing clones 1 and 7 (I, II). The differences in growth between the clones were, however, seen from the first growth measurements, before the emergence of the first uredia, and rust severity had no correlation with main shoot height, biomass or survival (Pearson correlation, $p>0.05$). Rust infection can thus be considered to have only a minor effect on the growth of herbal willow.

Though willow resistance to *Melampsora* rust seems to be highly clone-specific, the resistance may break down after a few years' cultivation (Dawson and McCracken 1994). The reproduction of the pathogen is also highly affected by weather conditions (Desprez-Loustau et al. 1998). In this

light, it is not possible to evaluate the suitability of the clones for cultivation on the basis of results from only two growing seasons. The rust resistance of a willow stand consisting of a mixture of varieties has been found to be higher than that of monoclonal stands (McCracken and Dawson 1994; 1997). Polyclonal stands and continuous selection and breeding of resistant plant material are probably needed for reliable herbal willow cultivation.

3.3. Herbivores – effective control of vole feeding with plastic mulch

Willows are a widely spread taxon in the northern hemisphere, serving as food and shelter for numerous herbivorous insects, small mammals and insectivorous birds (e.g. Ikonen et al. 2001; Gill 1992; Sipura 1999). Leaf damage caused by beetles and sawflies is common in monoclonal short rotation cultivation (Gruppe et al. 1999), but growth inhibition by insect feeding is rarely found in willow plantations (Abrahamson et al. 2002). Feeding by mammals, especially vole browsing during the winter-time may cause economically significant damage to small trees (Kanervo and Myllymäki 1970; Hanson and Larsson 1978; Rousi 1988)

Plastic mulch clearly reduced **vole feeding** in Kaavi during the winters 2001-2002 (II) and 2002-2003 (III). As the mulch-grown twigs were also avoided by the voles in the laboratory feeding trial (III), it seems that the mulch effect was not based mainly on a repelling effect. More likely it changed the quality of the willows so that they were less preferred by the voles. Mulch increased shoot diameter (I, III), and reduced vole browsing (III), indicating that the effect of the mulch on resistance is based on accelerated growth. In earlier studies with one-year-old birches, however, seedling size had no effect on vole browsing (Rousi et al. 1993).

Vole feeding varied amongst the willow clones in the laboratory and in the field (II, III). In the field, the clone effect on vole browsing was stronger during the winter 2002-2003 (III) than during 2001-2002 (II). This might be explained by twig diameter, which was more strongly affected by the clone in 2002 than in 2001 (data not shown).

Many of the earlier feeding trials with voles are based on pair-choice experiments and visual rating of feeding extent (e.g. Rousi et al. 1997; Kuokkanen et al. 2004). In trials of this kind, different pair combinations are introduced to the voles on different days, which may make the feeding dependent on time and thus level out the differences in vole preferences. In my laboratory feeding trial, all the different pair combinations were introduced at the same time leading to very clear differences in vole browsing amongst the plants (III). Unlike earlier studies (e.g. Rousi et al. 1997), the results of this multi-choice laboratory experiment showed quite similar feeding behaviour to that in the field, suggesting that this method might be more accurate than those reported earlier. Further development of the method would surely bring out new information on vole food selection.

In our study, **insect feeding** during the growing seasons 2001 and 2002 was not considered serious (II). Herbivore abundance may, however, become higher as the plantations age (Gruppe et. al 1999). Feeding was highly dependent on clone, and the ranking order of the clones was quite similar in Kaavi and Punkaharju. The strong clone effect on herbivore feeding indicates that the cultivation reliability of herbal willow may be enhanced by clone selection.

3.4. Willow phenolic chemistry – strict control of chemicals by genotype

Fertile hybrids of two or more willow species are easily formed in nature, and distinguishing between the species is not always unambiguous. The phenolic glucosides of the willows can, however, be used as indicators of willow taxonomy (Julkunen-Tiitto 1986; 1989). In this study, clones 9 and 10 contained tremulacin and ampelopsin in their leaves (Table 1). As these compounds are not characteristic of *S. myrsinifolia*, clones 9 and 10 were considered to be *S. myrsinifolia* x *phylicifolia* hybrids. In all, concentrations of phenolic glucosides were highly clone-dependent, and the effect of the cultivation method was weak (IV, Tables 1, 2).

The summarised concentration of **leaf salicylates** was high in *S. myrsinifolia* clones and in hybrid clone 10 (IV, Table 2) compared to earlier studies with naturally grown, field-cultivated and greenhouse-grown *S. myrsinifolia* (Julkunen-Tiitto 1989; Julkunen-Tiitto and Meier 1992; Turtola et al. 2005). The plant material used in the studies of Julkunen-Tiitto (1989) and Julkunen-Tiitto and Meier (1992) included the leaf blades and petioles, but in IV and in the study of Turtola (2005), the leaf samples were taken only from the leaf blades. The different sampling methods used in the studies may partly explain the different concentrations of salicylates. Salicortin was the main phenolic glucoside in the studied leaves. Hybridisation probably explains the low salicortin concentration in clone 9 (IV, Table 1).

The mean concentration of **leaf flavonoids** was higher in 2001 than in 2002 (IV, Table 1). In both years, flavonoid concentrations were high compared to earlier studies with very young micropropogated plantlets and greenhouse-grown saplings of *S. myrsinifolia* (Tegelberg and Julkunen-Tiitto 2001; Turtola et al. 2005). This may indicate that the concentration of flavonoids varies during willow ontogeny, although the concentration of the flavonoids is probably also influenced by the different growing environments.

The concentration of **condensed tannins in willow leaves** was clearly lower in 2001 compared to that in 2002 (IV, Table 2). Concentrations of condensed tannins in the leaves of *S. myrsinifolia* ranging between 0.3 mg/g and 50 mg/g have been reported in other studies (Ruuhola et al. 2001; Tegelberg and Julkunen-Tiitto 2001). In *S. myrsinifolia* clones, mulch reduced tannin concentrations in 2001, while in 2002 tannin concentrations were more dependent on the clone (IV). Tannin concentration is reported to be much higher in *S. phylicifolia* than in *S. myrsinifolia* leaves (Ruuhola et al. 2001), and the concentration of condensed tannins in the leaves of hybrid clone 9 was ten-fold compared to other clones in this study (Table 2).

In this study, picein was the main **phenolic glucoside in willow bark** (III). Its concentration was high compared to those found in earlier studies in the bark of *S. myrsinifolia* (Julkunen-Tiitto and Meier 1992) and of other willow species, such as *S. glauca, S. dasyclados* or *S. herbaceae* (Julkunen-Tiitto 1989). However, picein is reported also to be the main penolic in the bark of *S. reticulata* (Julkunen-Tiitto 1989). Concentrations of salicylates, especially of salicortin, measured from winter-dormant shoots (III) were clearly lower than those reported in *S. myrsinifolia* and measured in late summer (Julkunen-Tiitto and Meier 1992). Concentrations of **condensed tannins** were high in willow

Table 1. Mean concentrations (mg/g, dw.), standard errors of the means (SE.) and minimum and maximum values of leaf phenolics in naturally hybridised willow clones 9 and 10. Variation coefficient (V.C.) and 95% confidence interval (C.I.) for the V.C. show the relative variation within the data.

	2001						2002					
Phenolic compound	Mean	SE.	Min	Max	V.C. (%)	95% C.I.	Mean	S.E.	Min	Max	V.C. (%)	95% C.I.
Clone 9												
Salicylates												
Diglycoside of salicyl alcohol	1.08	0.02	0.84	1.42	13	4,22	1.13	0.05	0.40	2.05	33	20,46
Salicin	0.79	0.05	0.23	2.03	48	34,62	0.77	0.05	0.24	1.76	46	32,60
Salicortin	29.94	1.05	13.43	44.75	24	12,36	26.80	0.96	16.09	40.02	25	13,37
Tremulacin	3.12	0.11	1.54	5.45	24	12,36	1.37	0.11	0.20	2.76	54	40,68
Flavonoids												
Ampelopsin	14.51	0.88	6.43	43.37	42	28,56	1.22	0.29	0.00	14.32	nc	nc
Hyperin	5.02	0.13	2.65	7.71	18	7,29	3.15	0.10	0.25	4.21	22	10,34
Luteolin-7-glucoside	5.76	0.17	3.18	8.78	20	9,32	3.58	0.16	1.49	5.52	31	18,44
Other phenolic acids												
Chlorogenic acid	10.86	0.25	6.88	14.91	16	6,27	7.80	0.18	5.34	10.37	16	6,26
Clone 10												
Salicylates												
Diglycoside of salicyl alcohol	2.83	0.06	1.95	3.55	15	5,26	2.83	0.06	2.15	3.70	14	4,24
Salicin	4.54	0.17	2.80	8.13	25	13,38	3.13	0.15	1.09	6.10	32	19,46
Salicortin	113.56	2.62	83.09	174.27	16	6,26	73.94	2.31	48.94	112.34	22	10,33
Tremulacin	1.83	0.15	1.02	8.49	57	43,71	6.08	0.44	0.00	13.59	50	36,64
Flavonoids												
Ampelopsin	0.51	0.06	0.00	1.91	82	72,93	2.23	0.11	1.12	3.62	30	17,43
Hyperin	4.96	0.14	2.32	6.53	19	8,30	3.60	0.15	2.26	6.84	28	15,41
Luteolin-7-glucoside	5.70	0.22	2.05	10.14	27	15,40	3.28	0.15	1.22	6.01	33	19,46
Other phenolic acids												
Chlorogenic acid	19.42	0.48	11.95	25.84	17	7,28	11.63	0.40	0.00	17.17	24	12,36

Table 2. Mean concentrations (mg/g, dw.), standard errors of the means (SE.) and minimum and maximum values of compound groups in leaves of naturally hybridised willow clones 9 and 10. Variation coefficient (V.C.) and 95% confidence interval (C.I.) for the V.C. show the relative variation within the data. Letters 'nc' mean that due to high variation the value was not calculated.

	2001						2002					
Compound group	Mean	SE.	Min	Max	V.C. (%)	95% C.I.	Mean	S.E.	Min	Max	V.C. (%)	95% C.I.
Clone9												
Salicylates	34.93	1.13	18.37	52.62	22	11,34	30.07	1.05	17.62	44.28	24	12,36
Flavonoids	25.30	0.86	16.34	52.27	24	12,36	10.31	0.39	5.82	23.53	26	14,39
Condensed tannins	202.94	8.92	126.83	373.41	30	17,43	179.31	4.14	125.27	241.01	16	6,26
Clone10												
Salicylates	122.76	2.58	93.77	182.36	15	5,25	85.98	2.61	57.55	128.54	21	10,33
Flavonoids	11.17	0.31	6.88	15.52	19	8,31	9.86	0.27	7.01	15.42	17	6,28
Condensed tannins	26.16	4.19	10.06	196.98	nc	nc	17.60	1.21	3.73	52.63	48	34,62

bark (III). On the whole, concentrations of bark phenolics seemed to vary depending on the clone, whereas the effect of mulch on the chemicals was rather low (III).

Generally, fast-growing plants are assumed to allocate most of their resources to growth, and as a result of this, probably have less capacity to produce defensive chemicals (Bryant et al. 1983; Coley et al. 1985; Herms and Mattson 1992; but see also Hamilton et al. 2001; Tikkanen et al. 2003). In this study, phenolic compounds measured from willow leaves (IV) and bark (III) did not correlate with willow growth (I, II; Bonferroni adjusted Pearson correlation, $p>0.05$).

Nonetheless, concentrations of leaf salicylates and chlorogenic acid in 2001 and 2002 were highest in clone 8 (IV), which also had low total growth (II). At the other extreme, clone 1 with high total growth (II) and biomass (I) had low concentrations of leaf salicylates and chlorogenic acid, especially in 2002 (IV). Plastic mulch greatly increased the biomass of clone 1 (I), but in 2001 growth was increased at the expense of leaf salicylates (IV). Clones 4 and 7 with good growth (I, II) also had a relatively high concentration of phenolic glucosides in their bark (III) and leaves (IV). In clone 4 the biomass was greatly increased by plastic mulch and fertilisation (I), but this growth did not reduce leaf salicylates (IV). These results may be indications of the different resource uptake capacity and carbon allocation processes of the studied clones.

3.5. Chemical resistance – reducing vole browsing by bark salicylates and tannins

Generally, voles and other small mammals are considered to prefer food plants with a high content of nitrogen and low contents of phenolics and terpenoids. However, it has been hard to demonstrate any clear pattern for food selection amongst small trees, and earlier results from feeding trials with voles are for the most part contradictory (e.g. Hjältén and Palo 1992, Laitinen et al. 2002; but see Rousi 1989; Rangen et al. 1994). In this study, the browsing of *M. agrestis* was clearly reduced by the condensed tannins in willow bark (III). The correlation of vole browsing and bark salicylates was also strongly negative, but only amongst the browsed plants (III). This probably indicates that voles make their food selection in two steps: in the first step the voles select the twigs to be tasted on the basis of their exterior features, such as odour or twig dimensions. In the second step, the concentration of bitter tasting tannins and salicylates will determine whether the feeding is continued and how much of the bark is browsed.

Bark salicylate concentrations seemed to have a threshold value of around 10-15 mg/g, dw., below which salicylates did not limit vole feeding, but when the threshold value was exceeded, vole browsing was low (III). This threshold value might be related to the maximum content of salicylates that voles can digest and detoxify without limiting their food intake to less than their energy requirements (Dearing et al. 2005; Foley and Moore 2005). The results of this study show that selecting clones with a high concentration of salicylates for cultivation not only increases the yield quality, but may also reduce the risk of willows being damaged by voles.

The concentration of leaf phenolics in *S. myrsinifolia* is known to vary during ontogeny, but variation during the growing season is considered to be quite low (Hakulinen et al. 1999; Ruuhola and

Julkunen-Tiitto 2001). Production of phenolic compounds may be induced by herbivory or pathogen infection (e.g. Ruuhola et al. 2001; Fields and Orians 2006). Earlier studies, however, show that accumulation is not easily induced by tissue wounding (Julkunen-Tiitto et al. 1994). In this study, leaf analyses were carried out at the end of growing seasons (IV), while rust infected willows earlier in the growing season and insects fed on the plants during the whole growing season (II). Hence the results of the leaf analysis of this study give only an estimate for the chemical composition of the leaves during the growing seasons.

In earlier studies, the intensity of willow infecting *Melampsora* rust has been found to correlate negatively with leaf salicylates (Julkunen-Tiitto et al. 1994), while phenolics may also cause complete *Melampsora* resistance in young poplar leaves (Johnson and Kim 2005). High concentrations of salicylates may reduce feeding by generalist leaf beetles (Tahvanainen et al. 1985, but see Rowell-Rahier 1984; Rank 1992; Rank et al. 1998). Beetle feeding also seems to be controlled by other phenolic acids (Ikonen et al. 2000; Matsuda and Senbo 1986) and flavonoids (Matsuda and Matsuo 1985). In this study, there was no correlation between leaf phenolics and rust severity or insect feeding (Pearson correlation, $p>0.05$). However, insect feeding was high in Kaavi and in Punkaharju in hybrid clone 9 (II). This might have been due to the different leaf chemistry and also to other traits of the hybrid clone (IV).

The quality of food plants is widely known to change the population structure of herbivores and their enemies (e.g. Keith 1983; Bryant et al. 1985; Sipura 1999). As an example, a specialist leaf beetle *Phratora vitellinae* prefers food plants with high concentrations of salicylates. The beetles can use the salicylates as a precursor of saliclylaldehyde, an effective defence chemical against generalist predators (Pasteels 1983; Rowell-Rahier 1984; Rank 1992; Rank et al. 1998). Thus high availability of salicylate-rich food plants could favour the specialist herbivores, increasing their abundance. It is therefore obvious that the cultivation reliability of willows is a multi-faceted issue, requiring more long-term studies to show the effect of willow cultivation on herbivore population structure.

3.6. Total salicylates – high yields with increased biomass

The herbal medicine 'Salicis cortex' is defined as consisting of the ground dried bark of young branches or whole-dried pieces of young twigs of willow species (Anon. 2001). It is recommended that the drug contain not less than 1-1.5% of total salicylic derivatives based on definitions by the German Commission E and the European Pharmacopoeia, respectively. Willow extracts 'Salicis extractum' with standardised salicin content may also be used in phytotherapy (Schmidt et al. 2001; Eisenberg et al. 2002; Dabrowska-Zamojcin et al. 2002). Extracts may be made of any salicylate-containing plant parts. The salicin contents of commercial extracts usually vary between 8% and 12%, depending on the product. To ensure the high quality of the drugs and reasonable handling and transportation costs of raw material, only plant material with a high concentration of salicylates can be recommended for herbal production.

The willow material used for total salicylate analysis in this study contained all the above-ground parts of the plants, including the wood part, which contains almost no salicylates (Julkunen-Tiitto1989; Pohjamo et al. 2003). The

mean **concentration of total salicylates** was more than 1% in most of the clones and more than 1.5% in some clones (I). These concentrations were slightly lower than those in an earlier study with *S. myrsinifolia* (Julkunen-Tiitto and Meier 1992). The concentration, however, varied depending on the clone, indicating that some of the clones could meet the standards of salicylate concentration determined for Salicis cortex without removing the wood part. In general, plastic mulch and fertilisation seemed to slightly reduce the total salicylate concentration, but this reduction cannot be considered significant, as the effects of cultivation methods varied highly depending on the clone (I).

Total salicylate yield was doubled by the use of plastic mulch (I). Of the two most important yield components, total salicylate concentration and biomass, the latter seemed to be more important in determining the hectare yield of total salicylates. Clone effect on total salicylate yield was also high (I).

Salicylate potential, the estimated maximum yield of salicylates, was on average 78 kg/ha (Fig. 1), which was quite similar to the actual salicylate yield (I). The salicylate potential was nearly doubled by the plastic mulch compared to cultivation on bare soil. The potential also varied amongst the clones (Fig. 1, Table 3). The salicylate potential was slightly increased by fertilisation, but the extent of the fertiliser effect was clone-dependent (Fig. 1, Table 3).

Salicylate potential was calculated on the basis of chemical analysis of individual willow leaves (IV) and leaf biomass (I). Analyses were made on young, fully expanded leaves. However, the phenolic chemistry of the leaves is known to vary depending on leaf age and between the different parts of the leaves. As the harvested biomass consisted of leaves varying in age, the analysis of leaf blades of young leaves alone may not give a precise estimate of the salicylate potential of the harvested leaf mass. The concentration of bark salicylates at the time of harvest and the wood content of the harvested plant material were not analysed, and thus these results cannot be used for estimating the degradation of salicylates during the transportation and drying of the raw material that was used for the analysis of total salicylate concentration. However, these results highlight the importance of cultivation method in promoting growth (Fig. 1; I, IV).

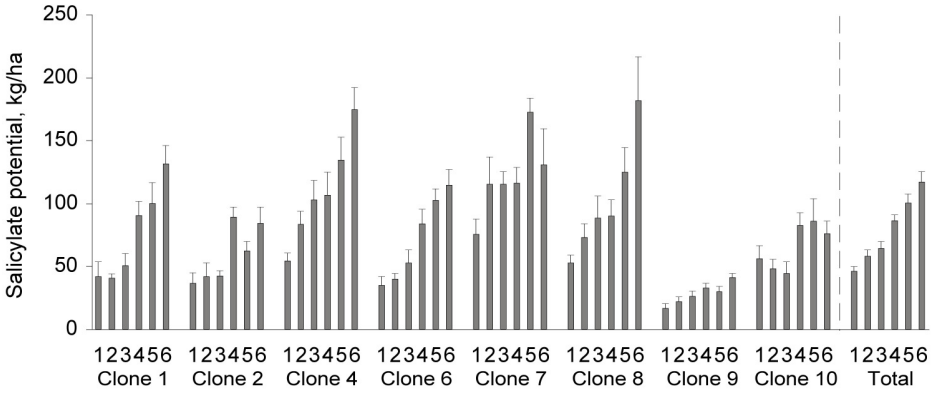
Table 3. Results of the analysis of variance for the effect of cultivation method and clone on the salicylate potential of herbal willows. Asterisks, *, ** and *** behind the *F*-values indicate that the effect was significant at the levels of $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Source of variation	df	F
Mulch (M)	1	72.183 ***
Fertilisation (F)	2	7.545 **
M*F	2	0.433 ns
Error for main plots	35	
Clone (C)	7	38.831 ***
M*C	7	3.408 **
F*C	14	3.235 ***
M*F*C	14	0.865 ns
Error for sub-plots	289	
Total	379	

Willow drugs have shown strong analgesic effects, which are considered not to be based only on salicin, but also on other constituents of the drug (Chrubasic et al. 2000; Schmid et al. 2001a). Especially willow flavonoids are considered to play an important role in the functioning of herbal drugs. Therefore, not only the total salicylate concentration, but also other components

of yield quality should be taken into account when developing cultivation techniques and pre-handling methods for herbal willow.

Figure 1 Salicylate potential of the willow clones cultivated by different methods (the numbers 1-6 on the x-axis refer to the cultivation methods explained in I). The bars represent mean values and the error bars indicate SE.



4. CONCLUSIONS

Willow growth can be increased considerably by the cultivation method, especially by combining soil tillage and plastic mulch. Willow chemistry, however, seems to be controlled more by genotype than by cultivation method. Nonetheless, salicylate yield is determined more by biomass than by concentration of salicylates. The different responses of the clones to cultivation methods suggest, however, that for profitable herbal willow cultivation, it is important to match the selected clones with an appropriate cultivation system.

Voles may cause severe damage to willows. Browsing is influenced by shoot diameter and concentrations of bark salicylates and condensed tannins. Voles prefer thin twigs when making a choice to taste the twig or not. After tasting, feeding continues in twigs containing a low concentration of salicylates and tannins. Thus, cultivation method, having a pronounced effect on growth, and clone selection, affecting bark chemicals, both regulate willow susceptibility to voles. *Melampsora* rust infected all willow clones, and there was high variation in rust severity amongst the clones, while the cultivation method had only a weak effect on rust.

This study highlights the importance of favourable growing conditions during the establishment of a willow plantation. The results of the study regarding the effect of plastic mulch on willow growth and salicylate yield during the first harvesting period following the stand establishment cannot be generalised to cover the whole 10-20 years cultivation period. As the pathogen structure of *Melampsora* may change and the specialist herbivores probably become more abundant during the aging of the willow plantation, the results of this study on damage by herbivores and

pathogens are only suggestive indicators of cultivation reliability. The study does, however, serve a starting-point for more long-term studies evaluating the cultivation reliability of herbal willow.

ACKNOWLEDGEMENTS

My sincere thanks are due to my supervisors Prof. Riitta Julkunen-Tiitto and Dr. Matti Rousi for their constructive and supportive comments on my manuscripts. I should especially like to thank Dr. Rousi for helping me to find the highlights and well-done parts of my work and to be happy with these. I also owe thanks to Dr. Satu Turtola, Ms. Ria Paunonen and Dr. Olli-Pekka Tikkanen for their help in the field and in the laboratory and for the constructive discussions and valuable comments on the manuscripts. Prof. Beat Meier and Mr. Veijo Tirkkonen also deserve thanks for their co-operation. Warm thanks to Ms. Outi Nousiainen and Ms. Sinikka Sorsa for their skillful and reliable work in the laboratory. I also want to thank Dr. Riitta Tegelberg, Prof. Heikki Henttonen and Mr. Asko Kaikusalo for their discussions, which helped me to find the interesting questions when planning the experiments and while analyzing the data. Many thanks go to Hanni and the staff of the Punkaharju Research Station of the Finnish Forest Research Institute, Maija, Jussi and his family, Johanna, Maarit, Elma, Eeva and the staff of the Mekrijärvi Research station for their help in the field and in the laboratory. I wish to thank Mr. Jaakko Heinonen for advising me with the statistical problems. I extend warm thanks to Ms. Joanna Goga, Ms. Rosemary Mackenzie and Mr. Kenneth Meaney, who kindly revised the English language. This work was financially supported by The Academy of Finland (project number 52784); Faculty of Mathematics and Science, University of Joensuu; GSForest - Graduate School in Forest Sciences and the Scientific Agricultural Society of Finland.

REFERENCES

Altieri, M. 1999. Agroecología. Bases Scientificas para una Agricultura Sustentable. Nordan-Comunidad, Montevideo, Uruguay. 339 p. In Spanish.

Anon. 2001. Willow bark Salicis cortex. Pp. 1602-1604 in: European Pharmacopoeia. The European Directorate for the Quality of Medicines and Health Care.

Anon. 2006. Yearbook of Farm Statistics. Information Centre of the Ministry of Agriculture and Forestry. Helsinki. 267 p.

Abrahamson, L., Volk, T., Kopp, R., White, E., Ballard, J. 2002. The Willow Biomass Producers Handbook. State University of New York, College of Environmental Science and Forestry, Syracuse. 31 pp.

Adegbidi, H., Volk, T., White, E., Abrahamson, L., Briggs, R and Bickelhaupt, D. 2001. Biomass and nutrient removal by willow clones in experimental bioenergy plantations in New York State. Biomass and Bioenergy. 20:399-411.

Braña, M., Del Río, L., Trive, N. and Salazar, N. 2005. La verdadera historia de la Aspirina. Anales de la Real Academia Nacional de Farmacia 71:813-819. In Spanish.

Bryant, J., Chapin III, F., Klein, D., 1983. Carbon/nutrient balance of boreal plants in in relation to vertebrate herbivory. Oikos 40:357-368.

Bryant, J., Wieland, G., Clausen, T. and Kuropat, P. 1985. Interactions of snowshoe hare and feltleaf willow in Alaska. Ecology 66:1564-1573.

Bullard, M., Mustill, S., Carver, P. and Nixon, P. 2002. Yield improvements through modification of planting density and harvest frequency in short rotation coppice *Salix* spp. - 2. Recourse capture and use in two morphologically diverse varieties. Biomass and Bioenergy 22:27-39.

Chrubasik, S., Eisenberg, E., Balan, E., Weinberger, T., Luzzati, R. and Conradt, C. 2000. Treatment of low back pain exacerbations with willow bark extract: A randomized double-blind study. American Journal of Medicine 109:9–14.

Clarkson, V. 1960. Effect of black polythene mulch on soil and microclimate temperature and nitrate level. Agronomy Journal 52:307-309.

Coley, P., Bryant, J. and Chapin III, F. 1985. Resource availability and plant antiherbivore defence. Science 230:895-899.

Dabrowska-Zamojcin E., Glinko, A., Samochowiec, L. and Kolodziej, B. 2002. Willow bark extract and aspirin – their potential for gastric injury in mice and other toxicity aspects. Focus on Alternative and Complementary Therapies 7:91.

Danfors, B., Ledin, S. and Roesenquist, H. 1997. Energiskogsodling. Handledning för odlare. JTI Informerar. Jordbrukstekniska Instituten, Uppsala, Sweden. 50 p. In Swedish.

Davies, R. 1988. Sheet mulching as an aid to broadleaved tree establishment I. The effectiveness of various synthetic sheets compared. Forestry 61:89-105.

Dawson, W. and McCracken, A. 1994. The effects of *Melampsora* rusts on the growth and development of *Salix burjatica* Korso in N. Ireland. European Journal of Plant Pathology 24:32-39.

Dearing, D., Foley, W and McLean, S. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annual Review in Ecology Evolution and Systematics 36:169-189.

Desprez-Loustau, M., Capron, G. and Dupuis, F. 1998. Relating germination dynamics of *Melampsora pinitorqua* teliospores to temperature and rainfall during overwintering. Forest Pathology 28:335-347.

Eisenberg, E., Chrubasik, S. Balan, E., Weinberger, T. and Luzzati, R. 2002. Willow bark extract for low back pain: a randomised double-blind study. Focus on Alternative and Complementary Therapies 7:91-92.

Fang, S., Xu, X., Lu, S. and Tang, L. 1999. Growth dynamics and biomass production in short-rotation poplar plantations: 6-year results for three clones at four spacings. Biomass and Bioenergy. 17:415-425.

Fields, M. and Orians, C. 2006. Specificity of phenolic glycoside induction in willow seedlings (*Salix sericea*) in response to herbivory. Journal of Chemical Ecology 32: 2647-2656.

Foley, W and Moore, B. 2005. Plant secondary metabolites and vertebrate herbivores – from physiological regulation to ecosystem function Current Opinion in Plant Biology 8:430-435.

Galambosi, B. 2006. Tuliko yrteistä elinkeino? Suomen yrttialan kehitys vuosina 1984-2004. Maaseutupolitiikan Yhteistyöryhmän Julkaisuja 5/2006, Maaseutupolitiikan Yhteistyöryhmä, Vammala. 102 p. In Finnish.

Gill, R. 1992. A review of damage by mammals in north temperate forests. 2. Small mammals. Forestry 65:281-308.

Green, S., Kruger, E., Stanosz, G. 2003. Effects of polythene mulch in a short-rotation, poplar plantation vary depending on weed-control strategies, site quality and clone. Forest Ecology and Management 173:251-260.

Gruppe, A., Fusseder, M., Schopf, R. 1999. Short rotation plantations of aspen and balsam poplar on former arable land in Germany: defoliating insects and leaf constituents. Forest Ecology and Management 121:113-122.

Hagerman A. 1995. Acid butanol assay for proanthocyanidins. Pp. 24-25 in: Hagerman A. (ed.) Tannin Analysis. Ohio, USA: Department of Chemistry, Miami University, Oxford.

Hakulinen, J., Julkunen-Tiitto, R. and Tahvanainen, J. 1995. Does nitrogen fertilization have an impact on the trade-off between willow growth and defensive secondary metabolism? Trees – Structure and Function 9: 235-240.

Hakulinen, J., Sorjonen, S. and Julkunen-Tiitto, R. 1999. Leaf phenolics of three willow clones differing in resistance to *Melampsora* rust infection. Physiologia Plantarum 105:662-669.

Hamilton, J., Zangerl, A., DeLucia, E. and Berenbaum, M. 2001. The carbon-nutrient balance hypothesis: its rise and fall. Ecology Letters 4:86-95.

Hansson, L. and Larsson, T-B. 1978. Vole diet on experimentally managed reforestation areas in northern Sweden. Holarctic Ecology 1:16-26.

Hansson, P-A., Svensson, S-E., Hallefält, F. and Diedrichs, H. 1999. Nutrient and cost optimization of fertilizing strategies for *Salix* including use of organic waste products. Biomass and Bioenergy 17:377-387.

Hedner, T. and Everts, B. 1998. The early clinical history of salicylates in rheumatology and pain. Clinical Rheumatology 17:17-25.

Herms, D. and Mattson, W. 1992. The dilemma of plants: to grow or defend? Quarterly Reviews in Biology 67:283-335.

Hiltunen, M. 2003. Feeding intensity of mountain hares (*Lepus timidus*) during winter in Finland. Mammalian Biology 68:48-52.

Hjältén, J. and Palo, T. 1992. Selection of deciduous trees by free ranging voles and hares in relation to plant chemistry. Oikos 63:477-484.

Houle, G. and Babeux, B. 1994. Fertilizing and mulching influence on the performance of four native woody species suitable for revegetation in subarctic Quebec. Canadian Journal of Forest Research 24:2342-2349.

Hytönen, J. and Kaunisto, S. 1999. Effect of fertilization on the biomass production of coppiced mixed birch and willow stands on a cut-away peatland. Biomass and Bioenergy 17:455-469.

Ikonen, A., Tahvanainen, J. and Roininen, H. 2001. Chlorogenic acid as an antiherbivore defence of willows against leaf beetles. Entomologia Experimentalis et Applicata 99:47-54.

Johnson, J. and Kim, Y. 2005. The role of leaf chemistry in *Melampsora medusae* infection of hybrid poplar: effects of leaf development and fungicide treatment. Canadian Journal of Forest Research 35:763-771.

Julkunen-Tiitto, R. 1986. A chemotaxonomic survey of phenolics in leaves of northern Salicaceae species. Phytochemistry 25:663-667.

Julkunen-Tiitto, R. 1989. Phenolic constituents of Salix: a chemotaxonomic survey of further finnish species. Phytochemistry 28:2115-2125.

Julkunen-Tiitto, R., Bryant, J., Kuropat, P. and Roininen, H. 1995. Slight tissue wounding fails to induce consistent chemical defense in three willow (*Salix* spp.) clones. Oecologia 101:467-471.

Julkunen-Tiitto, R., Hakulinen, J. and Meier, B. 1994. The response of growth and secondary metabolism to *Melampsora* rusts in field cultivated willow (*Salix*) clones. Acta Horticulturae 381:679-682.

Julkunen-Tiitto, R. and Meier, B. 1992. Variation in growth and secondary phenolics among field-cultivated clones of *Salix myrsinifolia*. Planta Medica 58:77-80.

Kanervo V. and Myllymäki, A. 1970. Problems caused by field vole, *Microtus agrestis*, in Scandinavia. EPPO Bulletin 5:11-26.

Keith, L. 1983. Role of food in hare population cycles. Oikos 40:385-395.

Kouwenhoven, J. 1997. Intra-row mechanical weed control – possibilities and problems. Soil and Tillage Research 41:87-104.

Kuokkanen, K., Niemelä,P., Matala, J., Julkunen-Tiitto, R., Heinonen, J., Rousi, M., Henttonen, H., Tahvanainen, J. and Kellomäki, S. 2004. The effects of elevated CO_2 and temperature on the resistance of winter-dormant birch seedlings (*Betula pendula*) to hares and voles. Global Change Biology 10:1504-1512.

Labrecque, M., Teodorescu, T., Babeux, P., Cogliastro, A. and Daigle, S. 1994. Impact of herbaceous competition and drainage conditions on the early productivity of willows under short-rotation intensive culture. Canadian Journal of Forest Research 24:493-501.

Laitinen, M-L, Julkunen-Tiitto, R., Yamaji, K., Heinonen, J. and Rousi, M. 2002. Variation in birch bark secondary chemistry between and within clones: implications for herbivory by hares. Oikos 104:316-326.

Matsuda, K. and Matsuo, H. 1985. A flavonoid, luteolin-7-glucoside, as well as salicin and populin, stimulating the feeding of leaf beetles attacking Salicaceous plants. Applied Entomology and Zoology 20:305-313.

Matsuda, K. and Senbo, S. 1986. Chlorogenic acid as a feeding deterrent for the Salicaceae-feeding leaf beetle *Lochmaeae capreae cribrata* (Coleoptera: Chrysomelidae) and other species of leaf beetles. Applied Entomology and Zoology 21:411-416.

McCracken, A. and Dawson, W. 1994. Experiences in the use of mixed-clonal stands of *Salix* as a method of reducing the impact of rust diseases. Norwegian Journal of Agricultural Science 18: 101-109.

McCracken, A. and Dawson, W. 1997. Growing clonal mixtures of willow reduce effect of *Melampsora epitea var. epitea*. European Journal of Forest Pathology 27:319-329.

McCracken, A. and Dawson, W. 2001. Pp. 111-120 in: Volk, T., Abrahamson, L., Ballard, J. (eds.) Proceedings of the Short Rotation Woody Crops Operations Working Group. State University of New York, College of Environmental Science and Forestry, Syracuse, NY, USA.

McCracken, A., Dawson, W., Watson, S and Allen, C. 2000. Pathotype composition in Melampsora epitea populations occurring on willow (*Salix*) grown in mixed and monoculture plantations. European Journal of Plant Pathology 106:879-886.

Meier, B., Sticher, O., Bettschart, A. 1985. Weidenrinden – Die Qualität. Deutsche Apotheker Zeitung 7:341-347. In german.

Morgan, R. 2005. Soil Erosion and Conservation. 3rd ed. Blackwell Publishing, Oxford, GB. 304 p.

Mortensen, J., Nielsen, K. and Jørgensen, U. 1998. Nitrate leaching during establishment of willow (*Salix viminalis*) on two soil types and at two fertilization levels. Biomass and Bioenergy 15:457-466.

Niemi, L., Wennström, A., Hjältén, J., Waldman, P. and Ericson, L. 2006. Spatial variation in resistance and virulence in host-pathogen system *Salix triandra-Melampsora amygdalinae*. Journal of Ecology 94:915-921.

Pasteels, M., Rowell-Rahier, M., Brakeman, J. and Dupont, A. 1983. Salicin from host plant as precursor of salicylaldehyde in defensive secretion of Chrysomelinae larvae. Physiological Entomology 8:307-314.

Pei, M., Royle, D. and Hunter, T. 1996. Pathogenic specialization in *Melampsora epitea* var. *epitea* on *Salix*. Plant Pathology 45:670-679.

Peldán, K. 1967. Suomen Farmasian Historia. Suomen Farmaseuttinen Yhdistys, Helsinki. 758 p. In Finnish.

Pierpoint, W. 1994. Salicylic acid and its derivatives in plants: medicines, metabolites and messenger molecules. Advances in Botanical Research 20:163-235.

Pohjamo, S., Hemming, J., Willför, S., Reunanen, M. and Holmbom, B. 2003. Phenolic extractives in *Salix caprea* wood and knots. Phytochemistry 63:165-169

Proe, M., Griffiths, J. and Craig, J. 2002. Effect of spacing, species and coppicing on leaf area, light interception and photosynthesis in short rotation forestry. Biomass and Bioenergy 23: 315-326.

Pywell, R., James, K., Herbert, I., Meek, E., Carvell, C. Bell, D. and Sparks, T. 2005. Determinants of overwintering habitat quality for beetles and spiders on arable farmland. Biological Conservation 123:79-90.

Ramstedt, M. 1999. Rust disease in willows – virulence variation and resistance breeding strategies. Forest Ecology and Management 121:101-111.

Rangen, S., Hawley, A. and Hudson, R. 1994. Relationship of snowshoe hare feeding preferences to nutrient and tannin content of four conifers. Canadian Journal of Forest Research 24:240-245.

Rank, J. 1992. Host plant preference based on salicylate chemistry in a willow leaf beetle (*Chrysomela aeneicollis*) Oecologia 90:95-101.

Rank, J., Köpf, A., Julkunen-Tiitto, R. and Tahvanainen, J. 1998. Host preference and larval performance of the salicylate-using leaf beatle *Phratora vitellinae*. Ecology 79:618-631.

Robinson, D. 1988. Mulches and herbicides in ornamental plantings. HortScience 23:547-552.

Rousi, M. 1988. Resistance breeding against voles in birch: possibilities for increasing resistance by provenance transfers. EPPO Bulletin 18:257-263.

Rousi, M. 1989. Susceptibility of winter-dormant *Pinus sylvestris* families to vole damage. Scandinavian Journal of Forest Research 4:149-161.

Rousi, M., Tahvananinen, J., Henttonen, H., Herms, D. and Uotila, I. 1997. Clonal variation in susceptibility of white birches (*Betula* spp.)

to mammalian and insect herbivores. Forest Science 43:396-402.

Rousi, M., Tahvanainen, J., Henttonen, H. and Uotila, I. 1993. Effects of shading and fertilisation on resistance of winter-dormant birch (*Betula pendula*) to voles and hares. Ecology 74:30-38.

Rowell-Rahier, M. 1984. The food plant preferences of *Phratora vitellinae* (Coleoptera: Chrysomelinae). Oecologia 64:375-380.

Ruuhola, T. and Julkunen-Tiitto, M-R. 2001. Salicylates of intact *Salix myrsinifolia* plantlets do not undergo rapid metabolic turnover. Plant Physiology 122:895-905.

Ruuhola, T., Tikkanen, O-P. and Tahvanainen, J. 2001. Differences in host use efficiency of larvae of a generalist moth, *Operopthera brumata* on three chemically divergent *Salix* species. Journal of Chemical Ecology 27:1595-1615.

Ruuhola, T., Sipura, M., Nousiainen, O. and Tahvanainen, J. 2001. Systemic induction of salicylates in *Salix myrsinifolia* (Salisb.). Annals of Botany 88:483-497.

Sage, R.1999. Weed competition in willow coppice crops: the cause and extent of yield losses. Weed Research 39:399-411.

Schmid, B, Lüdtke, R., Selbmann, H-K., Kötter, I., Tschirdewahn, B., Schaffner, W. and Heide, L. 2001a. Efficacy and tolerability of a standardised willow bark extract in patients with Osteoarthritis: Randomized placebo-controlled, double blind clinical trial. Phytotherapy Research 15:344-350.

Schmid B, Kotter I, Heide L. 2001b. Pharmacokinetics of salicin after oral administration of a standardized willow bark extract. European Journal of Clinical Pharmacology 57:387-91.

Setty, A. and Sigal, L. 2005. Herbal medications commonly used in the practice of rheumatology: mechanisms of action, efficacy, and side effects. Seminars in Arthritis and Rheumatism 34:773-784.

Sipura, M. 1999. Tritrophic interactions: willows, herbivorous insects and insectivorous birds. Oecologia 121:537-545.

Streck, N., Schneider, F. and Buriol, G. 1994. Modificações físicas causadas pelo mulching. Revista Brasileira de Agrometeorologia. 2:131-142. In Portugese, summary in English.

Tahvanainen, J., Julkunen-Tiitto, R. and Kettunen, J. 1985. Phenolic glycosides govern the food selection pattern of willow feeding leaf beetles. Oecologia 67:52-65.

Tegelberg, R. and Julkunen-Tiitto, R. 2001. Quantitative changes in secondary metabolites of dark-leaved willow (*Salix myrsinifolia*) exposed to enhanced ultraviolet-B radiation. Physiologia Plantarum 113:541-547.

Tikkanen, O.-P., Rousi, M., Ylioja, T. & Roininen, H. 2003. No negative correlation between growth and resistance to multiple herbivory in a deciduous tree, *Betula pendula*. Forest Ecology and Management 177:587-592.

Tolbert, V. and Schiller A. 1996. Environmental enhancement using short-rotation tree crops: Researchresults and directions. Pp. 217-224 in: BioEnergy '96: Proceedings of the Seventh National Bioenergy Conference, Southeastern Regional Biomass Energy Program, Nashville, TN, USA.

Turtola, S., Rousi, M., Pusenius, J., Yamaji, K., Heiska, S., Tirkkonen, V., Meier, B. and Julkunen-Tiitto, R. 2005. Clone-specific responses in leaf phenolics of willows exposed to enhanced UVB radiation and drought stress. Global Change Biology 11:1655-1663.

Vangessel, M., Wiles, L., Schweizer, E., Westra, P. and Wilson, R. 1998. Impact of timing and frequency of in-row cultivation for weed control in dry bean (*Phaseolus vulgaris*). Weed Technology 12:548-553.

Verwijst, T. 1996. Stool mortality and development of a competitive hierarchy in a Salix viminalis coppice system. Biomass and Bioenergy 10:245-250.

Weaver, S. 1984. Critical period of weed competition in three vegetable crops in relation to management practiced. Weed Research 24:317-325.

Weih, M. and Nordh, N-E. 2002. Characterising willows for biomass and phytoremediation: growth, nitrogen and water use of 14 willow clones under different irrigation and fertilisation regimes. Biomass and Bioenergy 23:397-413.

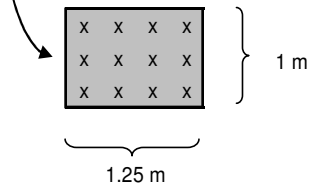
Zuin, V. and Wilegas, J. 2000. Pesticide residues in medicinal plants and phytomedicines. Phytotherapy Research 14:73-88.

ANNEX

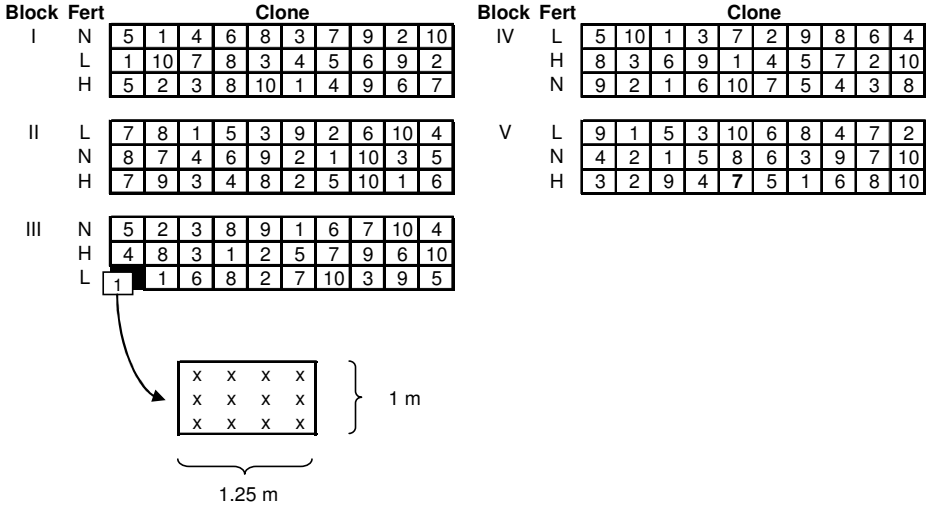
Annex 1. Experimental design of the cultivation experiment in Kaavi. Experimental area was split into eight blocks. Each block included six main plots (rows), treated with different combinations of mulch and fertliliser. Grey plots were treated with plastic mulch, open plots were unmulched. Letters N, L and H indicate no, low and high rates of fertiliser, respectively. Main plots were split into ten sub-plots. Each sub-plot included twelve cuttings belonging to the same clone (numbers 1-10 in within the boxes).

Block	Fert	Clones									
I	L	2	9	7	1	10	5	6	8	4	3
	L	10	6	4	5	7	8	3	1	9	2
	H	3	10	7	8	1	5	2	6	4	9
	N	9	2	6	10	5	4	8	7	1	3
	H	2	1	5	8	7	9	10	4	6	3
	N	8	2	6	10	1	9	4	3	5	7
II	L	1	2	5	3	8	10	7	6	9	4
	N	1	7	6	4	8	2	9	5	10	3
	H	6	10	3	5	7	9	4	2	1	8
	N	3	4	9	10	2	1	5	7	8	6
	L	10	9	2	5	1	3	6	8	4	7
	H	1	6	9	5	10	3	4	2	8	7
III	N	5	7	4	2	1	8	6	3	10	9
	N	9	10	1	7	6	8	2	5	3	4
	H	1	3	6	2	10	9	7	8	5	4
	L	3	8	4	5	7	6	9	1	10	2
	L	2	1	9	7	5	6	3	8	10	4
	H	6	10	4	8	5	7	9	2	3	1
IV	H	8	3	4	10	5	6	2	7	9	1
	N	1	9	2	3	7	4	5	10	6	8
	H	8	1	9	3	6	4	7	2	10	5
	N	6	9	5	2	10	3	4	7	1	8
	L	9	10	2	7	6	3	5	8	4	1
	L	1	4	8	3	9	2	6	7	5	10

Block	Fert	Clones									
V	N	2	4	1	3	9	10	5	8	7	6
	H	5	10	3	4	9	1	8	7	2	6
	L	5	3	6	7	1	10	8	9	4	2
	L	3	10	8	7	2	6	9	1	4	5
	N	9	4	3	2	5	6	8	7	10	1
	H	7	8	6	4	10	9	3	1	5	2
VI	H	10	2	8	6	5	9	4	7	1	3
	N	5	2	9	8	10	4	3	7	6	1
	L	3	10	4	8	7	1	2	5	9	6
	N	6	3	4	1	2	8	7	5	9	10
	L	10	2	5	1	7	3	4	6	8	9
	H	6	8	7	4	10	2	5	1	3	9
VII	H	7	8	3	5	1	2	6	4	9	10
	L	1	10	9	5	3	7	4	8	6	2
	H	3	10	2	7	5	9	8	4	6	1
	N	4	6	3	7	8	10	5	9	1	2
	L	10	6	2	7	5	4	3	8	9	1
	N	10	5	9	4	3	7	6	8	1	2
VIII	L	10	3	1	6	7	5	2	8	9	4
	N	1	10	9	4	8	2	6	5	3	7
	L	10	4	8	5	7	2	9	1	3	6
	H	5	8	4	3	2	9	10	1	7	6
	H	6	8	5	9	7	1	4	10	2	3
	N	10	9	8	4	5	7	2	3	1	6



Annex 2. Experimental design of the cultivation experiment in Punkaharju. Experimental area was split into five blocks. Each block included three main plots (rows), treated with no, low and high rates of fertiliser (letters N, L and H in the rows, respectively). Main plots were split into ten sub-plots. Each sub-plot included twelve cuttings belonging to the same clone (numbers 1-10 within the boxes).



Annex 3. Experimental design of the laboratory feeding experiment in Joensuu. Each vole (rows) received a different set of 10 twigs (squares) belonging to the different clones (indicated by the numbers in the squares) and grown in plastic mulch (grey squares) and in unmulched control (white squares). The whole procedure was replicated in three days.

Day 1

Vole	Twigs									
1	8	10	1	2	3	4	6	7	8	10
2	3	4	6	7	3	4	6	7	8	10
3	1	2	4	6	8	2	3	4	8	10
4	1	3	6	7	8	1	2	3	7	8
5	1	2	6	7	8	10	4	6	7	8
6	1	2	4	7	8	1	3	6	7	10
7	1	3	4	6	8	10	1	6	8	10
8	2	3	4	6	8	10	1	3	4	7
9	2	3	4	7	8	10	2	3	6	8
10	1	2	3	4	1	2	4	6	7	8
11	1	2	3	7	10	1	3	4	8	10
12	1	3	4	7	8	10	2	4	7	10
13	1	2	3	6	10	2	3	6	7	10
14	1	4	6	7	10	1	2	3	4	6
15	2	4	6	7	10	1	2	7	8	10
16	2	3	6	7	8	1	2	4	6	10

Day 2

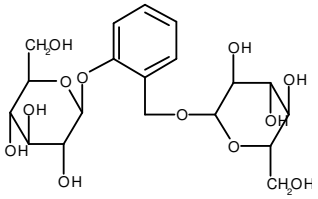
Vole	Twigs									
1	1	3	4	7	8	10	2	4	7	10
2	1	2	3	6	10	2	3	6	7	10
3	1	2	6	7	8	10	4	6	7	8
4	1	3	6	7	8	1	2	3	7	8
5	1	2	3	7	10	1	3	4	8	10
6	3	4	6	7	3	4	6	7	8	10
7	2	3	4	6	8	10	1	3	4	7
8	1	3	4	6	8	10	1	6	8	10
9	8	10	1	2	3	4	6	7	8	10
10	1	2	4	6	8	2	3	4	8	10
11	1	4	6	7	10	1	2	3	4	6
12	2	4	6	7	10	1	2	7	8	10
13	2	3	6	7	8	1	2	4	6	10
14	2	3	4	7	8	10	2	3	6	8
15	1	2	4	7	8	1	3	6	7	10
16	1	2	3	4	1	2	4	6	7	8

Day 3

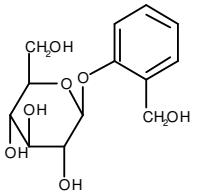
Vole	Twigs									
1	1	2	4	7	8	1	3	6	7	10
2	2	3	6	7	8	1	2	4	6	10
3	1	2	3	7	10	1	3	4	8	10
4	1	3	6	7	8	1	2	3	7	8
5	3	4	6	7	3	4	6	7	8	10
6	1	2	4	6	8	2	3	4	8	10
7	1	4	6	7	10	1	2	3	4	6
8	2	4	6	7	10	1	2	7	8	10
9	1	2	3	6	10	2	3	6	7	10
10	2	3	4	7	8	10	2	3	6	8
11	1	3	4	6	8	10	1	6	8	10
12	1	2	3	4	1	2	4	6	7	8
13	1	3	4	7	8	10	2	4	7	10
14	2	3	4	6	8	10	1	3	4	7
15	8	10	1	2	3	4	6	7	8	10
16	1	2	6	7	8	10	4	6	7	8

Annex 4. Molecule structures of the phenolic compounds found in the leaves and bark of studied willows.

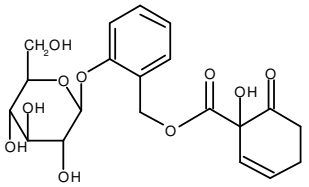
Diglucoside of salicyl alcohol



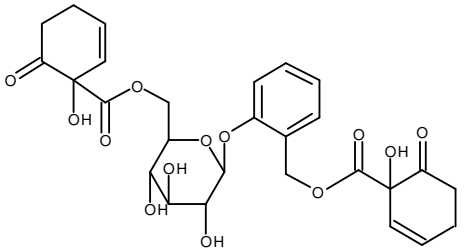
Salicin



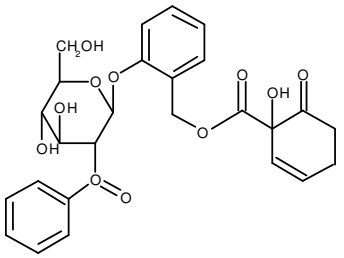
Salicortin



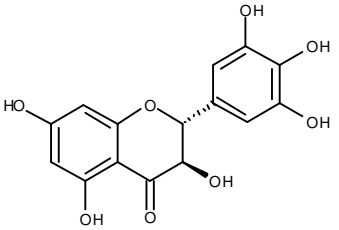
HCH-salicortin



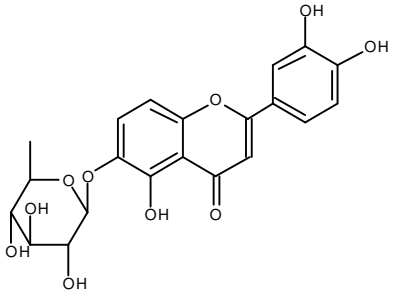
Tremulacin



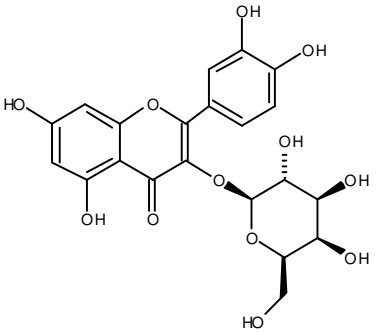
Ampelopsin



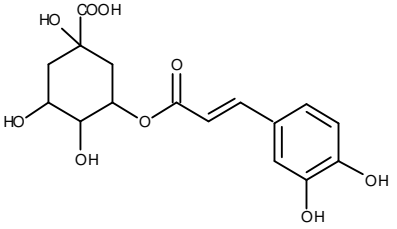
Luteolin-7-glucoside



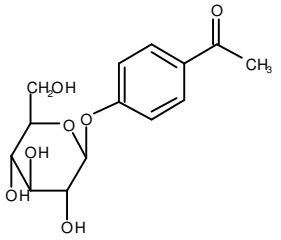
Hyperin (quercetin-3-galactoside)



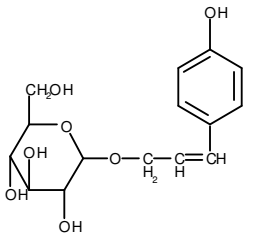
Chlorogenic acid



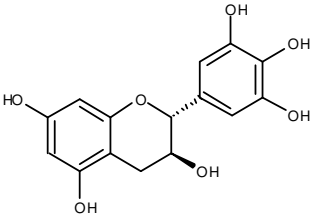
Picein



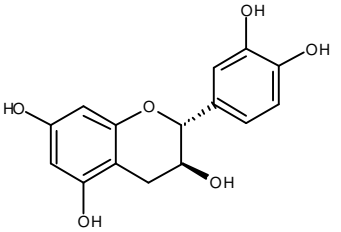
Triandrin



Gallocatechin



(+)-catechin



Condensed tannin
R=H or
R=OH