Allelopathic effects of bark phenols on epiphytic lichens

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Contents

List of symbols and abbreviations

HPLC: high perfomance liquid chromatography

RRT: relative retention time

TLC: thin layer chromatography

MS: mass spectroscopy

ESI: electrospray ionisation in MS

CI: chemical ionisation in MS

LD50 = IC50 : lethal dose/ inhibitory concentration, that inhibits 50% growth of soredia

spp.: different subspecies

Introduction

Allelochemicals are defined as plant secondary metabolites, that are released components from certain plant parts, that enhance or inhibit growth of plants of the same and other species in its surrounding (Molisch,1930). Today vegetational mapping and classification of plant communities do not imply allelopathic constraints of the substratum or solutes. Instead nutrient availability, espacially of nitrogen, phosphorus, kalium and trace elements are evaluated and widely used markers for plant growth under varying conditions. On the other hand, organic metabolites of the substratum are of interest as inhibitory residues in agricultural soils, or in evaluation of successional stages, e.g. after fire in different ecosystems.

Some physiological responses of allelochemicals, for example during seed germination can be attributed to general mechanisms, like osmosis or pH (Evenari, 1949), as has been analysed by germination tests with artificial solutions of defined pH and osmotic potential but without addition of secondary metabolites.

Still, none of the artificial solutions could account for the same strength of growth retarding effects similar to those obtained with solutions containing allelochemicals, indicating physiological interactions with target plant cells (Harborne, 1995; Evenari, 1949).

Our interest is directed towards allelochemical interactions between epiphytic lichens and bark phenolic allelochemicals.

Dürr, (1992) classified the preferences of different lichen species according to Ellenbergs system, using the criteria: light, humidity, nutrients, salt tolarance, pH and other general characteristics, while Barkman (1958) evaluates both physical, chemical and ecological factors in his extensive study on european epiphytic lichens. Although he already discussed the important role of total ectrolyte concentration, water capacity and pH of bark, he proposed a low impact of bark resins and tannines on lichen growth, while selective occurance of lichen – tree pairs were supposed to rely on specialized adaptation of lichens to tree allelochemicals.

Today, lichens and bryophytes are important monitoring organisms in the framework of environmental research. Eutrophication, air pollution, especially with SO_2 and the impact of heavy metals are described by cryptogamic plants, as they are very sensitive towards pollutants due to a lack of protective cuticular structures, their growing season, that extends to autumn and winter months and their slow growth.

Field monitoring of epiphytes has been described with respect to site selection and detailed procedures (VDI,2004)and the impact of eutrophication has been added to present guidelines, recently. (Franzen-Reuter, 2004).

The impact of tree age, annual precipitation rates of the study area and underlying allelochemical mechanisms have been evaluated during the present study, as natural implications for plant growth are important for our understanding of vegetational zonation.

Therefore, we analyzed allelopathic effects of bark phenols on epiphytic lichens on the basis of natural conditions and vegetation in different areas of North-Rhine-Westphalia (chapter 3), and on the microhabitat of a single tree (chapter 4).

Chemical and biochemical investigations on tissue distribution of endogenous phenols in the abundant lichen species: *Hypogymnia physodes* (chapter 1) and on allelopathic effects of natural bark phenols of the widely used tree species *Populus canadensis* acting on juvenile tissues of the lichen species: *Physcia tenella* in vitro (chapter 2) were carried out in order to understand the physiological basis of allelochemical interactions in epiphytes.

Aim of the study and Working programme:

1.) Analysis of natural concentrations of endogenous lichen phenols from epiphytic species in both reproductive (apothecia and soredia) and somatic tissues (main thalli).

2.) Development of an in-vitro test system for the evaluation of possible allelopathic effects of single bark phenols on reproductive lichen stages (soredia).

3.) Analysis of natural concentrations of bark phenols in trees of different age classes and at different sample sites in North-Rhine-Westphalia

4.) Analysis of natural concentrations of bark phenols in different parts of a single tree (height on the trunk, branch versus main trunk, stemflow areas versus dry areas)

1 Tissue distribution of phenols in epiphytic lichens:

Implications for growth and establishment of juvenile stages

1.1 Abstract:

In the present study we evaluated the distribution of endogenous lichen phenols within different tissue types in the epiphytic species *Hypogymnia physodes* (*Lecanorales, Ascomycetes*) growing on *Betula pubescens* in northern Finland. Both reproductive soredia and somatic thalline structures showed the same spectrum of phenols with a ratio of cortical atranorin to medullar physodic acids of 1: 4, but a significantly higher total content of phenols in soredial lobe ends (7,0 +/- 1,2% d.w.) compared to non soredial lobe ends (2,7 +/- 0,7% d.w.).

The influence of thallus age on total phenol contents was studied and varied between 6,0 % and 3,0% in whole thalli of different sizes and branching patterns. Water capacity of lobe ends was lower than water capacity of inner thalline structures: 95% versus 200%.

Physiological and ecological aspects of lichen phenol distribution and tissue development are discussed.

1.2 Introduction:

Lichen phenols comprise about 1 to 10 % of thallus dry weight, so that their total contents are much lower than concentrations of phenoles in higher plants. Lignin amounts to 20 % d.w. in tree tissues (Fengel, 1984). Although lichen thalli contain much less phenolic constituents than higher plants, the biological activity of their strongly hydrophobic phenolic metabolites is very high (Lawrey, 1995; Lauterwein, 1995).

The epiphytic lichen species *Hypogymnia physodes* is able to synthesize four phenolic metabolites, which can be found in the cortical (atranorin) and the medullar tissue (physodic acids) as excreted crystalls on the outer surface of fungal hyphae (Kauppi, 1990). Their biosynthesis requires both algae and fungi, although a variety of typical lichen phenols can be produced by mycobiont cultures, only (Yoshimura, 1994)

Depsides and depsidones function as UV protectants and feeding deterrents in the surface tissue layers - additionally a regulating role within symbiosis can be deduced from in-vitro experiments with isolated symbionts. Backor (1989) showed a pH dependend inhibition of algal cell cultures by lichen phenols and various studies showed their ability to inhibit fungal growth (Ruotsalainen, 1999; Halama, 2004; Vainshtein,1992).

Still, there are only few investigations on the distribution and concentration of lichen phenols in differentiated thallus structures, like reproductive apothecia or soredial lobe ends or in thalli of different age classes (Laakso, 1952; Manriqe, 1991). The focus on recent studies has been related to the UV protective role of phenols in differently exposed parts of podetia in *Cladoniaceae* and to a survey of a correlation between phenol content and sample sites at varying degree of incident radiation throughout Finland (Huovinen,1985).

Epiphyte vegetation establishes within the microhabitat of tree bark. Therefore allelopathic constraints of the adjacent substratum mediated by bark allelochemicals have to be evaluated with respect to the physiology and regulating role of endogenous secondary metabolites within reproductive structures and juvenile thalli.

Soredia are small (25-100um) balls of a few phycobiont cells wrapped in mycobiont hyphae. They originate in the medulla and algal layer and are released through pores or cracks in the upper surface of the thallus. In most species, soredia are produced in deliminated zones called soralia(Lawrey,1984).

We investigated the composition and total concentration of lichen phenols in reproductive and somatic thallus structures of the sorediate species *Hypogymnia physodes* in order to evaluate factors that are important for internal regulation of symbiosis and for allelopathic inhibition by phenols from internal and external sources.

1.3 Materials and methods

Sampling of lichen thalli:

Lichen thalli of *Hypogymnia physodes* were collected from *Betula pubescens* growing on the island Hailuoto in the Gulf of Bothnia near Oulu, Finland (64°45`N; 26° 00`E).

Five thalli were sampled from each tree at locations in mixed forest stands further than 5 km apart.

Lichen for the isolation of atranorin and physodic acids were sampled from trunks of *Pinus sylvestris* in a forest site with a stand of *Picea abies* and *Pinus sylvestris* near Muhos, thirty km east of Oulu.

Sample pretreatment:

Samples for isolation of lichen phenols were cleaned from bark residues and air dried for 5 days at room temperature. Storage of air dried thalli did not exceed two months.

Samples for tissue analysis were rehydrated over night in water saturated air at 5°C,

and then sprayed with deionised water and subsequently dissected using a stereo microscope.

Lobe ends were defined as the outer 2 mm wide periphery of thalli both in soredial and non soredial parts.

Dry weight was determined from subsamples after air drying for two days at room temperature.

Isolation of lichen phenols:

10 g of dried thalli were extracted for three days in a soxhlett extractor using ethylacetate as solvent.

After evaporation of the solvent at temperatures < 50 °C, the dry residue was subjected to flash chromatography (column: 25 cm x 5 cm) using silica gel (mesh 0,063 – 0,2) and the eluent:

ethylacetate 20 / acetone 15 / methylenchloride 6 / methanol 6 / water 4 .

Fractions were tested on TLC sheets Polygram Sil G/UV 40 x 80 mm, coated with 0,25 mm silica containing fluorescence indicator. (Macherey & Nagel). All solvents used were purchased from Merck and were of HPLC grade. Visualization of spots was done by spraying with 10% H_2SO_4 in ethanol.

Fractions obtained after evaporation of the eluent were of yellow – grey colour. The first fraction contained thermolabile, unknown apolar compounds and following fractions 10 to 120 mg of single lichen phenols. RRT (relative retention times) were: atranorin: 0,90; physodalic acid: 0,68, physodic acid: 0,59; hydroxy-physodic acid: 0,52. Chemical characterization was confirmed by RP-HPLC according to Huovinen (1985) and EI-MS.

pH of lichen extracts in water:

The pH was determined from air dried lichen thalli by immersion in destilled water in a ratio of 1 : 10 (w/v) over night. After filtration of lichen thalli pH was determined by a glass electrode.

Water capacity of tissues:

Water capacity was determined by determination of the ratio of weight at water saturation and weight after air drying. Water capacity = 100 (fresh weight – dry weight / dry weight)

Extraction and Analysis of small sample sizes:

Tissue fractions of 4 – 15 mg were weighed into tubes, 2 x 5 ml of diethylether was added and each extraction was performed by ultrasonication for 5 min at room temperature.
The combined extracts were filled to 10 ml and an aliquot used for photometrical and HPLC analysis according to Huovinen, 1985.
Photometric analysis was performed at 270 nm using isolated physodic acid mix as standard.
The ratio of atranorin to physodic acids was determined using TLC separation and quantification by digital imaging of spot fluorescence.

Analysis of total phenols:

Total phenols were analyzed in a modified price-butler method photometrically as iron-phenol complex at a wavelength of 700 nm from crude extracts. The standard used was atranorin (synth.)

1.4 Results and Discussion:

Analysis of total phenols in soredial lobe ends compared to non soredial lobe ends in thalli of the same age class revealed a significant difference in total contents, while there was no difference in the spectrum of phenolic constituents. Significant differences of total contents were obvious in all thalli analyzed (total number: 19), irrespective of sample site characteristics. While mean contents in soredial lobe ends were 7,0 % d.w. (+/- 1,2 %) mean contents in non-soredial lobe ends were 2,7 % d.w. (+/- 0,7%). (Table 1).

Nevertheless, the ratio of atranorin to physodic acids was about 1 : 4 (mean of 5 extractions) irrespective of reproductive or somatic thallus part analyzed, indicating the same ratio of cortical to medullar tissues both in soredial and non-soredial lobe ends. (Table 2)

Thallus age had a significant influence on total phenol contents. Thalli of three different diameters were investigated: Age class I (0,2 – 0,5 cm): 6 % d.w.; age class II (0, - 2,0 cm): 5 %. d.w. and age class III (2,0 – 5,0 cm): 3 % d.w. (mean of three sample extractions) (Table 3)

Water capacity of thalli was higher in the inner part of thalli in age class III compared to soredial lobe ends in the same age class of thalli: 200 % versus 95 % and pH of whole thallus extracts in water was 4,0. (single analysis with a biomass > 10 g)

We found twice as high phenol contents in soredial lobe ends compared to contents in non soredial lobe ends. This indicates a high biosynthetic activity and high stability of depsides and depsidones in soredial tissue. Previous studies showed , that lichen spore germination is inhibited by lichen acids of other species (Whiton, 1982) and bryophyte spore germination is inhibited by lichen acids from adjecent lichens species (Frahm, 2000) In the present case, inhibition of germination and growth by high contents of lichen acids of the same species to adjacent lichens or from parental plants towards propagules can be termed autotoxicity.

Here, increased contents of lichen phenols prevent premature germination at unfavorable conditions, e.g at insufficient air humidity and water supply. Furthermore, reproductive tissues with high phenol contents at exposed thallus structures, like apothecia, isidia and soredia are well defended against herbivores (Hyvärinen, 2000), e.g. snails (Hesbacher, 1995) or saprotrophic fungi (Lawrey, 1995).

A low internal pH and low water capacity in soredial tissues are additional factors, that prevent growth, increase the ability of dispersal of dry soredia and increase defense against fungal pathogens.

Environmental conditions change when soredia establish at bark microsites.

Biosynthesis of lichen acids may be of importance within the microhabitat of bark, that generally shows higher pH values than pH 4,0 in deciduous trees - for example pH 6,0 in *Populus canadensis* (Franzen-Reuter, 2004*)*. Here, biosynthesis of lichen acids within thallus tissues creates an internal pH that is different from bark pH and allows higher ability of defense against pathogens. Bark phenols and nutrients in stemflow are further allelochemical factors of importance during epiphytic lichen growth and establishment. Specific lichen - phorophyte associations have been described by Barkman, 1958 and we propose to analyse specific enhancement or inhibition of soredial growth in adapted and absent lichen species by whole extracts or isolated tree phenols at natural bark pH.

Acknowledgement: The present work describes preliminary experiments of the author for an extended cooperative study on the chemical ecology of three epipyhtic lichen species published elsewhere (Hyvärinen,2000). The present study has been carried out at the university of Oulu during 1998.

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1.5 Figures and Tables

Table 1.1

Phenol contents in *Hypogymnia physodes*

Sample site No.	**Total phenols % d.w.** **Soredial tissue**	**Total phenols % d.w.** **Non-soredial tissue**
1	8,3	3,0
2	6,8	2,2
3	5,8	2,0
4	6,8	3,4
Mean	**7,0 +/- 1,2**	**2,7 +/- 0,7**

Data are mean values of five extractions and analysis ; thallus diameter varied between 2 – 5 cm

Table 1.2

Phenol ratios in *Hypogymnia physodes* (reproductive / somatic tissues)

Sample No.	**Atranorin : Physodic acids** **Soredial tissue**	**Atranorin : Physodic acids** **Non-soredial tissue**
1	1 : 4,5	1 : 4,0
2	1 : 4,2	1 : 5,7
3	1 : 3,8	1 : 3,6
4,.	1 : 4,7	1 : 4,4
5	1 : 3,7	1 : 2,7
Mean	**1 : 4,2 (+/- 0,5)**	**1 : 4,1 (+/- 1,5)**

Table 1.3

Phenol ratios in *Hypogymnia physodes* (age classes)

Age class	Total phenols d.w.
I (0,2 – 0,5 cm)	6,0 +/- 1,0
II (0,5 – 2,0 cm)	4,8 +/- 0,5
III (2,0 – 5,0 cm)	3,0 +/- 0,5

Data are mean values of three extractions and analysis

2 In –vitro inhibition of soredial growth in the epiphytic lichen:

Physcia tenella (Lecanorales, Ascomycetes) **by a variety of bark phenols**

2.1 Abstract:

We tested the in-vitro growth rate of soredia from the common epiphytic lichen species: *Physcia tenella* at different concentrations of added phenols in an experimental design previously described by Hauck (2000) in order to evaluate the allelopathic effects of bark derived substances in stemflow.

The conditions used (pH = 6,0, endogenous phenols) were designed to resemble those found on bark of *Populus x canadensis*, as this tree species has been widely used for lichen mapping studies in Germany. Phenolic glycosides, flavonoids and tannines undergo hydrolytic decomposition in stemflow, resulting in a variety of monomeric phenolic acids, aldehyds and alcohols. Here we tested 11 phenolic substances of different biosynthetic origin in the concentration range between 10^{-6}M and 10^{-3}M . LD50 values were calculated for each substance - inhibition was highest for catechol, substituted phenolic glycosides, benzoic and ellagic acid and moderate for flavonoids, gallic acid, salicylic alcohol, salicylic aldehyd and low for salicylic acid.

2.2 Introduction:

Though it has been found, that lichen acids inhibit germination and growth of phanerophytes (Follmann, 1963) bryophytes (Frahm, 2000), algae (Backor, 1998), soil- and wood decaying and plant pathogenic fungi (Vainshtain, 1992, Halama, 2004) and other lichens (Whiton, 1984), the allelopathic effect of leachable organic compounds from phanerophytes on growth and establishment of lichens has been investigated in only a few studies (Pyatt, 1973; Ostrofsky, 1980). Nevertheless, especially epiphytic lichens may be influenced markedly by soluble substances in stemflow and throughfall.

Leachates originating from tree bark contain a variety of plant metabolites: Sugars and sugar alcohols, amino acids, metal- and nutrient ions and plant secondary metabolites, like phenoles, alkaloids, and others (Hauck, 2002, Fengel, 1984)

In allelophysiology, phenolic metabolites are investigated due to their high degree of species specifity (Hegnauer, 1962) and their endogenous role as protective agents against herbivores (Clausen, 1989), while their antimicrobial and antifungal activity has important implications in development of resistence against plant pathogens (Hakulinen, 1999). Soil properties, like microbial respiration, or inoculation rate with myccorhizal fungi are influenced by secondary metabolites in leaf litter decomposition (Olsen, 1971)

Endogenous lichen phenols are hydrophobic depsides, dibenzofuranes or anthroquinones. They are widely used for chemotaxonomical studies (Lawrey, 1984). Their localization within the lichen thalli shows typical medular and cortical substances (Kauppi, 1989), which differ in their hydrophobicity. They are qualitatively equally distributed throughout reproductive and somatic structures. Quantitatively, reproductive structures, like soredia or ascospores contain significantly higher amounts of phenols than somatic, thalline structures (Hyvärinen, 2000).

Wether this increased concentration determines lichen establishment in vivo is unknown. In vitro studies indicate inhibition of soredial growth by lichen acids of other lichen species (Whiton, 1977), supporting the theory of allelopathic interaction among lichen species.

The effects of water based bark extracts have been investigated on lichen ascospore germination. Both enhancement and inhibition have been found (Pyatt, 1973; Ostrofsky, 1980).

Our study is the first work using distinct organic compounds originating from tree bark in evaluating growth inhibition of epiphytic lichen soredia in vitro.

2.3 Materials and methods:

Plants: Saplings of *Populus x canadensis* were purchased from a tree nursery near Hannover in February 2004. Plants were two years old and had a stem length of about 1.50 m. After two days of plant storage at 10 °C, the stem was cut into pieces of about 3 cm length, and dried in an oven at a maximum temperature of 33 °C for 4 days. This material was used without further separation of wood and bark for isolation of salicortin and tremulacin. The epiphytic lichen *Physcia tenella* was collected in May on 60 years old cherry and apple trees near Gummersbach and stored in a paper bag at room temperature. Soredia for in vitro germination were used fresh one day after collection of thalli. Isolation of soredia was done by scrapping the tissue gently with a steel needle.

Chemicals:

All solvents and reagents used were purchased by Roth KG, Karlsruhe. Solvents used were of HPLC grade, reagents for preparation of nutrient solutions or for spectrophotometry were of analytical grade. Synthetic phenols used in tests had a purity of 98% to 99,5 %.

Culture medium:

A 0,8% agar was used containing 4ml/l of the following nutrient solution:

(0,1 g/l KCl; 1,5 g/l NH_4NO_3 ; 0,1 g/l $MgSO_4$; 0,5 g/l KH_2PO_4 ; 0,04 g/l$CaSO_4$ + 2 ml/l (1,35 g/l $FeCl_3$; 1,86 g/l EDTA) + 2ml/l of a trace element solution pH 4,6 (given in Hauck, 2002):

(containing: 2,68 g/l H_3BO_3, 1,18 g/l $MnCl_2x4H_20$, 0,22 g/l $ZnSO_4x7H_20$, 0,012 g/l MoO_3, 0,079 g/l $CuSO_4x5H_2O$)).

The agar solution was adjusted to pH: 6.0 with 1M HCl.

Chromatography:

Preparative column chromatography was done using a glass column with an internal diameter of 6 cm and a length of 34 cm (Volume = 960 cm^3) connected to a fraction collector. TLC analysis was done using Macherey & Nagel 10x5 cm Alugram SIL UV $_{254}$ sheets and the eluent described in the isolation procedure.

Isolation of Salicortin and Tremulacin:

175g of dried twigs of *Populus x canadensis* were extracted three times with 0,5 l methanol at room temperature for 1h in an ultrasonication bath. After evaporation of methanol at a maximum temperature of 50°C, the dark green, crude extract was subjected to preparative column chromatography using silica gel 60 (mesh.: 0,063-0,2 mm). The eluent used was: dichlormethan/ethylacetate/methanol/water (50/30/20/5). Fractions were evaporated to dryness, yielding a honey-coloured compound. Phenolic glycosides could be detected on TLC plates after spraying with 10% sulphuric acid in ethanol by their red coloured spots in contrast to other phenols with brown spots. Synthetic D-(-)-salicin was chosen as standard, which had a lower Rf value than isolated salicylates: tremulacin (own: RRT = 2,1 ; Lit.: Meier, (1988) : RRT = 2,3) and salicortin (own: RRT = 1,4 ; Lit.: Meier (1988): RRT = 1,4). Additionally, basic mass spectral data were obtained by direct injection of tremulacin and salicortin dissolved in methanol (see Annex). Mass spectra indicated slight deviation of real structures from given structures by side chain methylation or acetylation of tremulacin and salicortin. Still we continued to describe the isolates as “salicortin” and “tremulacin” in the following section.

Soredia-Agar test:

Aliquots of 150 ml of culture medium were prepared and sterilzed in erlenmeyer flasks, sufficient for preparation of 5 conventional steril petri dishes with a diameter of 8,5 cm. Phenols were weighed and added in solid form to the culture medium after cooling to 50 – 40°C. After solidification of the agar plates, 15 µl of suspended soredia in steril water were placed on top of the agar plate and spread onto the surface with sterile equipment. Plates were sealed with parafilm and incubated at 23 °C for 7 days at a natural light intensity lasting 12 hours in 24 hours. After incubation, the plates were unsealed and the upper plate lifted, in order to prevent counting mismatches due to visual interferences on the upper plate. Counting was done using a magnifying glass of 20 dpt (Eschenbach GmbH). Growing soredia could be detected by a small whitish ring of hyphae around the central core. Few contaminating fungi occurred, but did not interfere with soredial growth. Each concentration of added phenol was tested on 5 replicate plates. Altogether 11 compounds in four different concentrations: $5x\ 10^{-3}$ M; 5×10^{-4} M, 5×10^{-5} M and 5×10^{-6} M were tested. Concentrations refer to the final agar solution used in tests. Soredial growth rate was expressed as % growth compared to control plates without addition of phenoles.

LD50 values were calculated by doing graphical regression analysis of mean results for three different concentrations of each compound, representing high, medium and low inhibtion rates. Accuracy of the counting method was estimated to be 15 % using counting deviation from two investigations, weighing and dilution accuracy.

2.4 Results:

Table 1 shows LD50 values for all substances tested. The concentration, that is needed to obtain 50% inhibition of soredial growth differs from 10^{-3}M to 10^{-6}M. Scheme 1 shows the order of inhibitory action. Both hydrophobic (ellagic acid) and hydrophilic (benzoic acid) phenols are effective growth inhibitors at pH 6,0 with LD50 values lower than 10^{-4} M. Toxicity is highest for the main endogenous phenolic glycoside in young saplings of *Salicaceae*: tremulacin (LD50 = $<1 \times 10^{-6}$ M) and its main degradation products: catechol (LD50 = 1×10^{-5} M) and benzoic acid (LD50 = 2×10^{-5} M).

The biosynthetic precursors of tremulacin: salicortin and salicin are strong inhibitors with both LD50 values of 4×10^{-5} M.

Salicylic aldehyde, salicylic alcohol and (+)-catechin are moderate inhibitors with LD50 = 3×10^{-4}M

Salicylic acid and gallic acid are weak inhibitors of soredial growth compared to other salicylates: LD50 = 2×10^{-3} M.

2.5 Discussion:

In vitro tests of toxicity can only be model systems for environmental effects. Our choice of phenols represents a few characteristic substances of the whole spectrum of phenolic metabolites and biodegradation products occurring in bark of Salicaceae. We used substituted salicylates and their metabolic degradation products, ranging from the simple phenolic glycoside: salicin to catechol, benzoic acid and others (Ruuhola, 2003) Nevertheless, it has to be kept in mind, that all species specific secondary metabolites from *Salicaceae* have been isolated from young tissue, mainly leaves, twigs or bast (Julkunen, 1989). The spectrum of phenolic constituents in cork layers of aged trees of Populus spp. has not been investigated, so far. Comparable studies on cork extractives of *Quercus suber,* show a complex spectrum of extractable suberin, lignin-derived phenols and polysaccharides (Cordeiro, 2002)

Gallic and ellagic acid were included in our study, as they are widespread in woody plants though ellagic acid does not occur in *Salicaceae* (Hegnauer, 1962) .

Aromatic aldehydes and phenolic hydroxyls are biochemically reactive towards amino groups, both in biopolymers, like proteins, DNA and chitine and in related monomers, like amino acids, DNA bases or N-acetylglucosamine. Structural alterations of polymers and formation of toxic metabolites like aromatic amines inhibit cell division and differentiation. Furthermore, aromatic allelochemicals act as chelating agents, interacting with metal ions in biochemical complexes, like enzymes or membrane bound systems of energy and light acquisition (Parlar, 1991).

Highest toxicity in our test system was found for both hydrophilic (tremulacin, benzoic acid) and hydrophobic (ellagic acid) phenolic compounds. Previous studies focusing on antifungal components of *Populus* spp, indicated high toxicity both for phenolic glycosides and their degradation products: benzoic acid and catechol (Olsen, 1971; Butin, 1969) but did not analyze the effects of hydrophobic metabolites.

Uptake of allelochemicals into lichen algae or lichen fungi has to be evaluated under the specialized physiology of symbiosis. Lichen fungi are able to produce gelatinous layers around those hyphae, which are not in ultimate contact to algal cells. Furthermore, crystalline deposits of lichen acids and a chitine layer accompany polysaccharides - the major constituents of cell walls in lichen fungi. Algal cells in lichen symbiosis are surrounded by a trilaminar layer, containing an amorphous, polysaccharide derived matrix (Lawrey, 1984, Honegger, 1991).

Therefore it can be assumed, that allelochemicals acting on mature lichen thalli react with a variety of membrane polymers in a first step. Soredia are juvenile lichen thalli with less differentiated tissues, therefore highly susceptible to the uptake of allelochemicals.

Early stages of plant development are one of the main research areas in the field of allelophysiology (Evenari, 1949) and further work on lichen establishment and growth will have to show, wether studies on phanerophyte development are comparable to symbiotic organism.

Mature lichen thalli are subjected to allelochemicals from bark by direct attachment of lichen rhizines to the outer cork layer and by leachable components of bark in stemflow. 80 years old trees of *Populus x canadensis* show a bark pH of 6.0 (Franzen-Reuter, 2004). In lichen mapping studies focusing on air pollution, tree species with acidic and neutral to subneutral bark are analyzed in different groups as they host different lichen communities (VDI RL3957) Therefore our in vitro study evaluates the action of allelochemicals on tree species with subneutral bark, only: *Populus spp., Acer spp. , Fraxinus spp. , Ulmus spp. , or Malus spp*. Stability of phenolic glycosides and net charge of carboxylic acids, like benzoic or gallic acid will be altered at acidic pH, so that LD50 values obtained in our study can not be used to discuss allelopathy on trees with acidic bark. Furthermore, each tree species contains specific endogenous phenols in young bark and leaves, that contribute to stemflow chemistry by leaching processes. In *Salicaceae* phenolic glycosides represent less than 1% of dry weight of bark in saplings, while the amount of total phenols is about 5% (Julkunen, 1989). Salicylates, like tremulacin and salicortin are easily degraded in aqueous or buffered solution (Ruuhola, 2003). The resulting catechol, benzoic acid and salicin have been shown to be potent inhibitors of soredial growth in our test system. Therefore highest toxicity of the species specific tremulacin and salicortin in aqueous nutrient solution can be attributed to the combined action of their degradation products. Leachable degradation products from widespread tannines and proanthocyanidines, like gallic acid and catechin are less potent inhibitors of soredial growth (Table 1).

Physico-chemical parameters often named in relation to epiphyte growth are: Water holding capacity of bark, cation- and nutrient content. A prominent example of a tree lichen pair, that depends on nutrient rich bark near urban centres is: *Xanthoria parietina* on *Populus tremula* (Kuusinen,1994). Height on the tree trunk is another important factor determining site characteristics for epiphytes beneath snow cover in winter and in moist dripping zones during the growing season (Hyvärinen, 1992; Hauck, 2003). Ecological studies focus on forest stand characteristics, like shading from adjecent trees, tree age, (Hyvärinen, 1992) and soil properties (Gustaffson, 1995).

Hauck, (2002) found a negative relationship between the concentration of sulfate in bark tissues and vegetational cover with the common epiphytic lichen: *Hypogymnia physodes* on *Picea abies*, which he related to the influence of air pollution.

So far, ecological studies did not include the influence of allelochemicals into explanations about epiphytic lichen abundance.

Tertiary growth of the cork layer in aged trees leads to the formation of dead, suberinized and air filled cork cavities (Fengel, 1984) Concentration of total phenols in the outer 0,5 cm of cork layer of 80 years old *Populus x canadensis* amounts to about 6 mg/g dry weight (own data, not shown) corresponding to 3,5 x 10^{-5} mol gallic acid equivalents.

Inhibition of soredial growth in-vitro was measured at concentrations ranging from 10^{-6} M to 10^{-3} M in 0,8% agar – nutrient solution. Therefore our in-vitro test can be used as a model system for natural conditions occuring on the abundant tree-lichen pair: *Populus x canadensis* and *Physcia tenella.* Further studies focusing on allelophysiology of lichen propagules will have to consider both natural concentrations of allelochemicals in bark and corresponding vegetational patterns of epiphytic lichens.

Acknowledgment:

The author whishes to thank Isabelle Franzen-Reuter and Rolf Blöcher for their guidance during the in-vitro tests. Thanks also to my family for their support during bark and lichen sample preparation.

5 trees of the tree species used for bark sample preparation have been planted at their place instead of the preperation of Voucher specimens and for possible further studies.

Chemical isolation of bark phenols has been done in the group of Prof Manfred Grote at the department of chemistry University of Paderborn. Thanks to all members of the group for their kind welcome during my stay, espacially to Henning Stevens for obtaining MS spectra.

2.6 Figures and Tables

Table 2.1

LD50-values for selected phenols on the growth rate of Soredia from *Physcia tenella* in-vitro

Chemical substance	**LD50 (mol/l) x 10^{-6}M**
Tremulacin	< 1
Salicortin	40 (+/-10)
D-(-)-Salicin	40 (+/-10)
Salicylic acid	2000 (+/- 1000)
Salicylic alcohol	300 (+/-100)
Salicylic aldehyd	180 (+/-80)
Catechol	10 (+/- 3)
Gallic-acid	1700 (+/-800)
Ellagic-acid	20 (+/-5)
(+)-Catechin	400 (+/-100)
Benzoic acid	20 (+/-5)

Scheme 2.1

Order of toxicity in Soredia-agar test

tremulacin (10^{-6} M)

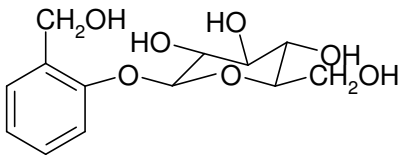
benzoic acid, ellagic acid, catechol, salicortin, salicin (10^{-5}M)

salicylic aldehyde, salicylic alcohol, catechin (10^{-4}M)

salicylic acid , gallic acid (10^{-3} M)

Figure 2.1

Chemical structures of salicylate glycosides



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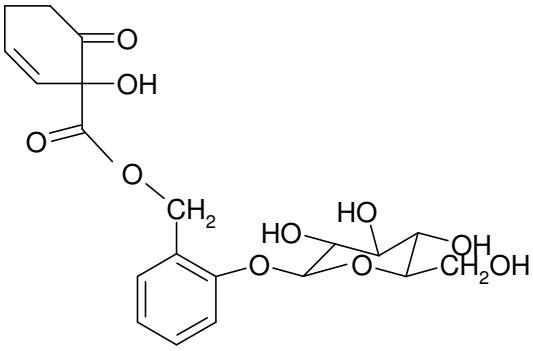

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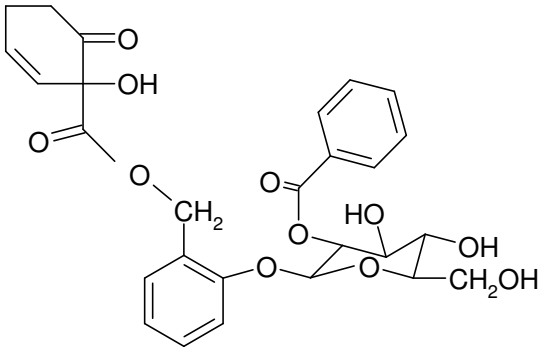
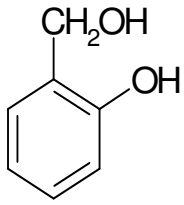


III

Figure 2.2 Chemical structures of bark phenols



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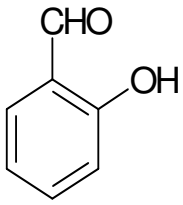

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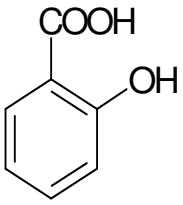

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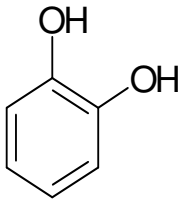
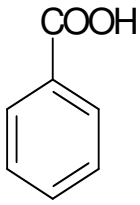


VII



VIII

I Salicin

II Salicortin

III Tremulacin

IV Salicylic alcohol

V Salicylic aldehyde

VI Salicylic acid

VII Catechol

VIII Benzoic acid

3 Bark phenols in relation to tree age and site factors:

Allelopathy on epiphytic lichen vegetation ?

3.1 Abstract:

Populus x canadensis is a tree species widely used for lichen mapping studies in Germany.

We studied the influence of tree age, height on the main trunk and sampling site characteristics on concentrations of total phenols in outer bark of *Populus x canadensis,* in order to evaluate possible allelopathic effects of leachable plant phenols on species richness and abundance of epiphytic lichens and bryophytes. Species richness correlated to site factors, but did not correlate to phenol content of bark. Epiphyte cover was inversely correlated to phenol content in bark, though exceptional sites showed no correlations. Concentration of total phenols decreased with tree age. Cork layers of 80 years old trees contained between 2 mg/g d.w. and 10 mg/g d.w., while 2 years old trees contained 30 mg/g d.w. in outer cork layers of bark. Variation in concentrations of phenols in trees of the same age class could be correlated to annual precipitation along an altitudinal transect throughout North-Rhine - Westphalia in central Germany. A low content of phenols correlated with high precipitation rates as a result of outwashing of easily leachable phenolic bark constituents. Epiphyte cover inceased on trees in montane regions with high precipitation rates and on old compared to young trees. Height and azimuth on the main trunk had significant influence on community structure of epiphytes, but no statistically significant influence on concentrations of phenols could be found within the basal trunk area. Ecological and physiological parameters acting on epiphytic communities are discussed.

3.2 Introduction:

Epiphytic lichens may be influenced by soluble substances in stemflow, as they lack protective cuticular tissues. In vitro studies indicate, that whole bark extracts exhibit antifungal activity both to soil saprophytic, mycorrhizal- and plant pathogenic fungi (Butin, 1969 ; Olsen, 1971, Yang, 2004). Both enhancing and inhibiting effects of bark extracts have been found for lichen ascospore and soredial growth in-vitro (Pyatt,1973 ; Ostrofsky, 1980; own data presented elsewhere). Tree response to pathogenic fungi include structural and induced mechanisms, both mediated by secondary plant phenolics. Epiphytic lichens and bryophytes are attached to outer cork layers of adult trees. Though their ecological niches differ markedly from parasitic plant pathogenes, they may be subjected to the same defense related allelochemicals.

While induced defense relies on pathways forming intracellular phenolic glycosides, coumarines and cinnamic acid derivates, structural defense is established continously during tree ageing by lignification. The latter aspect has to be evaluated using different time scales for different plant parts: leave tannine concentration increases during the growing season (Harborne, 1995), while the concentration of bark tannines changes significantly during several decades of tree growth. Furthermore allelopathic interactions mediated by secondary phenoles are well known for the vegetational process of litter decomposition in forest soils (Souto, 2000) and plant community succession (Harborne, 1995). The allelopathic effects of tree phenols on epiphytes have been studied only in a single case (Frahm, 2000). We followed the hypothesis, that old trees show increased epiphyte growth, partly favored by outwashing of inhibitory allelochemicals from outer bark. Furthermore, increased annual precipitation was expected to favor epiphyte growth by decreased concentrations of leachable allelochemicals. This hypothesis was investigated, using *Populus x canadensis*, a cultured tree species widely used for lichen mapping studies in Germany (Franzen-Reuter, 2003).

3.3 Materials and methods:

Bark sampling:

Two samples were taken from the outer 0,5 cm cork layer of *Populus x canadensis* at 30 cm, 85 cm, 1.25 cm and 1.55 cm on the north-west and south-east slope of the main trunk.

Samples were dried at 30 °C for three days and stored in paper bags at room temperature.

They were analyzed no later than 6 weeks after collection.

Samples for photometrical analysis were prepared by careful dissection of outer cork layers from cork attached lichens. Lichen tissue was excluded from any analysis and only bare cork layers were used. Small cork fractions of few mm diameter were scrapped of, resulting in similar sample sizes for extractions.

Sample sites:

Sampling sites were located near Düren (2540600/5629000), Königswinter (2584470/6167500) and Winterberg (3467500/5673200). Sites with *Populus x canadensis* in Düren were located at the edge of hayfields or small streets, while sites in Bonn were located near the river Rhine or at the edge of forests. Sites near Winterberg and Lennestadt were located near forests or meadows.

All trees investigated originated from cultured stands. Both solitairy trees and trees in stands were included. Tree age was estimated according to stem diameter at a height of 1.25 cm.

A stem girth of 150 – 230 cm was correlated to about 80 years; a stem girth of 350 – 420 cm to 140 years. For the transect study, six sample sites were chosen at locations between 90 m above sea height and 650 m above sea height. For the tree age study two age classes at sample sites near Bonn were chosen (80, 140 years). Samples for the two years old trees were taken from saplings growing along a small stream near Emmerthal. For the analysis of phenols related to height on the main trunk three height classes at one sample site near Bonn were taken (30 cm, 85 cm, 155 cm above ground). Analysis of species richness and correlation to trunk azimuth was done on six sample sites near Düren and Königswinter, which were more than 5 km apart. Each correlation analysis was based on a minimum of six samples taken from three different trees at one sample site.

Chemicals:

All solvents and reagents used were purchased by Roth KG, Karlsruhe.

Solvents used were of HPLC grade, reagents were of analytical grade.

Analysis of total phenols:

Total phenols were analysed photometrically as described in Hagerman (1995) using a modified Price-Butler method. Extaction of bark was done by 10 min ultrasonication of 25 mg bark in 2 x 3 ml 70% acetone at room temperature. The supernatants were combined and filled to 10 ml with 70% aceton. 0,5 to 1ml was used in the test. Gallic acid (3 – 50 µg/ml) was used as standard.

The procedure follows the preparation steps.:

1 ml sample solution

2 ml dest. Water

1 ml $K_3Fe(CN)_6$ 0,016M (510 mg ad 100 ml dest H_2O)

1 ml $FeCl_3$, 0,02M (320 mg ad 100 ml 0,1M HCl)

wait for 15 min

add 1 ml H_3PO_4 (20%)

Measurement of the green Fe-tannine complex formed was done using a Perkin Elmer spectrophotometer model lambda 11 at a wavelength of 700 nm.

Lichen species richness

Species richness was measured according to VDI guidelines (VDI, 2004). Measurement of epiphyte cover was done using five squares of 10 x 10 cm at a height of 100 to 150 cm on the main trunk in northwest and southeast slope resulting in 10 squares per tree.

Lichen and bryophyte abundance was estimated at a height of 100 cm to 155 cm on the main trunk in northwest and southeast slope using the following scale:

< 10% ; 10 – 20 % ; 20 – 30 % ; 30 – 50% ; 50 – 75% ; 75 – 90 % ; > 90%

Additionally, field estimation of epiphyte abundance was confirmed by using macrophotographs of all trees investigated.

Data treatment:

Mean values were calculated from data obtained from 3 to 12 trees per sample site.

Comparison of mean values was done using the t-test (95% confidence).

Correlation Data were analyzed using graphical plots in linear regression analysis and by calculating Pearssons regression coefficient.

3.4 Results:

Tree age is the most important factor determining concentrations of total phenols in outer bark of *Populus x canadensis*. While bark roughness and thickness increases, phenol content decreases considerably. Diagramme 1 shows concentrations of total phenols for 2, 80 and 140 years old trees. 140 years old trees contain 3,0 mg/g d.w. (+/- 0,9 mg/g d.w.) in its outer cork layer, while 2 years old trees reach 29,0 mg/g d.w. (+/- 4,0 mg/g d.w.). Height on the basal trunk (Diagramme 2) did not influence concentrations of total phenols significantly. Nevertheless, a structural differences between smooth and moist bark at 30 cm above ground and hard and dry bark in 155cm could be observed. Bryophyte cover in semi-dry areas (< 700 mm) near Euskirchen was restricted to trunk areas below 30cm and to the upper layer of twigs, while bryophytes in areas with high annual precipitation (> 1100mm) covered all height zones on the main trunk and twigs. External site factors, like sunshine and weathering in north-west and south-east direction did not influence concentrations of total phenols in trees of the same age class in a semi dry area (80 years old trees, data not shown), while increased annual precipitation (1100 mm versus 700 mm) caused a significant decrease in total phenols in outer bark (Diagramme 4).

Epiphytic lichens responded to external factors, as north-west and south-east azimuth of the trunk and total annual pecipitation rates, as these were important variables for the growth of different lichen communities (crustose versus foliose lichens in southeast slopes and in dry areas).

Neither differing phenol concentrations related to tree age, nor variation of phenol content within trees of the same age class (80 years) could account for lichen species richness (Diagramme 3).

Instead, epiphyte abundance and total cover increased in correlation to a) increased annual precipitation (Table 1), b) distance to industrial centres (Map 1) and c) decreased content of bark phenolic allelochemicals (Diagramme 4, 5). The combined action of all three factors were analyzed along an altitudinal transect throughout North-Rhine-Westphalia in Germany for the first time.

3.5 Discussion:

Secondary metabolites in plant tissues show a high degree of species specifity (Hegnauer, 1962), but also high variation in relation to a variety of biotic and abiotic factors. Tissue differentiation, defense against pathogens and herbivores, differing nutrient availablity and season are some of the most prominent causes of variation of concentrations in single substances (Harborne, 1995; Julkunen-Tiitto, 1989, Hakulinen, 1998). Light is related to the biosynthesis of plant secondary phenols by enzyme activation of the shikimic acid pathway and direct photochemical conversion of metabolites, especially in biosynthesis and degradation of lignin (Nuhn, 1990). Biosynthetic mechanisms have to be evaluated in the investigation of defense mediated by phenolic metabolites in leaves and current growth twigs. Cork evolves from tertiary growth, being comprised of dead suberinized cork cells in its outer layer. It functions as protective coat to the tree and due to its high content of suberin (40 – 50%) , a polymerized aliphatic acid, lignin, tannines and waxes (30%) and only 12 % of polysaccharides (Fengel, 1984) it is of low nutritional value to plant pathogenes. In contrast, it is resistent to weathering, deterrent against herbivores (Laitinen, 2004) and plays a role in tissue protection against UV radiation (Tegelberg, 2001). Outer bark is subjected to direct contact with its environment. Sunshine, rainfall, freezing and biological impact alter cork structure and composition of cork chemicals (Fengel, 1984).

We found high concentrations of phenols in cork tissue of young trees, while old trees showed decreased phenol contents, but compensated by development of thick and structured layers.

Organic extractives such as flavonoids or phenolic acids are located as crystalline deposits within cork cells, or they form part of the cell wall, like tannines (Fengel, 1984). Phenolic extractives from *Populus* bark are effective antifungal agents (Yang, 2004 ;Butin, 1969; Olsen,1971). We evaluated several site factors on outwashing of phenols from outer bark in order to analyze allelopathic constraints of phenolic bark components on epiphyte growth. While bark moisture and smoothness was highest on the stem basis (ground to 35 cm) and increased readily up to 155 cm, no statistically significant correlation of trunk height to bark phenol concentration could be found. Slope of the trunk, determining weathering influence could not be correlated to phenol content, either. Both results indicate, that phenolic constituents in outer bark of forest trees are strongly associated to cell wall structures (Fengel, 1984). Though exposed to weathering and moisture, tannines form part of the cork layer. Their ecological function can not be compared to biosynthetically inducable phenolic acids of early defense mechanism against fungal leave pathogenes nor to easily leachable leave litter phenols.

Though tannines are less effective feeding deterrents and antifungal agents, they impose stuctural strength to exposed tissues (Harbone, 1995; Tegelberg, 2001).

While whole cork layers are detached in trees with intense bark peeling (*Acer* spp, *Betula* spp) outer bark in *Populus x canadensis* shows highly structured layers with deep furrows. Direct rainfall causes stemflow in the inner part of single furrows. In regions with high annual precipitation (> 1100 mm/year) cork structure is smoother and furrows are flatened compared to trees in semi-dy regions (< 700 mm/year) , indicating both detachment of whole layers on the top of furrows and increased leaching of bark constituents in remaining cork layers. Our analysis of epiphyte growth along an altitudinal transect indicated considerable increase of epiphyte cover, especially of bryophytes with increasing site height above sea level and increasing annual precipitation. While in semi- dry regions bryophytes occurred on the lower part of the stem basis (< 35 cm above ground level) and on twigs, increased precipitation favored bryophytes on all parts of the main trunk.

Epiphytic lichens and bryophytes are attached to cork layers by their rhizines. Unlike parasitic epiphytes, like mistletoe (*Viscum alba*), they are autotrophes, which do not root into sieve or bast tissues of trees. Nevertheless, lichens and bryophytes are subjected to bark allelochemicals, that are primarily protective against plant pathogenic and wood destroying fungi. In vitro studies indicate inhibition of soredial growth by a variety of plant phenols (own data) and both inhibitory and enhancing effects of whole bark extracts on ascospore growth could be found (Pyatt, 1973 ; Ostrofsky, 1980). Field studies showed low survival for transplanted lichen thalli in regularly moist parts of the trunk. Thalli were imbalanced due to extreme algal compared to low fungal growth (Schuster, 1985).

In general, moisture conditions, rather than allelopathic constraints are used to described zonation of different epiphyte communities. The lowest 20-50 cm of the trunk are colonized by moisture tolerant bryophtes, the north-west slope of the trunk forms a suitable habitat for bryophytes and foliose lichens and the dry and sunny south-east slope hosts a higher percentage of crustose compared to foliose lichen species. Highly specialized adaptations to soil allelochemicals have been found for certain strains of mycorrhizal fungi cultivated in vitro (Souto, 2000) and on phanerophyte vegetation after for example forest fires, in gardening or in desert plant communities (Harborne, 1995) In contrast, allelopathy mediated by secondary phenoles in stemflow has not been studied so far.

Still, the effects of mineral composition have been analyzed by Hauck (2002) , who found ratios of Mn/Ca and Mn/Mg in dripzones to be essential for establishment of cyanolichens on bark of unusual

host trees and established concentrations of SO_4^{2-} to be important in imposing stemflow characteristics in temperate spruce forests (Hauck, 2002).

Eutrophication by nitrogenous air pollutants has been considered in new methods for lichen monitoring in Germany (Franzen-Reuter, 2003; VDI) as lichen abundance of nitrophileous species increased during recent years.

Detailed chemical analysis of single phenolic allelochemicals originating from different plant parts or stem areas with increased stemflow or the analysis of bark phenols at a trunk height in greater distance to ground level might reveal more specialized adaptation of single epiphyte species than has been possible in our pilot study.

Stemflow and stemflow characteristics within cork structures impose a characteristic vegetational pattern to trees. The abundant *Physcia tenella* on *Populus x canadensis* preferably grows in trunk areas with high probability of stemflow. This pattern may reflect areas of high dispersal and high survival rate of lichen propagules. Further factors influencing soredial dispersal are seasonal effects (Armstrong, 1991; Tormo, 2001), and moisture of the substrate during soredial establishment and during thallus growth (Schuster, 1995). Ecological factors imply the involvement of bark insects in dispersal of soredia. (own observations). Previous studies on allelopathic interactions mediated by plant phenolic metabolites between the host plant *Ecklonia radiata*, a sublitoral kelp and its attached epiphytes (Jennings, 1997) describe tissue specific levels of phlorotannines, but correlation to epiphyte growth is low and age dependend structural characteristics but not chemical factors are made responsible for enhanced growth of epiphytic species on certain plant parts. Still, this lack of correlation between epiphyte abundance and content of leachable allelochemicals may be due to a) high variation in plant metabolites among individual trees and b) site factors acting on both tree and epiphyte growth. Tree age and tree species diversity in old growth forests are generally accepted as most important factor for the establishment of a rich and diverse epiphyte vegetation in boreal (Hyvärinen, 1992 ; Hyvärinen, 1993 ; Kuusinen, 1994 ; Gustaffson, 1995 ; Boudreault, 2000 ; Benson, 2002) temperate (Eckhardt, 2003); mediterranian (Loppi, 2004) and tropical forests (Zotz, 2003).

Our study shows a statistically significant decrease of bark phenols in aged trees and in trees subjected to high annual precipitation, closing a gap between biochemical processes acting on single tree tissues and biological field data, which correlate increased epiphyte cover with site characteristics, like stand age.

Our further work will be focused on the biochemistry of phenolic allelochemicals and the ecology of different epiphyte communities in microhabitats on different plant parts of an individual tree. As a result of our study, lichen mapping studies in germany (VDI) will include detailed guidelines for selection of sampling sites with similar conditions of annual precipitation.

3.6 Figures and Tables

Map 3.1: Sampling Sites in Central Europe

E : Euskirchen (160m above sea level) ; W : Winterberg (840 m above sea level)

Table 3.1

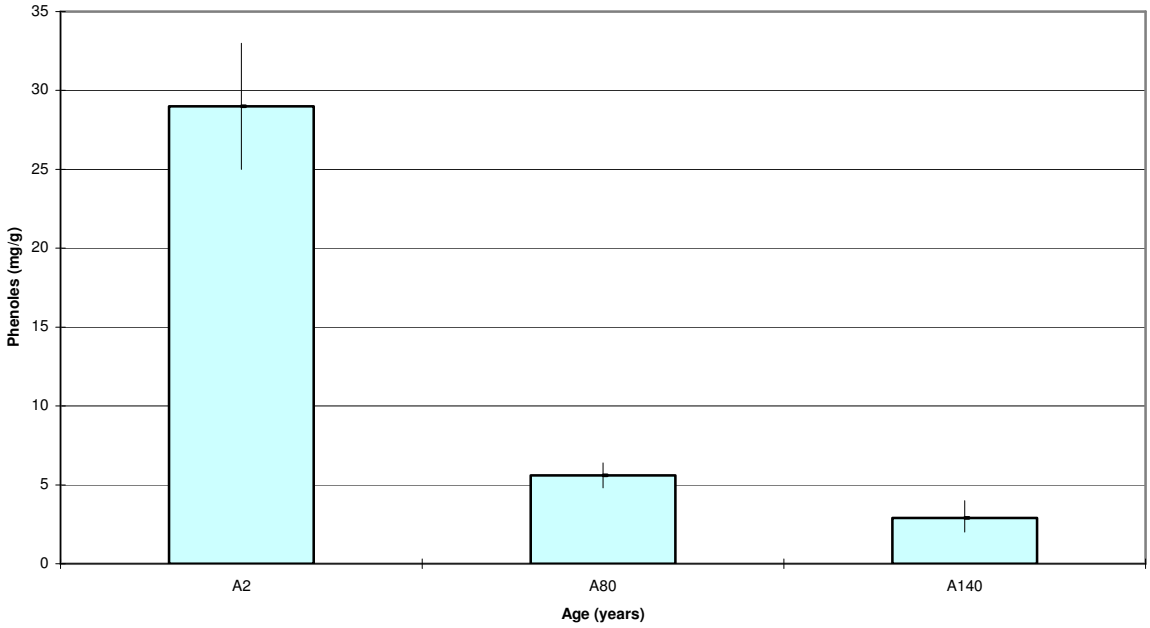
Climatic characteristics of sampling areas:

Site/climate	**Bonn (90m)**	**Euskirchen (160m)**	**Lennestadt (300m)**	**Winterberg (840m)**
Mean annual Temp.	11,2	11,4	9,9	6,2
Mean annual precipitation	922	653	1128	1283
Days with precipitation > 0,1mm	208	203	243	238
Days with ground snowcover > 50%	4	7	15	98

Source: Deutscher Wetterdienst, Essen ; Jahresreport 2000

Diagramme 3.1

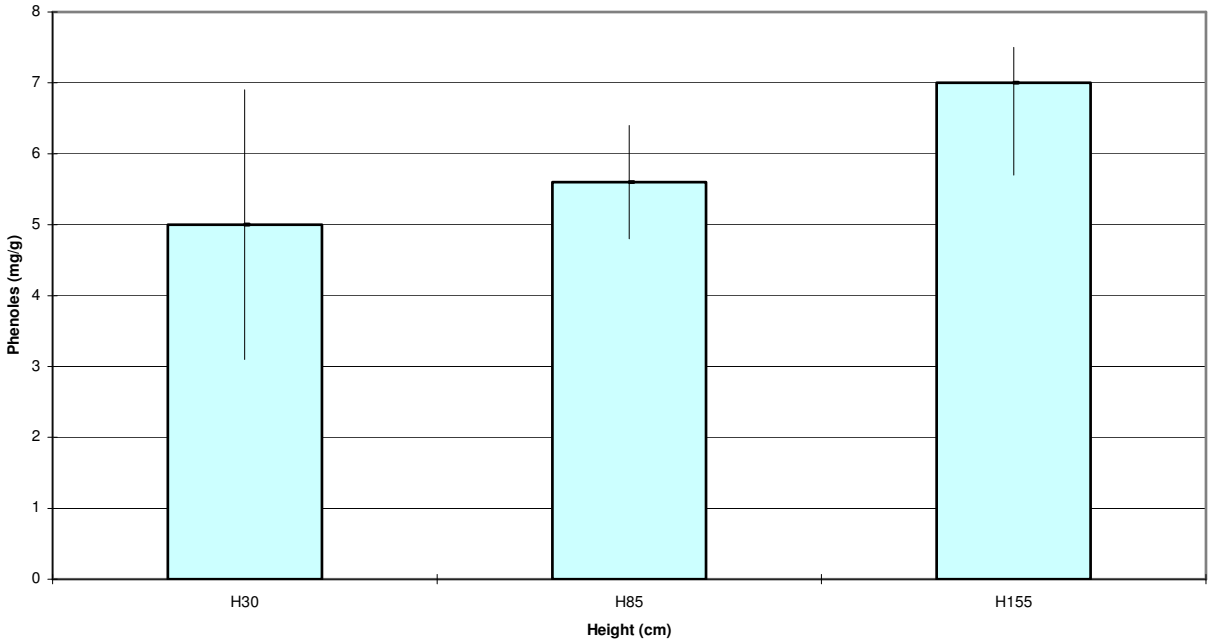
Tree age versus phenol content



Mean values are calculated from data obtained from five trees. Bark samples were taken at 1.25 cm on the main trunk.

Diagramme 3.2

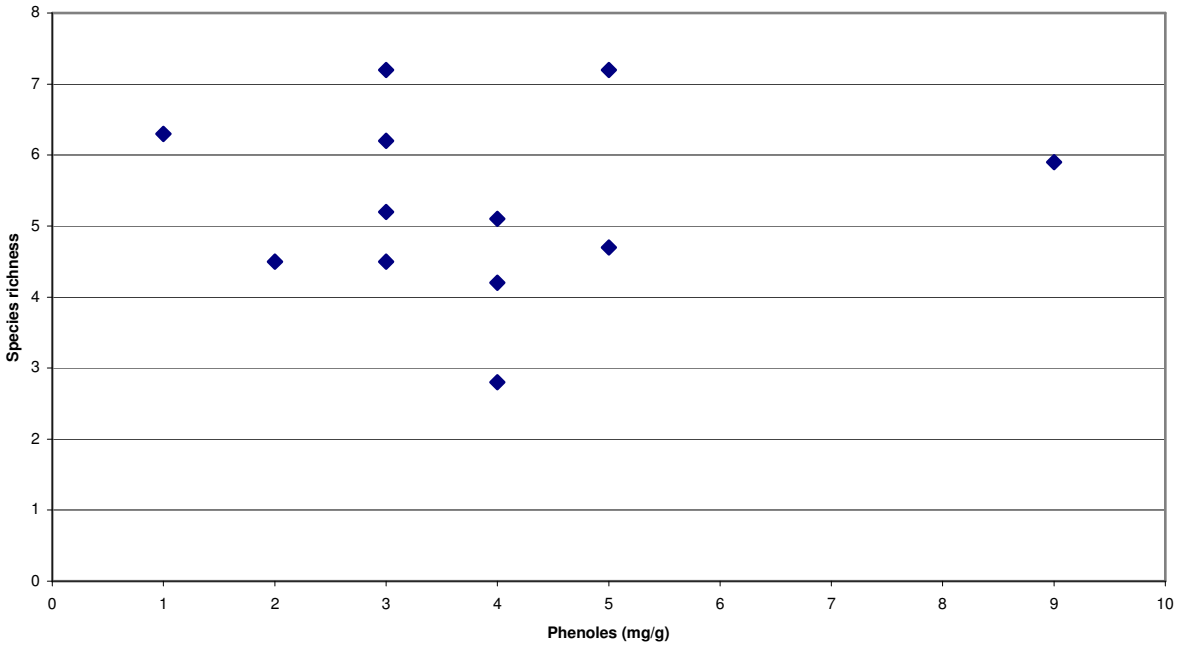
Trunk height versus phenol content



Mean values were obtained from data obtained from five trees at one sample plot (Königswinter). Bark samples were taken at the northwest slope of the main trunk.

Diagramme 3.3

Correlation of phenol content and lichen species richness

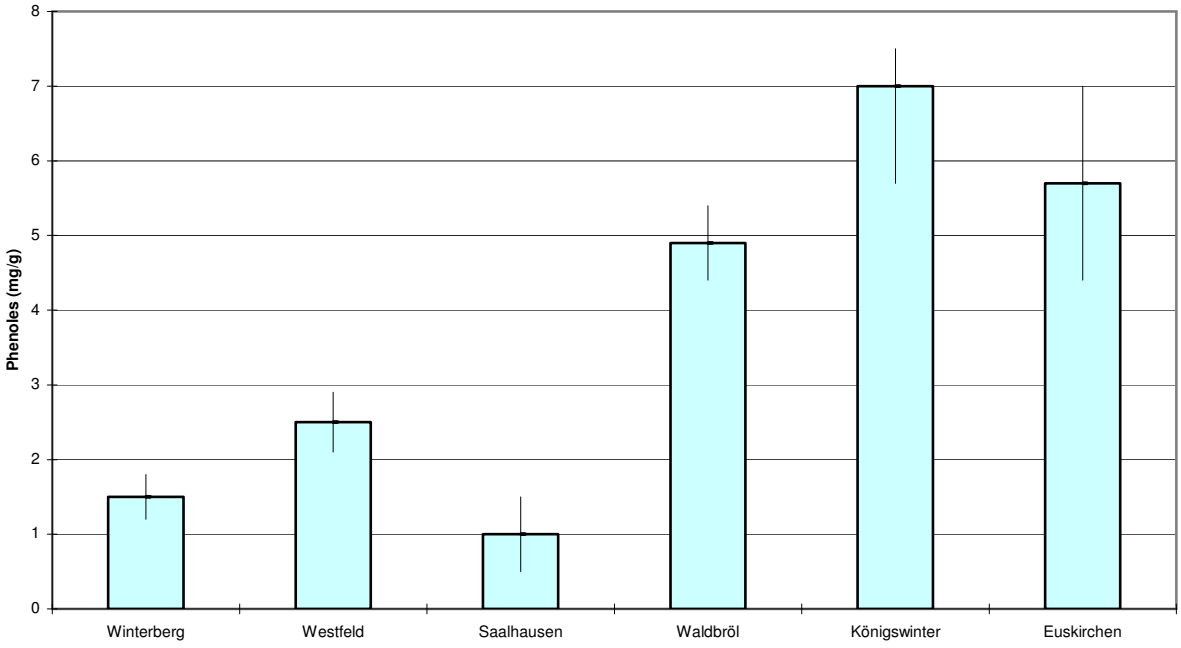


Data were taken at one climatic site at different sample plots near Düren.

Each phenol concentration represents the mean of two samples (bark sample northwest slope and bark sample southeast slope) taken from one tree.

Diagramme 3.4

Site characteristics versus phenol content



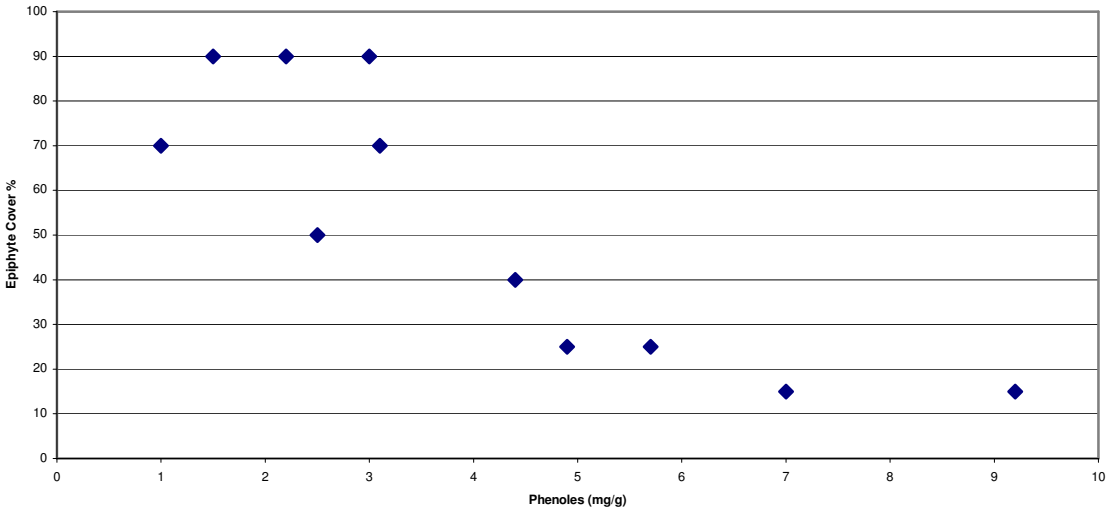
Mean values are calculated from data obtained from five trees at each sample plot.

Samples were taken on the northwest slope at 1.25 cm on the main trunk.

The sampling sites: Winterberg, Westfeld and Saalhausen are located higher than 400 m. above sea level and annual precipitation exceeds 1100 mm. Waldbröl, Königswinter and Euskirchen are located between 90 m and 300 m above sea level and annual precipitation is lower than 900 mm.

Diagramme 3.5

Correlation of phenol content and epiphyte cover



Correlation Data (linear) : Cover% = - 10,4 x c (Phenoles) + 94,7 ; Pearsson-Coefficient: r = - 0,85

Each data point represents the analysis of three trees at one sample site. Trees at each site were less than 50 m apart. Both northwest and southeast slopes on the main trunk are included.
Tree age was about 80 years at all sample plots. Sampling sites near Bonn and sites near Winterberg are included.

Acknowledgment:

The author whishes to thank Isabelle-Franzen Reuter for her help in sample site selection and for data on lichen species richness at sample sites near Bonn.

Chemical analysis has been done in the group of Prof Manfred Grote at the department of chemistry University of Paderborn.Thanks to all members of the group for their kind welcome during my stay.

4 Crown architecture and bark chemistry in *Quercus robur:* Determinants of epiphyte vegetation ?

4.1 Abstract:

Vegetational mapping studies in epiphyte research generally exclude stem areas with high probability of stemflow. These areas are characterized by high epiphyte cover, which is explained by high nutrient loads during rainfall. Still, these exceptional sites may serve as model for general mechanisms acting on epiphyte communities. We analyzed additional chemical and structural site factors in stemflow areas on a single tree (*Quercus robur*). We found bark tannine concentrations to be reduced by ten times compared to stem areas without stemflow (1-3 mg/g d.w. (stemflow area) versus 10 – 25 mg/g d.w. (dry stem area)) This was true both for stemflow areas at the southeast and northwest slope of the tree. Structure and hardiness of outer cork layers differed markedly. We therefore discuss allelopathic factors originating from tree bark in addition to nutrients in explaining vegetational cover with epiphytic bryophytes and lichens.

4.2 Introduction:

Epiphyic lichens and bryophytes are strongly dependend on microclimatic conditions of the host tree. Different parts of a tree form distinct habitats with great variation in moisture content, incident radiation, rainfall, nutrient availability and concentrations of leachable alellochemicals.

While ecological- and physico-chemical parameters (Harris, 1971; Jahns, 1983; Eckhardt, 2003; Loppi, 2004) found wide attentention in epiphyte research, allelopathic interactions have been investigated to much less extent. In most cases, the focus in studies on allelopathy lies on the interaction of lichens or lichens and bryophytes as competitors (Whiton, 1984; Frahm, 2000).

The host tree is regarded as indifferent system without any mode of allelochemical interactions with epiphytic species. This point of view is supported by the fact, that lichens and bryophytes do not use tree tissue as source of nutrients. Unlike parasitic plants, like mistletoe (Viscum alba) they obtain nutrients from stemflow, dust, fog or insect residues in throughfall (Frahm, 2001).

The annual growth rate of lichens and bryophytes is small (Hakulinen, 1966) and their ecological niches are characterized by low nutrient loads and continuous cycles of wet and dry periods. Both factors are essential for the normal function of photosynthesis and metabolism (Farrar, 1976).

Water is taken up by the whole organism, without any discrimination of soluble substances , which makes them effective cation exchangers for nutrients, but also effective accumulators of heavy metals and radionuclides (Masuch, 1992; Frahm, 2001)

Different hypothesis are used to explain generally high epiphyte cover in stemflow areas:

- high input of nutrients and water favour growth and dispersal ;
- stemflow causes outwashing of bark allelochemicals (organic and inorganic substances) and favor growth and establishment due to a decrease in habitat toxicity.
- certain conditions of light and temperature favor growth of adapted epiphyte species on certain zones of the trunk.

While the allelochemical effects of inorganic ions, especially manganese have received much attention during recent years (Hauck, 2003), organic bark extractives have not been analyzed in connection with lichenological field data.

We investigated epiphyte cover and concentrations of total phenols in basal stemflow areas of *Quercus robur* and the upper and lower part of a single branch. The sample site was located in North-Rhine-Westphalia in an area with high annual precipitation.

Further physico-chemical parameters acting on epiphyte communities are disussed.

4.3 Materials and Methods

Bark sampling and site description:

Bark samples were taken in october 2004 from a solitairy *Quercus robur* (circumference: 480 cm) located near Gummersbach at 340 metres above Sea height in North-Rhine-Westphalia, Germany. The area receives annual precipitation of 1000 to 1300 mm (Deutscher Wetterdienst, 2000). Forest sites are characterized by high abundance of cultured *Picea abies* and more natural sites are mixed stands of *Betula pubescens* , *Sorbus aucuparia and Ilex aquifolium*. *Fagus sylvatica* and *Quercus* spp. occur only randomly in forests, but have been planted more frequently at the edge of forests and meadows.

The investigated tree grows at the edge of a meadow (southeast to northwest direction) but is affected by a small road on its north side. The distance between asphalt layer and tree is small (20 cm to 40 cm). The north side is shaded in its lower layers by a stand of small trees on the other roadside (mainly *Salix caprea* and *Corylus avellana*). Large trees in the near vicinity (< 10m) of the tree are absent. A planted stand of *Larix decudia* along the road on the north side imposes no shading effect on the oak tree. Samples for stem analysis were taken from the outer 0,5 cm cork layer at 1.50 cm on different microsites on the main trunk. Sites with stemflow on the north side originated from a cutted branch at a height of about 3 m above ground, that caused continuous spread of water droplets to the lower parts of the trunk during rainfall. The southeast side was affected by stemflow caused by branches, but to less extent. Dry areas and areas with stemflow could easily be recognized even after short periods of rain by visual inspection of moisture and colour of bark. Samples for branch analysis were taken on the upper and lower side of one branch (circumference = 20 cm), which was growing on the south-east side of the tree at a height of 2,30 m above ground.

Bark sample praparation:

Samples were dried at 30 °C for three days and stored in paper bags at room temperature.

Samples for photometrical analysis were prepared by careful dissection of outer cork layers from cork attached lichens and bryophytes. Lichen tissue was excluded from any analysis and only bare cork layers beneath lichen and bryophye tissue were used. Small cork fractions of few mm diameter were scrapped of, resulting in similar sample sizes for extractions.

Biological mapping:

The circumference of the tree (480 cm) was described as x- Axis and a height of 150 cm used as standard height on the y-Axis. Bryophyte and lichen cover were estimated as % cover on the species group level.

Lichen species were determined according to Kremer, 1991 and Moberg, 1982.

Areas with stemflow were determined in october 2004 during rainfall lasting for more than two hours.

Chemical Analysis:

Total phenols were analysed photometrically as described in Hagerman (1995) using a modified Price-Butler method. Extraction of bark was done by 10 min ultrasonication of 25 mg bark in 2 x 2 ml 70% acetone at room temperature. The supernatants were combined and filled to 10 ml with 70% aceton (sample solution). 0,5 to 1ml was used in the test. Gallic acid (3 – 50 µg/ml) was used as standard. The procedure follows the preparation steps.:

1 ml sample solution

2 ml dest. Water

1 ml $K_3Fe(CN)_6$ 0,016M (510 mg ad 100 ml dest H_2O)

1 ml $FeCl_3$, 0,02M (320 mg ad 100 ml 0,1M HCl)

wait for 15 min

add 1 ml H_3PO_4 (20%)

Measurement of the green Fe-tannine complex formed was done using a Perkin Elmer spectrophotometer model lambda 11 at a wavelength of 700 nm

4.4 Results and Discussion:

Concentrations of total phenols were significantly higher in dry areas of the trunk compared to areas with stemflow during rain. Differences were high and varied between 5:1 on the southeast side and 15:1 on the north-west side. On the north-west side stemflow areas hosted closed cover of bryophytes while dry areas were mostly covered by crustose and few foliose lichens.

On the south-east side crustose lichens could be found on both stemflow and dry areas above a a height of 35 cm. Below 35 cm the basal trunk area hosted bryophytes. The inner parts of the cork layer on the south-east side differed in cover with crustose lichens – while more than 90 % of stemflow areas were covered with crustose lichens, dry areas were covered by only 50%. The top of cork layers on the south-east side were almost devoid of lichens in both stemflow and dry areas, due to high degree of bark peeling. Both, the west and east slope of the tree were habitats of foliose lichens, like *Parmeliopsis* spp. and *Parmelia* spp. Both sides showed scattered occurrence of few foliose thalli and general occurrence of crustose species. Bryophytes on the east and west slope of the tree occurred on basal areas (< 50 cm). A single habitat on the north side hosted *Evernia prunastri* on a 20 x 20 cm square in a closed cover of bryophytes. Cork structure in the bryophyte area was smooth, almost desintigrated on the upper 2 mm. Cork of the dry areas was generally harder, though on the south-east side both areas showed bark peeling on the top of furrows.

Twigs were covered with bryophytes in stem near areas of the upper part, and additionally with foliose lichens in stem far regions on the upper part. The lower part of the twig was covered with crustose lichens. Cover generally exceeded 75% on both parts of the twig.

In general, crustose lichens with lower ratio of surface to volume are adapted to higher incident radiation and to low water availability, as they are able to take up and absorb water more efficiently, while foliose lichens can be found in areas with low incident radiation and high probability of water saturation (Jahns, 1983, Moberg, 1982). Nevertheless none of the mentioned species can exist in ecological niches without cycles of wetting and drying. Transplanted lichen thalli in extraordinarily moist areas are easily overgrown by algae, resulting in imbalanced symbiosis (Schuster, 1985). Light is important in zonation of different lichen species in relation to trunk height, as has been shown by Harris (1971) for zonation of *Parmelia* spp. on oak and for epiphyte diversity on deciduous versus evergreen trees (Loppi, 2004).

Chemical characteristics of bark, especially in aged trees, can not be seperated from exogenous factors. Outer cork layers are no longer biosynthetically active and abiotic constraints, here light and moisture as well as biotic impact, e.g. microbial biofilms alter the composition of cork layers.

Though bark secondary phenols are synthesized in biosynthetically active tissues, their chemical structure changes during the formation of bark and cork layers. In *Quercus robur* catechines are transported via the phloem downwards to bark tissues (Hatheway, 1959).

In a second step phenoloxidases produce dimeric and polymeric phlobatannines starting from catechines, like (+)-gallocatechine. Concentrations of Tannines in outer bark of *Fagaceae* are given in Hegnauer, 1966 and vary between 20 mg/g d.w. and 160 mg/g d.w.

In our study, we found concentrations of tannines in outer cork layers of the tree of investigation to be 1 mg/g to 25 mg/g. Comparison of concentrations of natural products from different varieties, plant parts or plants at different age or sites are problematic. Furthermore tannine contents may have been determined by different analytical methods. We previously showed (Koopmann, 2005) that most of all, tree age and annual precipitation of the study area had a significant influence on tannine concentrations in outer cork layers of 2 – 140 years old *Populus x canadensis.* The present study has focused on the comparison of chemical characteristics in different parts of a single tree. We were able to show , that the process of outwashing from rain droplets is able to account for great differences of tannine concentrations even within very small microsites on the trunk (1 mg/g in stemflow areas versus 10 mg/g – 25mg/g in dry areas). Bryophytes occupy sites with lowest concentrations of tannines (1 mg/g – 4 mg/g) and highest degree of shading and moisture (north-west side on the trunk with high stemflow during rain and the upper parts of the branches). Trunk areas with higher contents of allelochemicals, higher incident radiation and lower moisture content are typical lichen habitats. Nevertheless, also lichens prefer microsites with low contents of allelochemicals and increased moisture and shelter, as can be seen on the south-east side of the tree, where highest cover of crustose lichens inside cork furrows occurs in stemflow areas. The foliose lichen species: *Evernia prunastri* grows in an intermediate area, together with bryophytes. We therefore propose to evaluate vegetational zonations not only with reference to abiotic factors - light or nutrients - acting directly on epiphyte growth and dispersal, but to add allelopathic constraints, which may not only result in growth depression and competition, but are important factors (both positive and negative) in shaping plant community structures.

4.5 Figures and Tables

Table 4.1

Concentrations of total phenols in *Quercus robur*

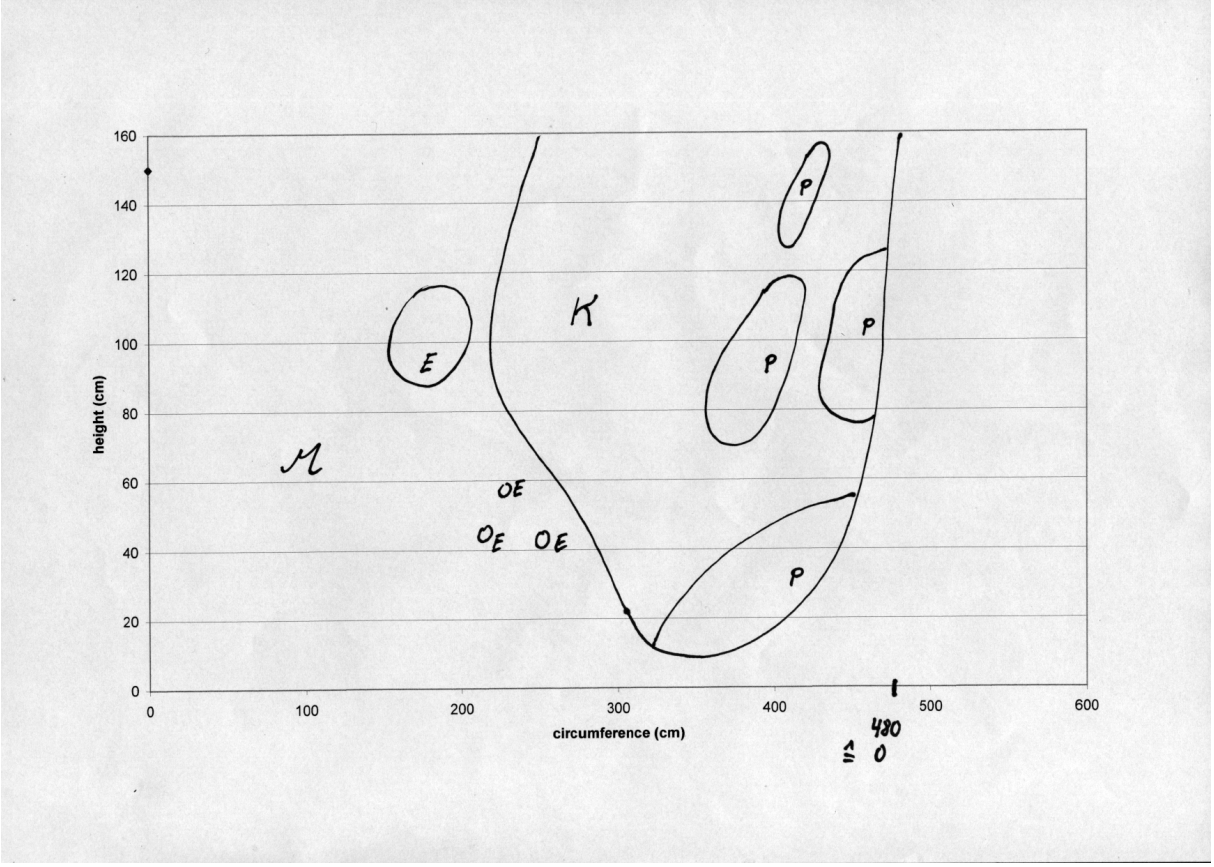
Plant part	**Total phenols (mg/g)**
Main trunk / north-west / stemflow area	1,1 (+/- 0,2)
Main trunk / east / dry area	17,5 (+/- 7,5)
Main trunk / south-east / stemflow area	3,7 (+/- 0,3)
Twig / upper side	3,0 (+/- 0,3)
Twig / lower side	3,8 (+/- 0,8)

Data are mean values of the analysis of five bark samples, taken at a height of 150 cm above ground.

Samples for twig analysis were taken at a distance of 1,0 m from the main trunk.

Diagramme 4.1

Biological mapping of epiphytic lichens and bryophytes on *Quercus robur*



E: Evernia prunastri; P: Parmelia / Parmeliopsis spp.; K: crustose lichens ; M: bryophytes

100 cm = north ; 220 cm = east ; 340 cm = south ; 460 = west

Total circumferene of the tree: 480 cm

5 Summary

Secondary metabolites in the epiphytic lichen *Hypogymnia physodes* have been studied focusing on the within tissue localization of phenols. Though total phenol contents were higher in sorediate compared to non-sorediate thallus structures (7 % d.w.vs. 3 % d.w.), the spectrum of phenolic constituents did not differ between tissues, indicating the same ratio of cortex to medulla in both tissue types analyzed. Higher phenol contents, pH 4,0 and a lower water capacity in soredia compared to thalline structures inhibit preliminary germination, act as feeding deterrents and in perception of abiotic site factors. External allelopathic phenols were investigated in an in-vitro test system using single bark phenols from a *Salicaceae* (*Populus x canadensis*) that hosts the lichen species *Physcia tenella*.

We found species specific bark phenols (phenolic glycosides) and their degradation products (catechol, benzoic acid and salicylic aldehyde) to be most important inhibitors (LD50 = 10^{-6}M – 10^{-5}M) of soredial growth at natural bark pH = 6.0. General metabolites of tannine and lignin turnover, (gallic acid, catechine, salicylic acid) showed less inhibitory strength (LD50 = 10^{-4}M – 10^{-3}M). As epiphytic lichens are only slightly attached to the outer cork layers and do not root deeply into the phloem, they are solely subjected to leachable phenols in stemflow.

We therefore investigated contents of phenols in different stem areas on trees of different age classes (2 – 140 years) and found a correlation between increased epiphyte cover and decreased contents of leachable phenols in the outermost 0,3 cm of cork layers of old compared to young trees. Aside tree age, annual precipitation was the most important site factor acting on cork phenol contents in trees of the same age class. Sample sites studied in central Germany showed precipitation levels between 600 mm/year and 1300 mm/year. Height on the trunk was studied on the basal stem area and we found phenol contents to increase slightly with height. Zonation of lichens and bryophytes was described additionally. Bryophytes were most vulnerable to increased phenol contents as a result from both the precipitation and zonation study.

Our results are of importance for site selection in environmental mapping studies. Sample sites for the important mapping tree species: *Populus x canadensis* should be located at the same height above sea level in oder to achieve site characteristics with similar annual precipitation level. Another factor already mentioned in VDI guidelines is the exclusion of direct stemflow areas on the trunk. Here, epiphyte cover is generally high. In addition to increased water and nutrient supply, we were able to show, that phenol contents in cork layers of stemflow areas are about ten to twentyfive times lower than contents in dry areas.

Apart from the basal trunk, the upper and lower side of a branch were analyzed and showed small deviations in contents of allelochemicals, the lower side containing about 25 % less total phenols than the upper side. The upper part of the branch was covered with bryophytes and foliose lichens, while the lower part was covered with crustose lichens.

As a conclusion, crown architecture does not only impose zonation of epiphytes by alteration of incident light, but alters rainfall specifically, resulting in zonation of moisture and bark chemicals.

The influence of bark phenols on epiphytes may result from general defence mechanisms, that are primarily directed against plant pathogenic fungi. Both trees and lichens develop species specific mechanisms of protection against saprotrophic fungi. Nevertheless, all defense related allelochemicals may act in interspecies selection that favor a certain vegetational community with adapted structures.

Outlook:

Further work on the allelopathic effects of bark phenols on epiphytic plants could be directed towards the analysis of specialized tree- lichen or bryophyte pairs, investigations of bark constituents at different stem heights or the chemical analysis of stemflow.

Botanical studies could be directed to the occcurence of allelopathy in different geographical and ecological habitats and its effects on epiphyte communities.

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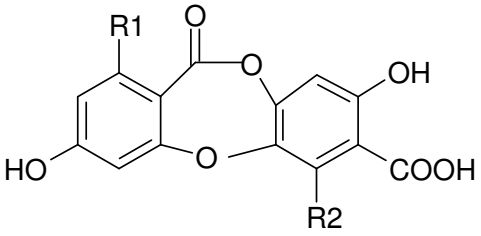
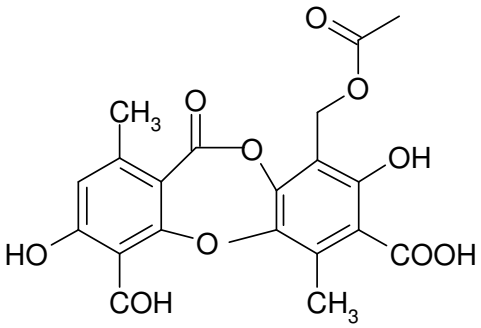
A1: Phenols in lichen tissues

A1/1: Chemical Structures of Lichen phenols in *Hypogymnia physodes*

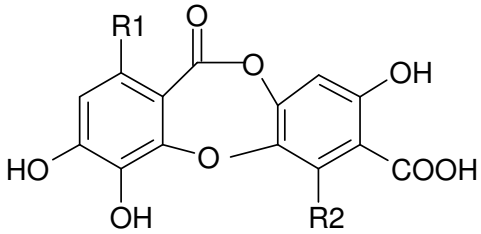


R1 = $-CH_2-CO-C_5H_{11}$
R2 = $-C_5H_{11}$

Physodic Acid

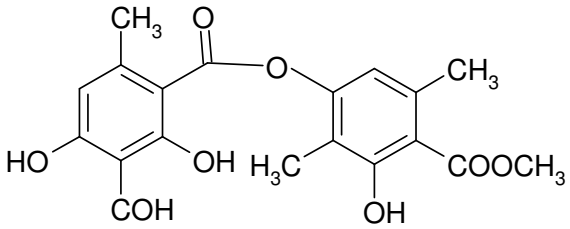


Physodalic acid



R1 = $-CH_2-CO-C_5H_{11}$
R2 = $-C_5H_{11}$

Hydroxyphysodic acid



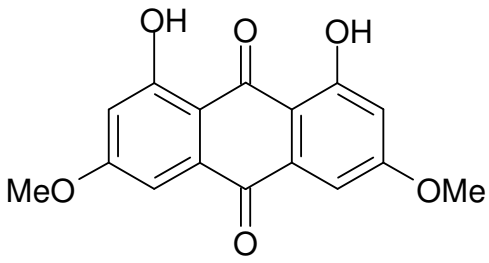
4 Atranorin

A1/2

Hypogymnia physodes growing on Betula pubescens (near Oulu)

A1/3

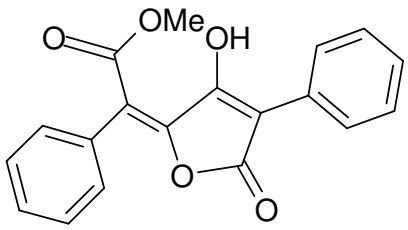
Chemical structure of the main lichen phenol in *Xanthoria parietina*



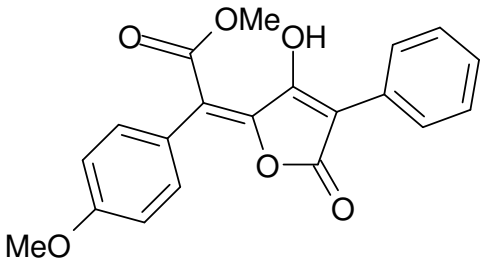
Parietin

A1/3

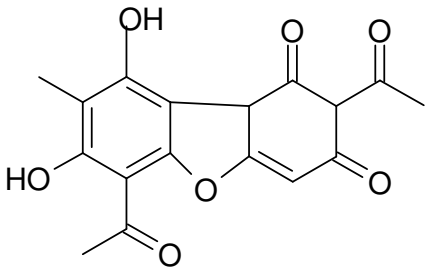
Chemical structure of lichen phenols in *Vulpicida pinastri*



Vulpinic Acid



Pinastric Acid



Usnic acid

A1/4

Xanthoria parietina growing on Populus tremula (near Muhos)

A1/5

Vulpicida pinastri growing on Betula nana (near Oulu)

A1/6

Chemical characterization and analysis of lichen phenols

Methods:

TLC (*Hypogymnia physodes):*

Silica Gel 60, Macherey & Nagel; F254

Eluent: Ethylacetat 20 / Aceton 15 / Methylenchlorid 6 /Methanol 5 /Water 4

Visualization: spraying with 10% sulfuric acid in ethanol and heating at 105°C for 3 min

TLC (*Vulpicida pinastri)*

Silica Gel:60, Riedel de Häenl F24

Eluent: n-Hexan 130 / Diethylether 80 / Formic acid 20

Visualization: spraying with 10 % sulfuric acid in ethanol and heating at 105 °C

HPLC 1 : (Hypogymnia physodes)

according to Huovinen (1985), Acta Pharm Fenn.94 : 99-112

HPLC System: Pharmacia LKB

Pump: model 2248, detector: UV-VIS model VWM 2141, autosampler 2157

Column: Inertsil ODS 3 , 150 x 4 mm (with precolumn occasionally)

Eluent: A: 100 % methanol , B: 0,09g phosphoric acid (85%) / 100 ml water ; filtrated and degassed

Gradient: 20% methanol -> 1 % methanol -> 100 % methanol (within 85 min.)

Flow rate: 1 ml/min

Detection: 280 nm

HPLC 2 (*Vulpicida pinastri / Xanthoria parietina*)

Instrument: see above

Column: Lichrocart 125-4 (RP18)

Eluent: isocratic: 100 % methanol

Detection: 270 nm (usnic acid) / 394 nm (vulpinic / pinastric acid)

Flow rate: 1 ml/min

UV-VIS:

Beckmann DU 60

Scan mode: 220 – 340 nm

MS:

Kratos MS 80 FF

EI and CI-NH_3 mode ; direct inlet system ; samples were air dried prior analysis

Phenols in *Hypogymnia physodes*

Atranorin ($C_{19}H_{18}O_8$)
(synthetic Sigma)
MW: 374,3 g/mol
RRT (TLC) : 0,90
RT (HPLC) : 70 min
m/z (CI-MS) : 375, 164, 136

Physodic acid ($C_{26}H_{30}O_8$)
(isolated from *Hypogymnia physodes,* collection in Oulu*)*
MW: 470,5 g/mol
RRT (TLC): 0,59
RT (HPLC): 56 min
m/z (CI-MS): 426, 370, 248

Physodalic acid ($C_{20}H_{16}O_{10}$)
(isolated from *Hypogymnia physodes,* collection in Oulu*)*
MW: 416, 33 g/mol
RRT (TLC): 0,68
RT (HPLC): 62 min
m/z (CI-MS): 356, 314

Hydroxy-Physodic acid ($C_{26}H_{30}O_9$)
(isolated from *Hypogymnia physodes,* collection in Oulu*)*
MW: 486,5 g/mol
RRT (TLC): 0,52
RT (HPLC): 49 min
m/z (CI-MS): 443, 264, 207

Identification was mainly based on comparison of Rf values in TLC with literature about organic synthesis of different physodic acids (J. Elix,Canberra).

Phenols in *Xanthoria parietina:*

Parietin ($C_{16}O_6H_{13}$)

MW: 301,3 g/mol

Chemical characterization was not performed, as the substance was obtained as standard
HPLC analysis was done using isocratic elution and extraction of lichen thalli in methanol instead of DMF 20/ethylacetate 40/acetone 40 due to the better solubility of parietin in methanol.compared to other lichen substances..

Phenols in *Vulpicida pinastri*

Vulpinic acid ($C_{19}H_{14}O_5$)
(isolated from Vulpicida pinastri)
MW: 322,3 g/mol
RRT (TLC): 0,56
Rt (HPLC): 0,85 min
m/z (CI-MS):322, 290, 145

Pinastric acid ($C_{20}H_{16}O_6$)
(isolated from *Vulpicida pinastri,* collection in Oulu)
MW: 352,3 g/mol
RRT (TLC): 0,49
Rt (HPLC): 0,85 min (coelution with vulpinic acid)
m/z (CI-MS): 370, 353, 320

Usnic acid ($C_{18}H_{14}O_5$)
(synthetic Sigma)
MW: 344,3
RRT (TLC): 0,60
Rt (HPLC): 1,1 min (baseline separation from pinastric/ vulpinic acid)
m/z (EI-NH3): 345, 260, 233

A 2 In vitro experiments

A2/1

Physcia tenella growing on Malus spp.

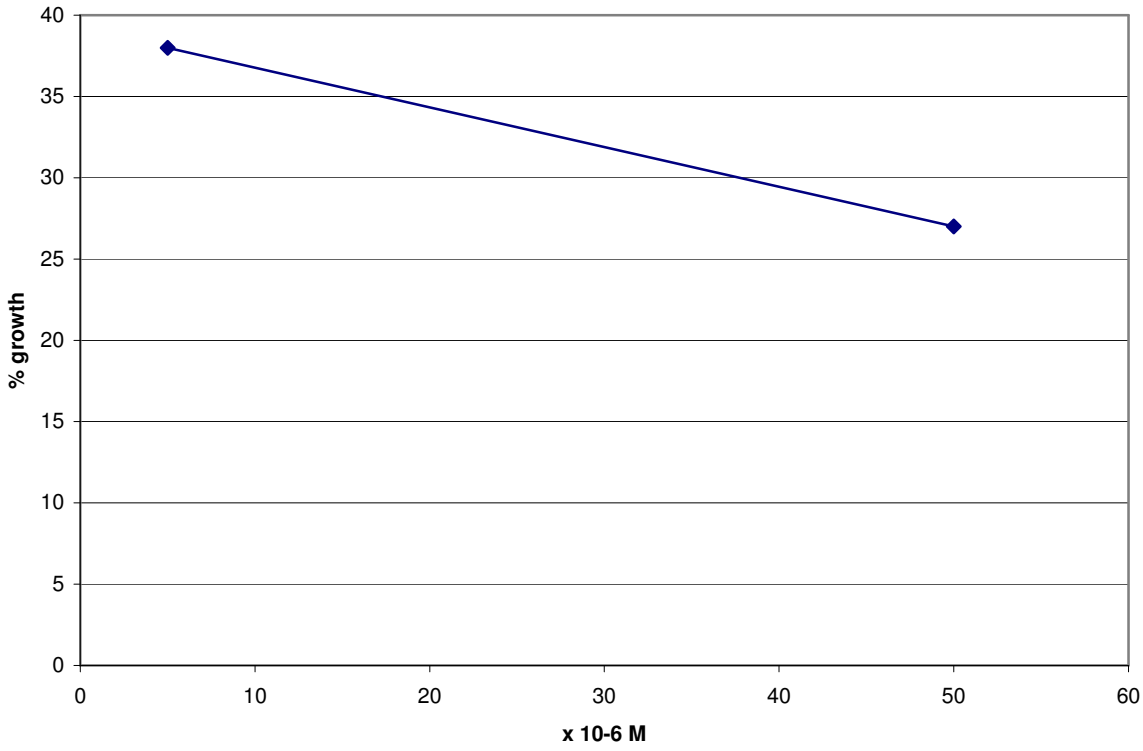
A2/2

In-vitro inhibition of soredial growth by a variety of bark phenols:

Diagramme: % growth compared to control versus phenol concentration of the substratum

1

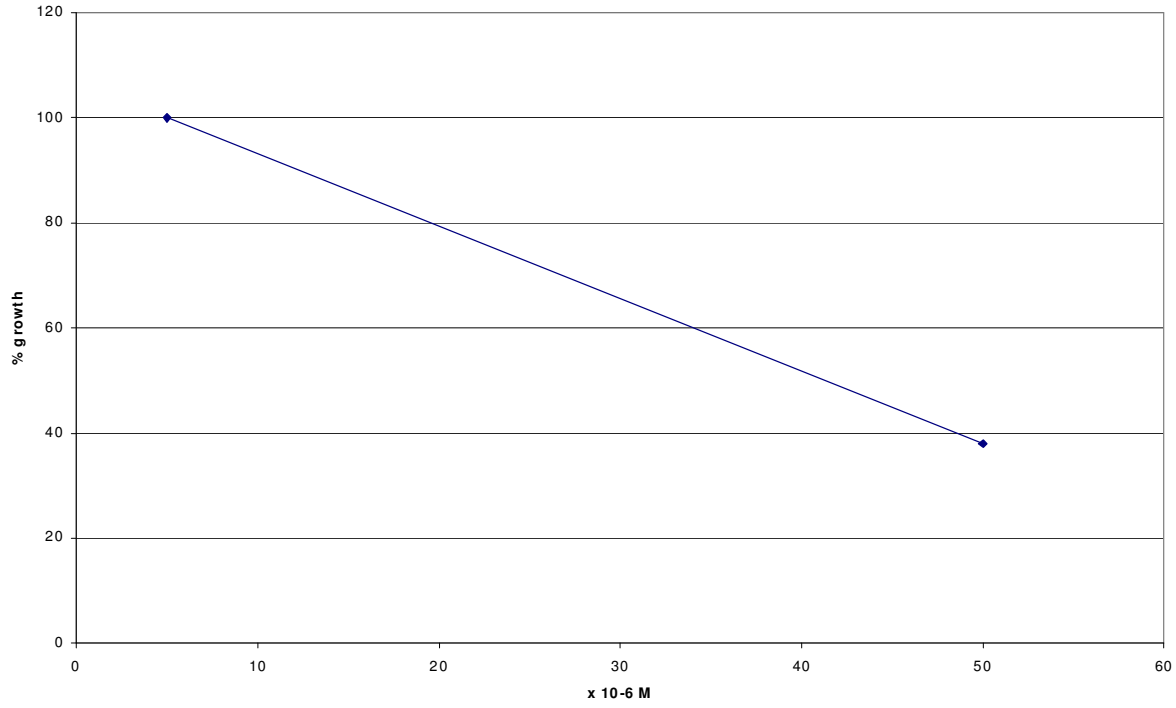
Tremulacin



Each data point represents the mean of five replicate in vitro tests

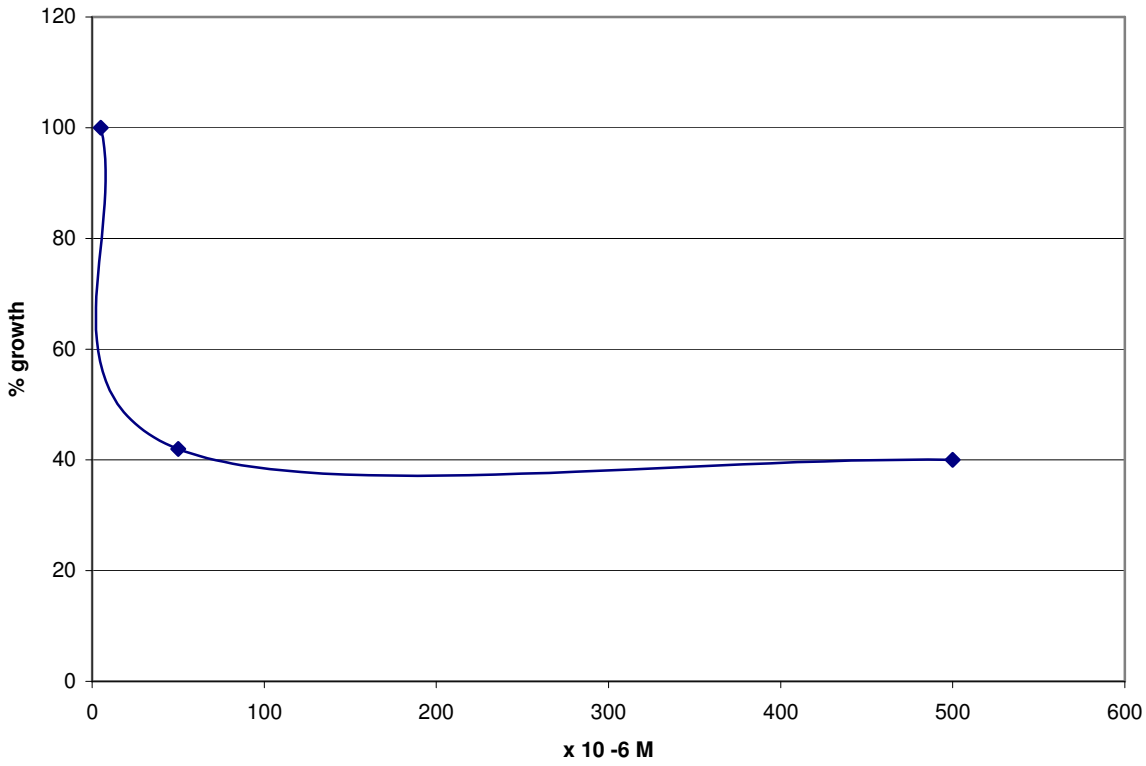
2

Salicortin



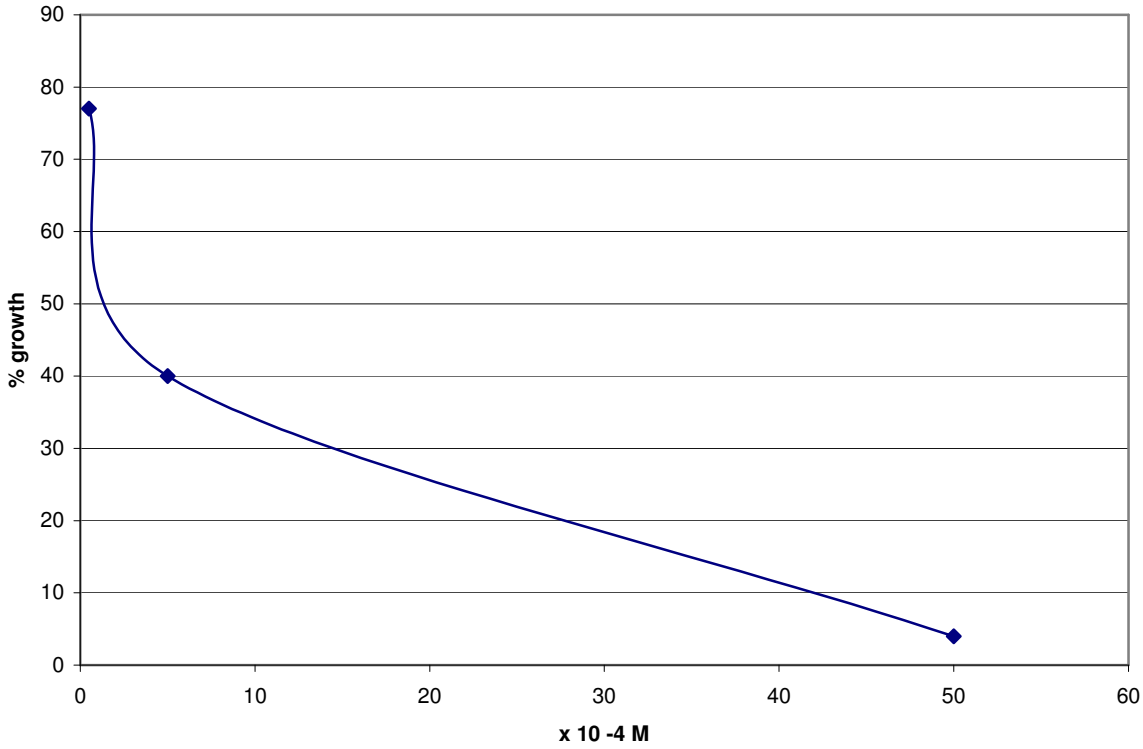
3

Salicin



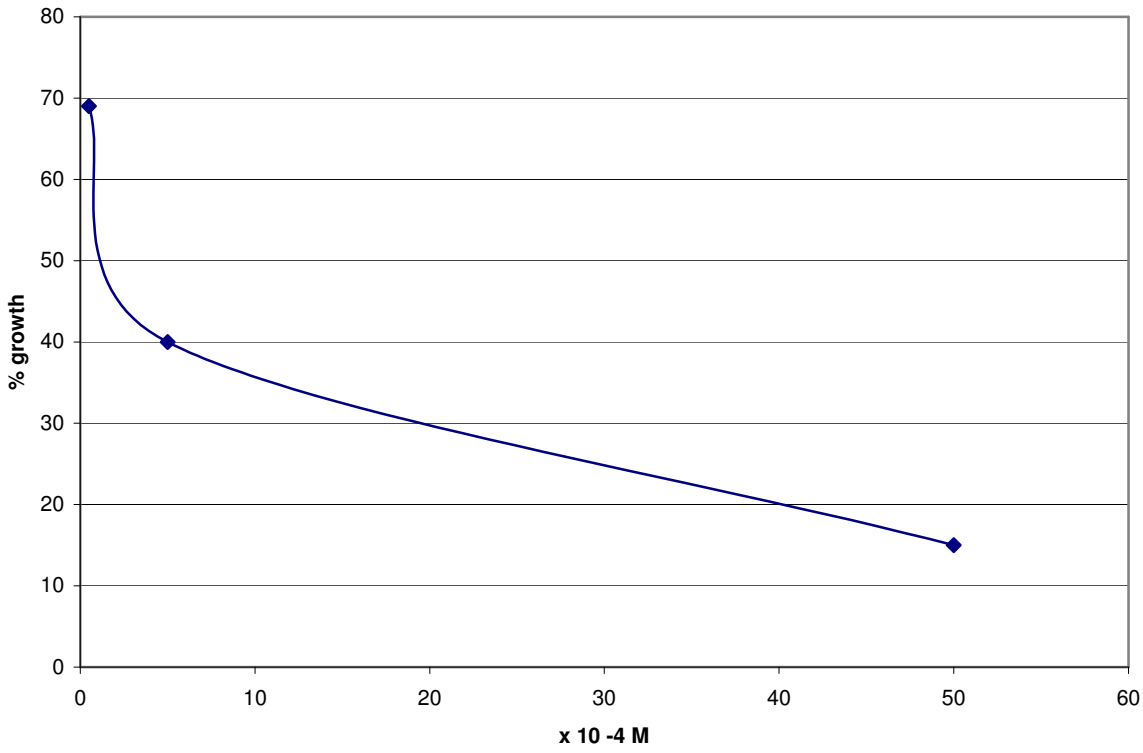
4

Catechin



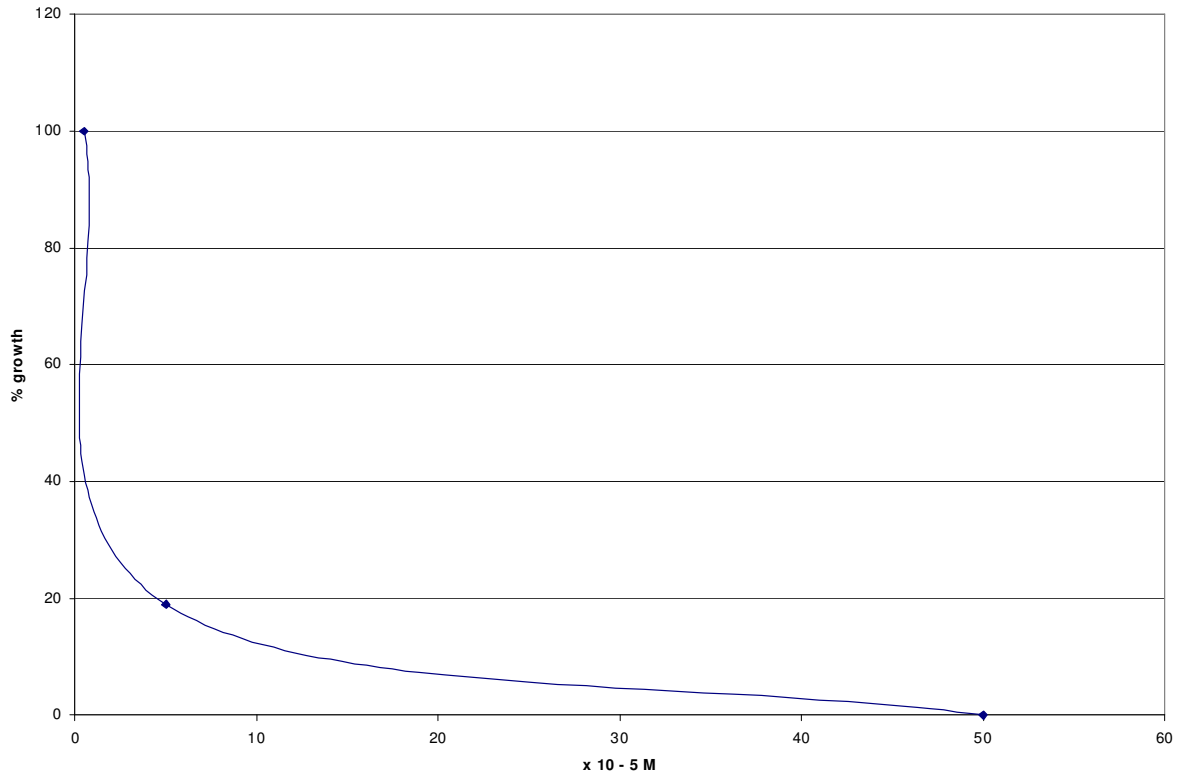
5

Salicylic alcohol



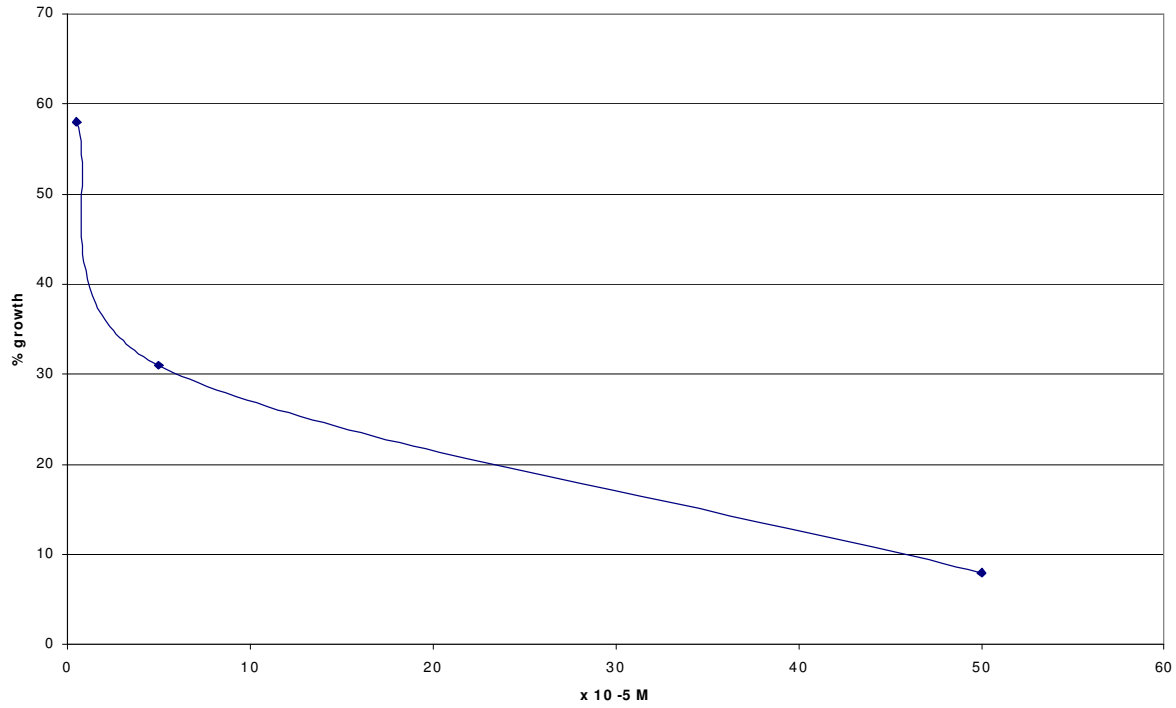
6

Benzoic acid



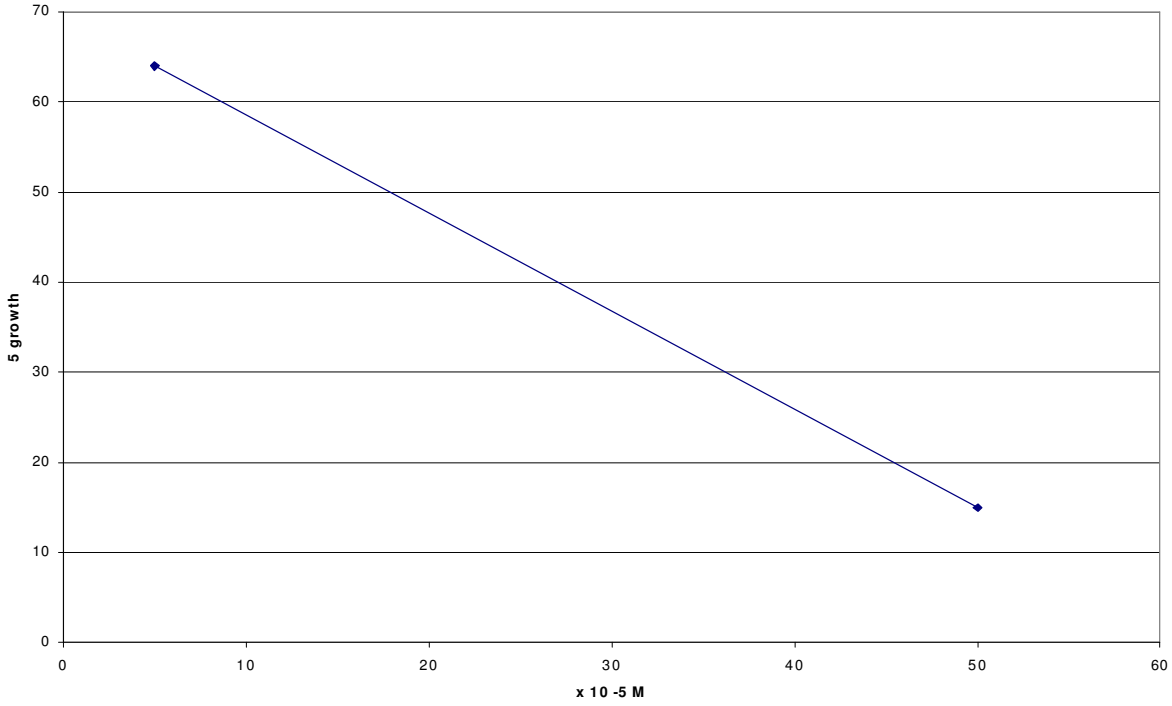
7

Catechol



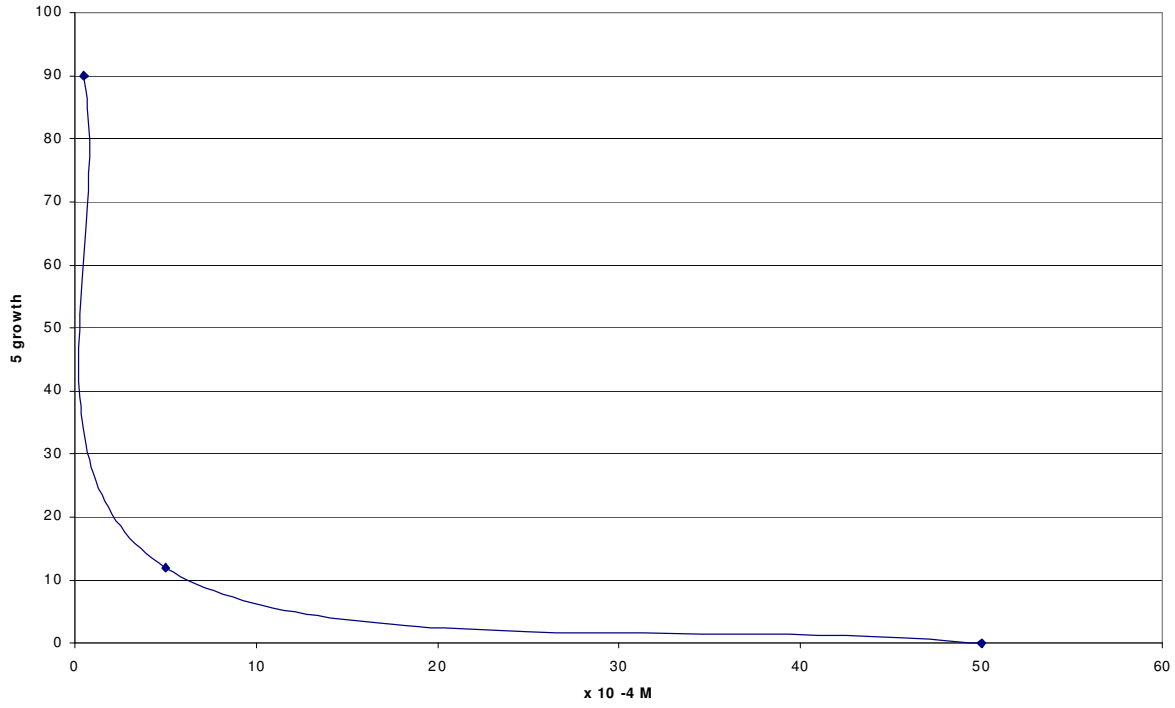
8

Gallic acid



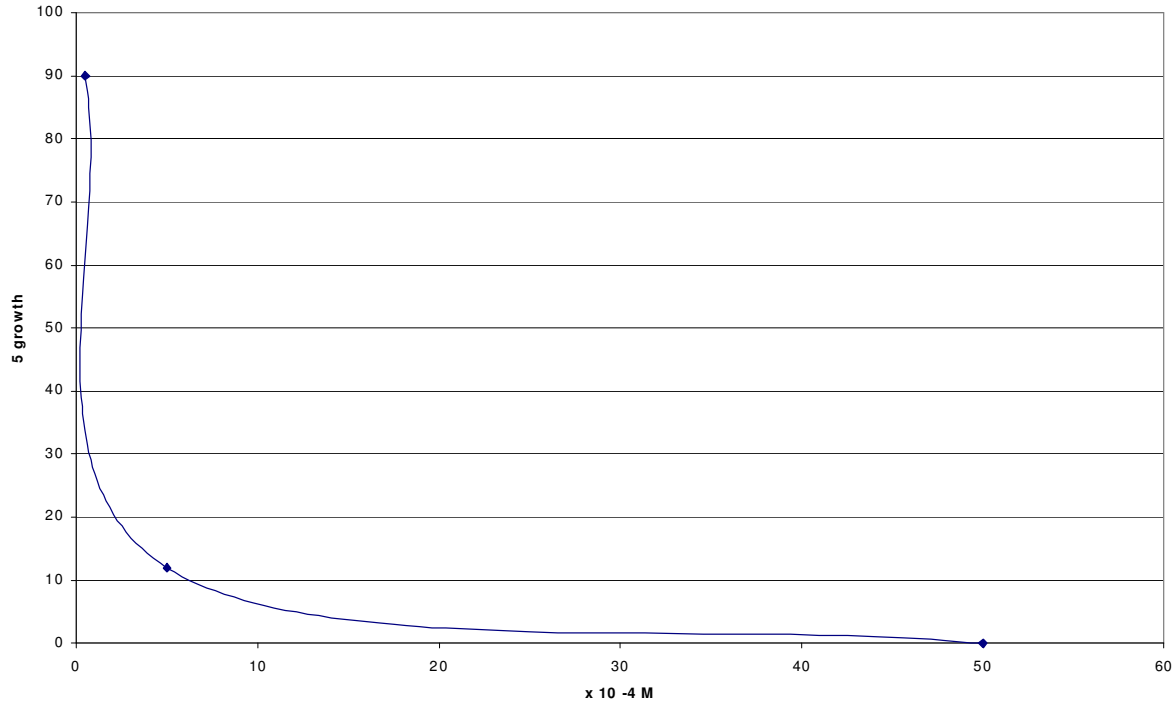
9

Ellagic acid



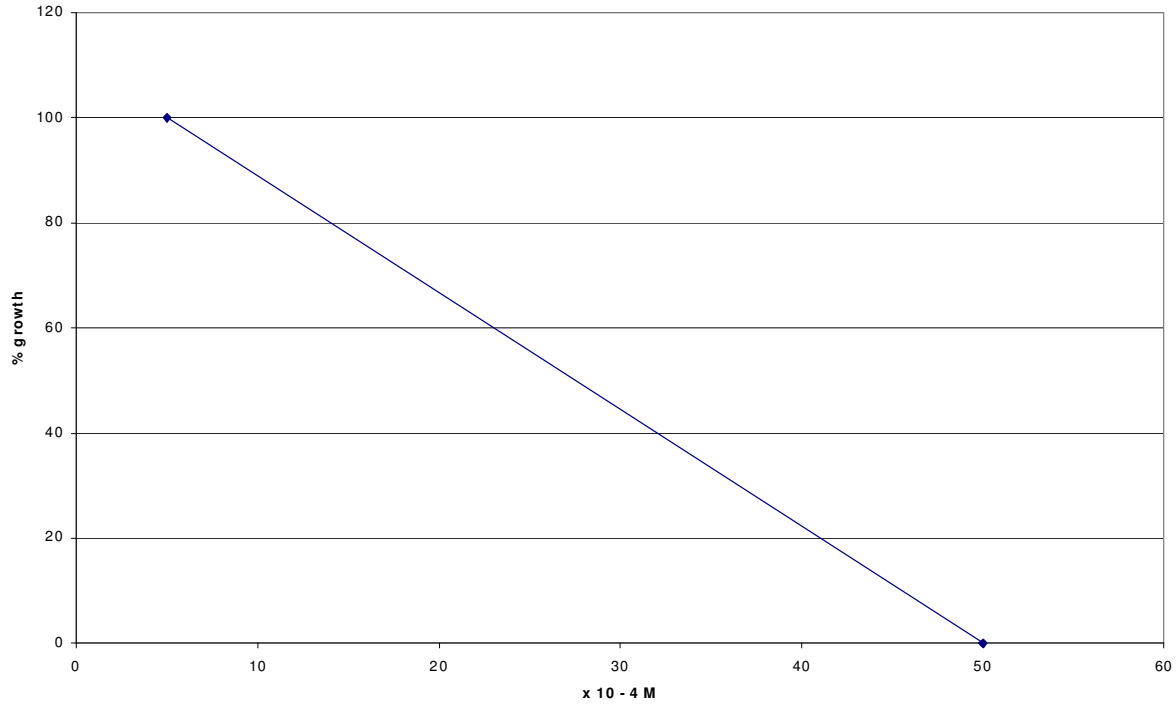
10

Salicylic aldehyde



11

Salicylic acid



A2/3

Mass spectral data of samples: Salicortin and Tremulacin

(Analysis was done by H. Stevens, University of Paderborn)

System:

LCQ-Advantage (Thermo Finnigan)

Electrospray Ionisation (ESI) negative mode $(M-H)^-$

Mass range: 80-2000 m/z

Sheat gas flow rate: 60 arb

Auxillary gas flow rate: 4,5 arb

Ion spray voltage: 5 kV

Capillary temperature: 280 °C

Tube Lens: - 20 V

Lens voltage: 14,25 V

Multipole Offset: 7 V

Results:

Sample: “Salicortin” (in methanol): m/z: 447,0 ; 439,1 ; 423,0

Sample: “Tremulacin”(in methanol): m/z: 585,0 ; 481,0

Salicin MW = 258,2 g/mol

Salicortin MW = 423,4 g/mol

Tremulacin MW = 528,5 g/mol

Acetyltremulacin MW = 570,5 g/mol

A3 Lichen Monitoring and Correlation with bark chemistry

A3/1

Sample site near Königswinter

(Populus canadensis and Populus nigra near the river Rhine)

A3/2

Sample Site near Königswinter

(*Populus canadensis* in a plantation near a forest site)

A3/3

Sample site near Saalhausen

A3/4

Populus canadensis bark at Winterberg

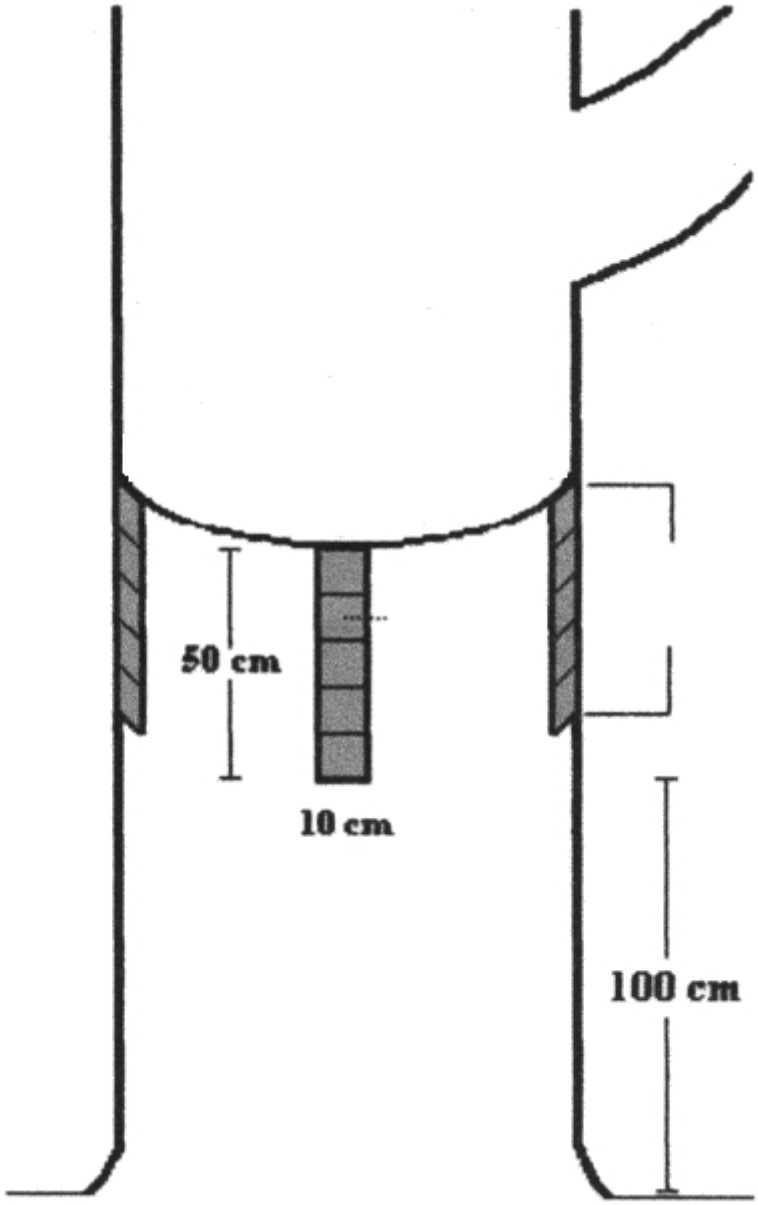
A3/5

Populus canadensis bark at Düren

A3/6

Diagramme of lichen mapping arrangement

(taken from Franzen-Reuter, 2004)



Danksagung

Die vorliegende Arbeit gliedert sich in ein Forschungsprojekt des Nees Instituts für Biodiversität, Bonn, das im Jahr 2003 mit dem Themenvorschlag von Prof Jan-Peter Frahm begann, sowie ein zweites in den Jahren 1998 und 1999 durchgeführtes Forschungsprojekt der Finnischen Akademie, das an der Universität Oulu bearbeitet wurde.

Mein ganz besonderer Dank gilt den Betreuern der Arbeit: Prof Jan-Peter Frahm, Nees Institut der Universität Bonn und Prof Manfred Grote, Fakultät für Naturwissenschaften der Universität Paderborn für ihre stete Diskussions- und Hilfsbereitschaft.
Meinen Coautoren Dr Isabelle Franzen-Reuter und Dipl.-Chem. Henning Stevens danke ich für die konstruktive und freundschaftliche Zusammenarbeit.
Diese Arbeit wäre nicht gelungen ohne ein besonders gutes Betriebsklima. Hierfür bedanke ich mich bei allen beteiligten Mitarbeitern und Kollegen.

In der Zeit von 1998 bis 1999 hatte ich die Möglichkeit als Lizentiatsstudent an der Universität Oulu in Finnland grundlegende Kenntnisse in Lichenologie zu erwerben.
Für die Aufnahme als Gaststudent, die finanzielle Förderung durch finnische Institutionen, sowie die langjährige Korrespondenz bis zur Veröffentlichung der dort durchgeführten Arbeiten bedanke ich mich bei allen Beteiligten.

Meiner Familie, Freunden und Kollegen danke ich für ihre Unterstützung und Kritik.

Eidesstattliche Erklärung

An Eides statt versichere ich, dass ich diese Arbeit selbst und ohne jede unerlaubte Hilfe angefertigt habe, und dass ich diese oder eine ähnliche Arbeit noch an keiner anderen Stelle zur Prüfung vorgelegt habe.

Ricarda Koopmann

Bonn, Juni 2005