The Lichenologist **39**(6): 567–572 (2007)
doi:10.1017/S0024282907005907 *Printed in the United Kingdom*

In vitro inhibition of soredial growth in the epiphytic lichen *Physcia tenella* (Ascomycetes: *Lecanorales*) by a variety of bark phenols

Ricarda KOOPMANN, Henning STEVENS, Isabelle FRANZEN-REUTER, Jan-Peter FRAHM and Manfred GROTE

Abstract: The in-vitro growth rate of soredia from the epiphytic lichen species *Physcia tenella* was tested at different concentrations of phenols added in an *in vitro* experiment designed to evaluate the allelopathic effects of bark derived substances in stem flow. The conditions used were designed to resemble those found on bark of *Populus* × *canadensis*, as this tree species has been widely used for lichen mapping studies in Germany. Bark phenolic glycosides, flavonoids and tannins undergo hydrolytic decomposition in stem flow, resulting in a variety of monomeric phenolic acids, aldehydes and alcohols. Eleven phenolic substances of different biosynthetic origin in the concentration range between 10^{-6} M and 10^{-3} M in the culture medium were tested. IC50 values were calculated for each substance. Inhibition was highest for catechol, substituted phenolic glycosides, benzoic and ellagic acids, moderate for flavonoids, gallic acid, salicylic alcohol, salicylic aldehyde and low for salicylic acid. The tests show that bark phenols in natural concentrations inhibit soredial growth of the test species *Physcia tenella* and therefore have an influence on the growth of lichens on trees.

Key words: *in vitro* test, salicylates, soredia

Introduction

In allelophysiology, plant derived secondary phenolic metabolites are studied because of their role as germination inhibitors (Evenari 1949; Follmann & Nakagawa 1963; Frahm *et al.* 2000), while their antimicrobial and antifungal activity have important implications in development of resistance against plant pathogens (Butin & Loeschke 1969). The allelopathic effects of endogenous organic lichen compounds on the *in vitro* growth of algal cells and on bryophytes have already been shown (Backor *et al.* 1998; Frahm *et al.* 2000). Epiphytic lichens may be influenced markedly by soluble, bark-derived substances in stem flow and throughfall.

Leachates originating from tree bark contain a variety of plant metabolites of different biosynthetic origin: sugars and sugar alcohols, amino acids, nutrient ions and secondary metabolites, such as phenols or alkaloids (Fengel & Wegener 1984). Barkman (1958) mentions bark chemicals as one of several factors in his classification of ecological and chemical factors, which are important for epiphytic lichen growth. Unlike the impact of bark pH or total electrolyte capacity, variations in secondary metabolites in bark have been little studied (Gauslaa 1985; Bates & Brown 1981) with the few studies that have been undertaken focusing on ecological data in relation to total tannin contents of bark. In general, abundance and composition of epiphytes are strongly correlated to mineral elements, pH and nutrients in bark (Gaussla 1985; Gustaffson & Erikkson 1995). However,

R. Koopmann, I. Franzen-Reuter and J. P. Frahm: Nees Institute for Biodiversity of Plants, University of Bonn, Meckenheimer Allee 170, D-53115 Bonn, Germany. Email: frahm@uni-bonn.de
H. Stevens and M. Grote: Department of Natural Science, University of Paderborn, Warburger Strasse 100, D-33098 Paderborn, Germany.

there are contradictory field results on the relationships between epiphyte cover and tannin concentrations in bark, which may vary with tree age and annual precipitation rates (Gaussla 1985; Bates & Brown 1981; Koopmann 2005). The effects of water-based bark extracts on lichen ascospore germination have been investigated (Pyatt 1973; Ostrofsky & Denison 1980) and both enhancement and inhibition were found, depending on the extract tested, the method of extract preparation and the lichen species tested.

Physcia tenella (Scop.) DC. (*Lecanorales*, Ascomycetes) is an abundant epiphyte on the tree species *Populus* × *canadensis* (L.) Moench, so that we expected differential responses of soredial growth to phenolic metabolites in *Salicaceae* and used it as a test species. In the present study we have used distinct organic compounds known from *Salicaceae* to evaluate growth inhibition of lichen soredia of the test species *in vitro*. Of the substances used, salicortin and tremulacin were extracted from tree bark. Additional phenolic metabolites such as salicin, salicylic alcohol, salicylic aldehyde, salicylic acid, catechin, catechol, gallic acid, ellagic acid and benzoic acid were purchased as synthetic compounds (Fig. 1).

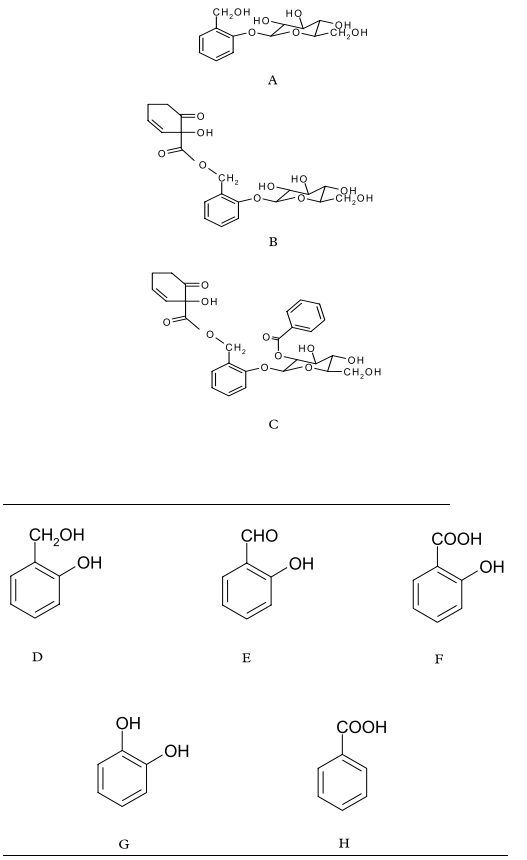

FIG. 1. Chemical structures of salicylates and their metabolites used in the soredia growth test. A, salicin; B, salicortin; C, tremulacin; D, salicylic alcohol; E, salicylic aldehyde; F, salicylic acid; G, catechol; H, benzoic acid.

Material and Methods

Material

For isolation of salicortin and tremulacin, two to three year old saplings of *Populus* × *canadensis* were purchased from a tree nursery near Hannover in February 2004. After two days of plant storage at 10 °C, the stems were cut into pieces of about 3 cm in length (total maximum stem length was 1·50 m), 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.

The epiphytic lichen *Physcia tenella* was collected in May from 60 year old cherry and apple trees near Gummersbach (51 °06′N, 7 °40′E) and stored in a paper bag at room temperature. Soredia for *in vitro* germination were used fresh one day after collection of thalli, and were isolated by scraping the tissue gently with a steel needle. The lichen was collected from tree species other than from *Populus*, as they were easily accessible and we did not expect to see any difference in growth characteristics between *Physcia tenella* soredia obtained from different trees.

Isolation of Salicortin and Tremulacin

Dried twigs (175 g) of *Populus* × *canadensis* were extracted three times with 0·5 l methanol at room temperature for 1 h 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 solvent used was dichlormethane/ethyl acetate/methanol/water (50/30/20/5). Fractions were evaporated to dryness, yielding a honey-coloured compound with a purity <85% (estimate from TLC spot intensity). Phenolic glycosides could be detected on TLC plates by their red coloured spots after spraying with 10% sulphuric acid in ethanol in contrast to other phenols with brown spots. Synthetic D-(-)-salicin was chosen as a standard (relative retention time = 1·0), which had a lower Rf value than the isolated salicylates: tremulacin (this study: RRT = 2·1 ± 0·2; Meier (1988): RRT = 2·3 ± 0·2) and salicortin (this

https://doi.org/10.1017/S0024282907005907 Published online by Cambridge University Press

TABLE 1. *Soredial growth of* Physcia tenella *in the* in vitro *growth assay*

Substance	% growth*			
	5×10^{-6} M	5×10^{-5} M	5×10^{-4} M	5×10^{-3} M
Tremulacin	38 (± 10)†	27 (± 10)	n.a.	n.a.
Salicortin	100 (± 25)	38 (± 10)	34 (± 9)	n.a.
Salicin	100 (± 25)	n.a.	40 (± 10)	31 (± 8)
Catechin	100 (± 25)	77 (± 20)	40 (± 10)	4 (± 2)
Salicylic alcohol	100 (± 25)	69 (± 17)	40 (± 10)	15 (± 4)
Benzoic acid	100 (± 25)	19 (± 5)	0 (± 2)	n.a.
Catechol	58 (± 15)	31 (± 8)	8 (± 2)	n.a.
Gallic acid	n.a.	100 (± 25)	64 (± 16)	15 (± 4)
Ellagic acid	100 (± 25)	19 (± 5)	12 (± 4)	n.a.
Salicylic aldehyde	n.a.	90 (± 23)	12 (± 4)	0 (± 2)
Salicylic acid	n.a.	n.a.	100 (± 25)	0 (± 2)

*Data are expressed as mean of five replicates ± standard error; † % growth measured at 1×10^{-6} M; n.a. not analyzed.

study: RRT = 1·4 ± 0·2; Meier *et al.* (1988): RRT = 1·4 ± 0·2). EI-MS spectra were used to confirm basic structural features (data not shown).

Preparative column chromatography was carried out using a glass column with an internal diameter of 6 cm and a length of 34 cm (=960 cm^3) connected to a fraction collector.

TLC analysis was done using Macherey & Nagel 10 × 5 cm Alugram SIL UV 254 sheets and the eluent described in the isolation procedure.

Assessment of soredial growth

A 0·8% agar was prepared containing 4 ml of the following nutrient solution: 0·1 g l^{-1} KCl; 1·5 g l^{-1} NH_4NO_3; 0·1 g l^{-1} $MgSO_4$; 0·5 g l^{-1} KH_2PO_4; 0·04 g l^{-1} $CaSO_4$; 2 ml l^{-1} (containing 1·35 g l^{-1} $FeCl_3$; 1·86 g l^{-1} EDTA); 2 ml l^{-1} of a trace element solution pH 4·6 (Hauck *et al.* 2002*a*) (containing: 2·68 g l^{-1} H_3BO_3, 1·18 g l^{-1} $MnCl_2.4H_2O$, 0·22 g l^{-1} $ZnSO_4.7H_2O$, 0·012 g l^{-1} MoO_3, 0·079 g l^{-1} $CuSO_4.5H_2O$). The agar solution was adjusted to pH 6·0 with 1 M HCl.

Aliquots of 150 ml of the culture medium were prepared and sterilized in Erlenmeyer flasks, sufficient for preparation of 5 conventional sterile Petri dishes with a diameter of 8·5 cm. Phenols were weighed and added in solid form to the culture medium after cooling to 40–50 °C. A total of eleven compounds each at four different concentrations (in the final agar solution): 5×10^{-3} M; 5×10^{-4} M, 5×10^{-5} M and 5×10^{-6}M were tested. Each concentration of added phenol was tested on five replicate plates. After solidification of the agar plates, 15 µl of suspended soredia in sterile water was added to the surface of the agar and spread with a sterile loop. Plates were sealed with parafilm and incubated at 23 °C for 7 days under a 12 h light/12 h dark regime using a natural light intensity. Growing soredia characterized by a small whitish ring of hyphae around the central core, were counted and soredial growth rate was expressed as % growth compared to control plates without the addition of phenols.

The inhibitory concentration resulting in 50% soredial growth compared to control ("IC50") was calculated from regression lines using the mean results for three different concentrations of each compound, representing high, medium and low inhibition rates.

Results

All phenolic compounds tested inhibited the growth of soredia from *Physcia tenella* but there were marked differences between compounds in relative toxicity (Tables 1 & 2). Concentrations needed to obtain 50% inhibition of soredial growth varied from 10^{-3} to 10^{-6} M. Both hydrophobic (ellagic acid) and hydrophilic (benzoic acid) phenols are effective growth inhibitors at pH 6·0 with IC50 values $<10^{-4}$ M. Toxicity is highest for the main endogenous phenolic glycoside in young saplings of *Salicaceae*, tremulacin (IC50 = $<1 \times 10^{-6}$ M) and its main degradation products, catechol (IC50 = 1×10^{-5} M) and benzoic acid (IC50 = 2×10^{-5} M). The biosynthetic precursors of tremulacin, salicortin and salicin, both with IC50 values of 4×10^{-5} M are strong inhibitors. Salicylic aldehyde, salicylic alcohol (Fig. 2) and (+)-catechin are moderate inhibitors with IC50 values varying

https://doi.org/10.1017/S0024282907005907 Published online by Cambridge University Press

TABLE 2. *IC50-values for selected phenols on the* in vitro *growth assay on soredia of* Physcia tenella

Chemical substance	IC50 (mol l^{-1}) $\times 10^{-6}$ M
Tremulacin	<1
Catechol	10 (± 3)
Benzoic acid	20 (± 5)
Ellagic-acid	20 (± 5)
Salicortin	40 (± 10)
D-(-)-Salicin	40 (± 10)
Salicylic aldehyde	180 (± 50)
Salicylic alcohol	300c (± 100)
(+)-Catechin	400c (± 100)
Gallic-acid	1700c (± 300)
Salicylic acid	2000c (± 500)

IC50 values are given as 50% inhibition compared to growth on control plates.

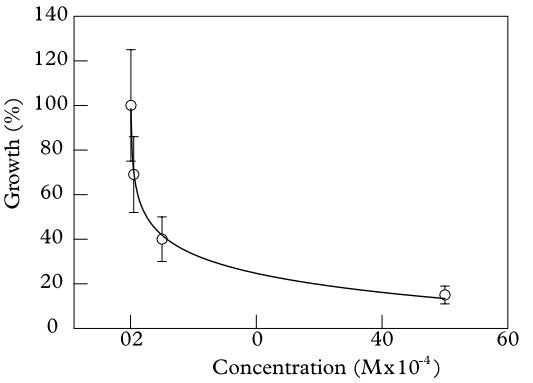

FIG. 2. IC curve of salicylic alcohol.

from 1·8–4 $\times 10^{-4}$ M. Gallic acid and salicylic acid with IC50 values of 1·7 $\times 10^{-3}$ M and 2×10^{-3} M, respectively, are weak inhibitors of soredial growth when compared to the other salicylates.

Discussion

In this study we used substances representative of the whole spectrum of phenolic metabolites and their biodegradation products occurring in bark of *Salicaceae*. We also added the naturally occurring substituted salicylates (salicortin, tremulacin and salicin) and their degradation products (catechol, benzoic acid and salicylic aldehyde) to the agar medium. Nevertheless, it has to be kept in mind that all species-specific secondary metabolites reported from *Salicaceae* have been isolated from young tissue, mainly leaves, twigs or bast (Julkunen-Titto 1989). The spectrum of phenolic constituents in cork layers of *Populus* spp. of known age has not yet been investigated. Comparable studies on cork extracts of *Quercus suber*, show a complex spectrum of extractable suberin and lignin derived phenols and polysaccharides (Cordeiro *et al.* 2002). We used two year old trees for isolation of phenolic constituents since major phenols from twigs and saplings are chemically well characterized, and, because these constituents are likely to produce biodegradation products in aged cork layers and in stem flow. Gallic and ellagic acid were included in our study, because they are widespread in woody plants though ellagic acid does not occur in *Salicaceae* (Hegnauer 1962).

The 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 *et al.* 1971; Butin & Loeschke 1969) but did not analyze the effects of hydrophobic metabolites.

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

Lichen fungi can produce gelatinous layers around hyphae, which are not in ultimate contact to algal cells. Algal cells in

lichen symbiosis are surrounded by a trilaminar layer, containing an amorphous, polysaccharide derived matrix (Lawrey 1984; Honegger & Bartnicki-Garcia 1991). Therefore it can be assumed, that allelochemicals acting on mature lichen thalli initially react with matrix compounds. In contrast, soredia are composed of undifferentiated algal and fungal cells (Lawrey 1984) and therefore are 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 are necessary to reveal, whether studies on phanerophyte development are relevant for the lichen symbiosis.

Mature lichen thalli are exposed to allelochemicals from bark by direct attachment of lichen rhizines to the outer cork layer and by leachable components of bark in stem flow. Eighty year old trees of *Populus* × *canadensis* have a bark pH of 6·0 (Franzen 2001). In lichen mapping studies focusing on air pollution, tree species with acidic and neutral to subneutral bark are considered as a distinct group, because they host different lichen communities (VDI 2005). Stability of phenolic glycosides and net charge of carboxylic acids, such as benzoic or gallic acid will be altered at acidic pH, so that IC50 values obtained in our study can not be used to indicate allelopathy on trees with acidic bark. Furthermore, each tree species contains specific endogenous phenols in bark, that contribute to stem flow chemistry by leaching processes. In *Salicaceae* phenolic glycosides represent less than 1% of dry weight of bark in saplings, while the quantity of total phenols is about 5% (Julkunen-Titto 1989). Salicylates, such as tremulacin and salicortin are easily degraded in aqueous or buffered solution (Ruuhola *et al*. 2003). The resulting catechol, benzoic acid and salicin were shown to be potent inhibitors of soredial growth in our test system. Therefore the 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 widely occurring tannins and proanthocyanidines, such as gallic acid and catechin, are less potent inhibitors of soredial growth (Table 1). Tertiary growth of the cork layer in old trees leads to the formation of dead, suberinized and air filled cork cavities (Fengel & Wegener 1984). The content of total acetone soluble phenols in the outer 0·5 cm of the cork layer of 80 year old *Populus* × *canadensis* amounts to about 0·6% by mass (Koopmann 2005) corresponding to 4×10^{-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 agar–nutrient solution. Our *in vitro* test can be used as a model system for natural conditions occurring on outer bark of *Populus* × *canadensis*. Further studies might be directed to an analysis of susceptibility of rare and abundant lichen species towards certain bark allelochemicals and the relationship between bark allelochemicals and epiphyte cover.

We thank Brian O'Shea for correcting the English text.

References

Backor, M., Hudak, Repcak, M., Ziegler, W., Backorova, M. (1998) The influence of pH and lichen metabolites (vulpinic acid and (+)-usnic acid) on the growth of lichen photobiont *Trebauxia irregularis* cultures. *Lichenologist* **30:** 577–582.

Barkmann, J. J. (1958) *Photosociology and Ecology of Cryptogamic Epiphytes*. Assen: van Gorcum.

Bates, J. W. & Brown, D. H. (1981) Epiphyte differentiation between *Quercus petraea* and *Fraxinus excelsior* trees in a Maritime area of South West England. *Vegetatio* **48:** 61–70.

Butin, H. & Loeschke, V. (1969) Nachweis fungistatischer Stoffe in der Pappelrinde. *Naturwissenschaften* **19:** 451–452.

Cordeiro, N., Neto, C. P., Rocha, J., Belgacem, M. N., Gandini, A. (2002) The organosolv fractionation of cork components. *Holzforschung* **56:** 135–142.

Evenari, M. (1949) Germination inhibitors. *Botanical Reviews*. **15:** 153–194.

Fengel, D. & Wegener, C. (1984) *Wood-Chemistry, Ultrastructure, Reactions*. Berlin: De-Gruyter.

Follmann, G. & Nakagawa, M. (1963) Keimhemmung von Angiospermensamen durch Flechtenstoffe. *Naturwissenschaften* **50:** 696–697.

Frahm, J. P., Specht, A., Reifenrath, K. & Leon-Vargas, Y. (2000) Allelopathic effects of crustaceous lichens on the germination of bryophytes and vascular plants. *Nova Hedwigia* **70:** 245–254.

Franzen, I. (2001) Epiphytische Moose und Flechten als Bioindikatoren am Westrand des Ruhrgebietes. *Limprichtia* **16:** 1–85.

Gauslaa, Y. (1985) The ecology of *Lobarion pulmonariae* and *Parmelion caperatae* in *Quercus* dominated forest in south-west Norway. *Lichenologist* **17:** 117–140.

Gustafsson, L. & Eriksson, I. (1995) Factors of importance for the epiphytic vegetation of aspen *Populus tremula* with special emphasis on bark chemistry and soil chemistry. *Journal of Applied Ecology* **32:** 412–424.

Hauck, M., Paul, A., Mulack, C., Fritz E. & Runge, M. (2002*a*) Effects of manganese on the viability of vegetative diasporas of the epiphytic lichen *Hypogymnia physodes*. *Environmental and Experimental Botany* **47:** 127–142.

Hegenauer, R. (1963) *Chemotaxonomie der Pflanzen*. Basel: Birkhäuser.

Honegger, R. & Bartnicki-Garcia, S. (1991) Cell wall structure and composition of cultured mycobionts from the lichens *Cladonia macrophylla*, *Cladonia caespititia*, and *Physcia stellaris* (*Lecanorales*, *Ascomycetes*). *Mycological Research* **95:** 905–914.

Julkunen-Tiitto, R. (1989) *Distribution of certain phenolics in* Salicaceae. PhD. Thesis, University of Joensuu, *Publications in Sciences* No 15.

Koopmann, R. (2005) *Allelopathic effects of bark phenols on epiphytic lichens*. Ph.D. Thesis, University of Bonn.

Lawrey, J. D. (1984) *Biology of Lichenized Fungi*. New York: Praeger.

Meier, B., Julkunen-Tiitto, R., Tahvanainen, J. & Sticher, O. (1988) Comparative high performance liquid and gas liquid chromatographic determination of phenolic glycosides in *Salicaceae* species. *Journal of Chromatography*. **442:** 175–186.

Olsen, R. A., Odham, G. & Lindeberg, G. (1971) Aromatic substances in leaves of *Populus tremula* as inhibitors of myccorhizal fungi. *Physiologia Plantarum* **25:** 122–129.

Ostrofsky, A. & Denison, W. C. (1980) Ascospore discharge and germination in *Xanthoria polycarpa*. *Mycologia* **72:** 1171–1179.

Parlar, H. & Angerhöfer, D. (1991) *Chemische Ökotoxikologie*. Berlin: Springer.

Pyatt, F. B. (1973) Lichen propagules. In *The Lichens* (V. Ahmadjian & M. Hale, eds.): 117–145. New York: Academic Press.

Ruuhola, T., Julkunen-Tiitto, R. & Vainiotalo, P. (2003) In vitro degradation of willow salicylates. *Journal of Chemical Ecology* **29:** 1083–1097.

VDI Richtlinie 3957 Blatt 13 (2005) *Biologische Messverfahren zur Ermittlung und Beurteilung der Wirkung von Luftverunreinigungen auf Flechten (Bioindikation)*. Berlin: Beuth.

Accepted for publication 19 *January* 2007