Mycoscience (2011) 52:413–418
DOI 10.1007/s10267-011-0118-3

SHORT COMMUNICATION

Bioactivity of secondary metabolites and thallus extracts from lichen fungi

Mark Kowalski · Georg Hausner · Michele D. Piercey-Normore

Received: 4 August 2010 / Accepted: 31 March 2011 / Published online: 17 April 2011

Abstract Fungal secondary compounds and total extracts are known to affect growth of bacteria, fungi, and plants. This study tested the effects of purified compounds and total extracts from three lichens on the growth of two plant pathogens, *Ophiostoma novo-ulmi* ssp. *americana* and *Sclerotinia sclerotiorum*. Usnic acid showed no reduction in relative growth rates (RGR), whereas vulpinic acid reduced RGR for both fungi and atranorin reduced RGR of *S. sclerotiorum* only. However, purified vulpinic acid showed stronger effects than total extracts on fungal growth. The results suggest that these lichens show further promise as a source for bioactive compounds against fungi.

Keywords *Cladonia amaurocraea* · *Hypogymnia physodes* · Plant pathogen · *Vulpicida pinastri*

More than 800 secondary compounds have been discovered from lichen-forming fungi, and most of these are unique to lichens (Huneck and Yoshimura 1996; Miao et al. 2001), being produced entirely within the fungal partner (Culberson et al. 1985; Culberson and Armaleo 1992). Lichen secondary compounds have historically been used in the taxonomy of lichens (Hawksworth 1976), and many have known ecological functions or medicinal properties (Lawrey 1986; Huneck 1999). In some cases the purified compound alone was bioactive, and in other cases the total extract was reported to be bioactive, suggesting a synergistic effect with other compounds in the extract. Among common secondary metabolites, atranorin has been shown to inhibit spore germination of lichenized fungi (Whiton and Lawrey 1984). Atranorin is produced in the cortex of *Hypogymnia physodes* where it is thought to change the light wavelength so as to promote photosynthesis (Rao and LeBlanc 1965). *Hypogymnia physodes*, one of the most common tree lichens in conifer forests in North America, also produces five other secondary compounds (Culberson et al. 1977; Brodo et al. 2001). Usnic acid, another secondary compound, has been shown to inhibit biofilm formation of *Staphylococcus aureus* (Francolini et al. 2004) and to inhibit growth of eight fungal and Oomycota species in the genera *Pythium*, *Phytophthora*, *Rhizoctonia*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Stagonospora*, and *Ustilago* (Halama and Van Haluwin 2004). Usnic acid is produced by many species of *Cladonia* including *Cladonia amaurocraea,* which is a ground-dwelling boreal forest species that produces barbatic acid in addition to usnic acid. Vulpinic acid, another secondary compound, is a deep yellow metabolite that is poisonous to mollusks and insects (Elix and Stocker-Wörgötter 2008) and has also been reported to inhibit spore germination of some lichenized fungi (Whiton and Lawrey 1984). Vulpinic acid is produced by *Vulpicida pinastri* along with two other important secondary compounds. *V. pinastri* is common in moist habitats on trees and sometimes rocks in the boreal forest of North America (Brodo et al. 2001).

The bioactive nature of lichen secondary compounds suggests possible strategies to manage plant pathogens, which may include interference with different developmental stages, such as prevention of spore germination and inhibition of sexual reproduction, or interference directly with mycelial growth. These strategies are perhaps

M. Kowalski · M. D. Piercey-Normore (✉)
Department of Biological Sciences, University of Manitoba, Winnipeg, MB R3T 2N2, Canada
e-mail: pierceyn@cc.umanitoba.ca

G. Hausner
Department of Microbiology, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

achievable with application of a purified bioactive compound or a suite of components with a synergistic effect (reviewed in Huneck 1999). The effects of lichen secondary metabolites on mycelial growth can be determined by measuring the growth of the mycelial mass over time (relative growth rate, RGR) or by measuring the prevention of growth near the source of an inhibitory compound (inhibition).

Ophiostoma novo-ulmi ssp. *americana* Bras. et Kirk, a causative agent for a severe wilt disease (Dutch elm disease), has devastated American elm populations in North America (Brasier 2000). The fungus is also found in Europe and Eurasia and has recently been reported from Japan (Masuya et al. 2010). The application of plant breeding and fungicides to control *O. novo-ulmi* ssp. *americana* has been expensive and limited in use (Brasier 2000), and few efforts have been devoted to resistance breeding of native elms. Alternative control strategies would be economically and biologically beneficial for preserving urban forests composed of *Ulmus* sp. *Sclerotinia sclerotiorum* (Lib.) de Bary, commonly referred to as "white mold" of many agricultural crops (e.g., beans, canola, carrots, lettuce, sunflowers) and some woody ornamentals, devastates crops and causes spoilage of fruits and vegetables during storage (Agrios 2005). Although there are several fungicides available for *S. sclerotiorum*, the cost of these chemicals can again be high and timing of application is usually very important (Saharan and Mehta 2008). A combination of biological control agents or fungicides with secondary metabolites might be valuable in developing effective integrated control strategies against this disease (Budge and Whipps 2001; Saharan and Mehta 2008).

In this study we examine whether compounds from three lichen-forming fungi (*C. amaurocraea*, *H. physodes*, and *V. pinastri*) are useful as potential antifungal compounds. The effects of three secondary metabolites (atranorin, usnic acid, vulpinic acid) and total lichen thallus extracts on the mycelial growth and inhibition of two unrelated plant pathogens (*Sclerotinia sclerotiorum* and *Ophiostoma novo-ulmi* ssp. *americana*) were studied. It was hypothesized that (1) all three compounds would reduce the RGR and inhibit growth of these plant pathogenic fungi; (2) that the higher concentrations of the compounds would show greater reduction in RGR and greater inhibition than the lower concentrations; and (3) that the purified compounds would show greater reduction in RGR and greater inhibition than the thallus extracts.

The three compounds and total extracts were isolated from the lichen-forming fungal species *Cladonia amaurocraea* (Flörke) Schaerer (Canada, Manitoba, Wapusk National Park; 58°31′16.6″ N, 93°34′22.9″ W; 2008, *Normore 8652* (WIN(C)), *Hypogymnia physodes* (L.) Nyl. (Canada, Manitoba, Wapusk National Park; 58°30′37.7″ N, 93°28′16.4″ W, 2008, *Normore 8565* (WIN(C)), and *Vulpicida pinastri* (Scop.) J.-E. Mattsson and M.J. Lai (Canada, Manitoba, Sherridon Rd., 54°42′24.7″ N, 101°33′53.1″ W; 2008 *Normore 8728* (WIN(C)). These lichens were selected because they are common in North America (Brodo et al. 2001) and the secondary metabolites are well known and readily isolated. The two plant pathogenic fungi are *Sclerotinia sclerotiorum* (WIN(M)) 1647, isolated from *Daucus carota* L. in Manitoba), and *Ophiostoma novo-ulmi* ssp. *americana* (WIN(M)) 903, isolated from *Ulmus americana* Planch. in Manitoba. These pathogens were selected for this study because their growth in culture was suitable for measuring growth rates over a period of 1–2 weeks, and the radial growth on the medium allowed for accurate growth measurements. A total of ten treatments were tested: three purified compounds at two concentrations each (crudely 20 mg/ml and 10 mg/ml from silica-coated plates); total extracts of the three lichens *C. amaurocraea*, *H. physodes*, and *V. pinastri* at a concentration of 20 mg/ml total extract; and a control consisting of acetone, which was the solvent for all treatments. Each treatment was tested on the two plant pathogenic fungi and was replicated five times for each pathogenic fungus with three inoculations per Petri dish.

Usnic acid, atranorin, and vulpinic acid were isolated from *C. amaurocraea*, *H. physodes*, and *V. pinastri*, respectively. To obtain 50–100 mg dry weight of the target compounds, clean tissues of the lichens were ground with 25 ml acetone, and the crude extracts obtained were applied to preparative thin-layer chromatography (TLC). The developing solvent was toluene/dioxane/glacial acetic acid (180:45:5) (Orange et al. 2001). A "purified" extract is defined as the extract retrieved from the TLC isolation even though traces of other compounds may be present. A "crude" extract refers to the entire complement of compounds soluble in acetone from the entire lichen thallus. No TLC was performed to obtain the thallus crude extract.

Test fungi were cultured on malt yeast agar (MYA) (20 g malt extract, 2 g yeast extract, 15 g agar per 1,000 ml distilled water) at 20°C for 24 h in the dark. A 50-µl portion of test extract or acetone as negative control was dropped on a sterilized triangular filter paper placed on the center of the culture plate; 2-mm fragment of mycelium was then inoculated onto the plate at a 1-cm distance from each of the three edges of the filter paper. Colony diameters were measured every 2 days (Soylu et al. 2007), and the inhibition zone was recorded as the distance between the filter paper and the leading front of mycelial growth. The colony area was calculated based on the diameter measurements of mycelial growth and area $= \pi ab$ (where $\pi = 3.14$; $a =$ major radius; $b =$ minor radius). Growth curves were produced by log10 transforming the average

values for each treatment, and these values were plotted against time. The relative growth rates were then calculated from the interval between day 2 and day 4 because this interval represents the exponential growth phase, providing the most accurate representation of growth (Hunt 1978). Because colony growth was not consistent along the edge of the filter paper for the inhibition assays, three measurements were averaged for each side of the filter paper.

The RGR between the treatments were assessed using a two-way analysis of variance (ANOVA) (Sokal and Rohlf 1995) with a 95% confidence interval implemented in Microsoft Excel (ver. 12.2.5) to examine the relationship between the compound (atranorin, usnic acid, vulpinic acid) and the concentration (10 or 20 mg/ml) for each of *O. novo-ulmi* ssp. *americana* and *S. sclerotiorum*. Another two-way ANOVA examined extract type (total extract and purified extract) and lichen species (*V. vulpicida*, *H. physodes*, *C. amaurocraea*) to test the relationship between lichen species and whether the total extract or a single compound affected growth. An interaction suggests these are not independent of one another. Each treatment was also compared to the control treatment to determine if a significant difference occurred, using a *t* test with a 95% confidence interval. The same treatments were analyzed for the inhibition measurements.

Diagnostic secondary metabolites for the three lichen species are well known (Culberson et al. 1977), with Rf values and spot characteristics described by Orange et al. (2001). *Cladonia amaurocraea* produces usnic acid (Rf = 0.70) and barbatic acid (Rf = 0.44). *Hypogymnia physodes* produces atranorin (Rf = 0.75) and chloroatranorin (Rf = 0.74). Five additional compounds are produced with Rf values less than 0.55 in *H. physodes*. *Vulpicida pinastri* (=*Cetraria pinastri*) produces vulpinic acid (Rf = 0.71), pinastric acid (Rf = 0.70), and usnic acid (Rf = 0.70). Therefore, it is likely that the usnic acid isolated from *C. amaurocraea* is pure, but the isolated atranorin from *H. physodes* may contain small amounts of chloroatranorin, and the isolated vulpinic acid from *V. pinastri* may also contain some usnic and pinastric acids. To identify these compounds, the Rf values and the spot characteristics were compared with those in Orange et al. (2001).

The first analysis in Table 1 tested the hypothesis that the type of compound (atranornin, usnic acid, vulpinic

Table 1 Summary of analysis of variance (ANOVA) results for relative growth rates (RGR) of *Ophistoma novo-ulmi* ssp. *americana* and *Sclerotinia sclerotiorum* showing effects of purified compound (atranorin, usnic acid, vulpinic acid) and concentration (20 and 10 mg/ml) in one series of tests; and comparing lichen species (*Cladonia amaurocraea*, *Vulpicida pinastri*, *Hypogymnia physodes*) and extraction type (purified, crude extract) in the second series of tests

Source	*O. novo-ulmi* ssp. *americana*			*S. sclerotiorum*		
	df	*F* ratio	*P* value	*df*	*F* ratio	*P* value
Compound	2	65.258	<0.0001	2	174.950	<0.0001
Concentration	1	3.326	0.0807	1	1.096	0.3060
Interaction	2	1.003	0.3817	2	2.598	0.0950
Lichen	2	13.861	<0.0001	2	20.362	<0.0001
Extraction	1	0.389	0.5390	1	21.450	0.0001
Interaction	2	28.022	<0.0001	2	6.343	0.0061

A *P* value less than 0.05 indicates a statistically significant difference between treatments

Table 2 Summary of *t*-test results for comparison of mean relative growth rates (RGR, $mm^2/mm^2/days$) among treatments for *Ophistoma novo-ulmi* ssp. *americana* and *Sclerotinia sclerotiorum*

Treatment	Mean relative growth rate ($mm^2/mm^2/days$)	
	O. novo-ulmi ssp. *americana*	*S. sclerotiorum*
Acetone control	0.49	0.53
Usnic acid (20 mg/ml)	0.46	0.53
Usnic acid (10 mg/ml)	0.40	0.48
Atranorin (20 mg/ml)	0.45	0.37*
Atranorin (10 mg/ml)	0.40	0.37*
Vulpinic acid (20 mg/ml)	0.21*	0.18*
Vulpinic acid (10 mg/ml)	0.21*	0.20*
Total extract (*Cladonia amaurocraea*)	0.30*	0.49
Total extract (*Hypogymnia physodes*)	0.37	0.46
Total extract (*Vulpicida pinastri*)	0.37	0.41*

For both species, treatments were compared with the acetone controlAsterisk (*) indicates a significant difference from the control at $a = 0.05$ based on a *t* test

acid) and the concentration (20 or 10 mg/ml) had an effect on fungal growth. Results show that the type of compound had a significant effect on growth but the concentration of the compound did not affect growth. The second analysis tested the hypothesis that lichen species (*C. amaurocraea*, *H. physodes*, *V. pinastri*) and extraction type (purified or total extract) had an effect on mycelial growth. Although type of extract had no effect on mycelial growth, the lichen species did have an effect. That both compound and lichen species had a significant effect on mycelial growth is supported by the taxonomic concept of chemical species within lichens (Hawksworth 1976). The analysis in Table 2 tested the hypothesis that each compound and each extract could limit mycelial growth in the plant pathogens. Table 2 shows that vulpinic acid alone and the total extract from *C. amaurocraea* reduced the growth of *O. novo-ulmi*. However, two compounds alone (atranorin and vulpinic acid), as well as the total extract from *V. pinastri*, reduced the growth of *S. sclerotiorum*. Figure 1 shows that vulpinic acid reduced RGR for both species (Fig. 1a–d) whereas atranorin reduced RGR only of *S. sclerotiorum*. The data also show that vulpinic acid inhibited growth of both pathogens and the crude extract from *C. amaurocraea* inhibited growth of *S. sclerotiorum* (Fig. 1e–f).

The decrease in RGR of *O. novo-ulmi* ssp. *americana* and the inhibition of *S. sclerotiorum* growth with the total

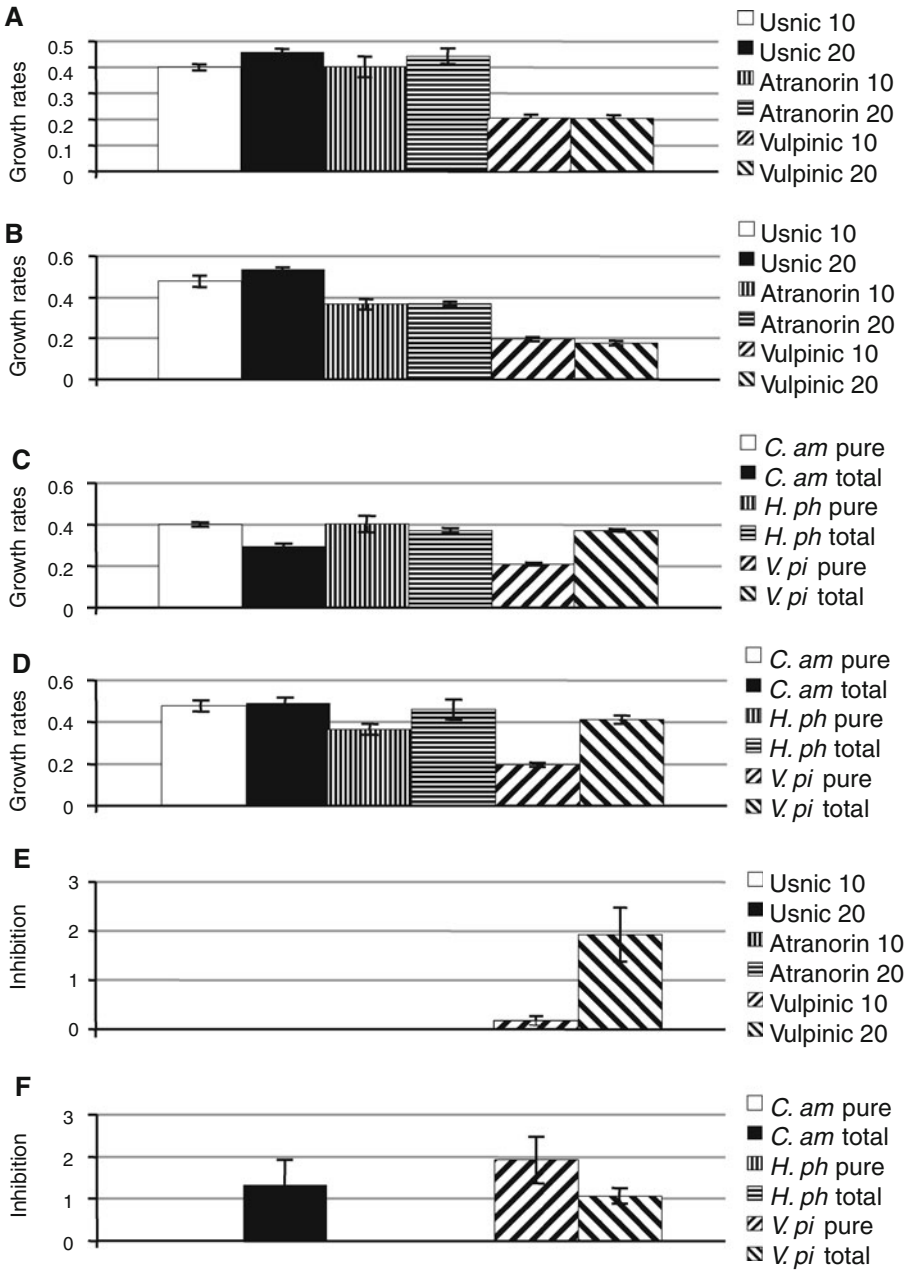

Fig. 1 Comparison among treatments showing relative growth rates (mm^2/mm^2/day) of *Ophistoma novo-ulmi* ssp. *americana* at both concentrations for each of the three purified compounds (**a**); relative growth rates of *S. sclerotiorum* for both concentrations for each of the three purified compounds (**b**); relative growth rates of *O. novo-ulmi* ssp. *americana* for pure and total thallus extracts (**c**); relative growth rates of *S. sclerotiorum* for pure and total thallus extracts (**d**); growth inhibition (mm) for *S. sclerotiorum* for both concentrations for each of the three purified compounds (**e**); and growth inhibition (mm) for *S. sclerotiorum* for pure and total thallus extracts (**f**). *C. am*, *Cladonia amaurocraea*; *H. ph*, *Hypogymnia physodes*; *V. pi*, *Vulpicida pinastri*

extract from *C. amaurocraea* suggest that other components within the thallus extract, in addition to usnic acid, affected mycelial growth. This finding is supported by Halama and Van Haluwin (2004), who reported greater inhibition of fungal growth in the total extract treatment and only slight inhibition by purified usnic acid. Previously, usnic acid was shown to inhibit fungal growth at concentrations greater than 32 μg/ml (Lauterwein et al. 1995) and with 50 μg of the chemical added to the plate (König and Wright 1999). The concentrations used in this study were greater than the reported effective concentrations, but the extraction method likely included impurities and fine silica particles that inflated dry weight measurements. Although no inhibition was observed with the purified atranorin treatment or the total *H. physodes* extract for *S. sclerotiorum*, the RGR of *S. sclerotiorum* decreased with the purified atranorin treatment in this study. Atranorin alone was previously shown to be a weak growth inhibitor (Lauterwein et al. 1995; Halama and Van Haluwin 2004), but the crude extract of *H. physodes* was effective against several plant pathogenic fungi (Halama and Van Haluwin 2004). The purified vulpinic acid compound showed greater activity than the total extract from *V. pinastri*, which is the opposite result of that shown by usnic acid and the extract from *C. amaurocraea*. Our findings are supported by previous reports that vulpinic acid, at 50 μg/plate, inhibited the growth of several fungi (König and Wright 1999).

The greater growth inhibition observed in *S. sclerotiorum* compared to *O. novo-ulmi* spp. *americana* in many of the treatments in this study is consistent with findings by Land and Lundström (1998). They reported that yeasts, molds, blue stain fungi, and rot fungi behave differently when exposed to total extracts from *Nephroma arcticum*. Total extracts from species of *Cladonia*, *Hypogymnia*, and *Evernia* have also been reported to differentially reduce mycelial growth of various ascomycete and basidiomycete species, depending on the species tested (Halama and Van Haluwin 2004).

The results showed that some lichen fungi produce bioactive secondary metabolites, which were either purified compounds or combinations with other components contained in the total extract. Although optimal concentrations of their antifungal activities were not determined in this study, the relative evaluation between total extracts and purified compounds may provide a direction for assessing antifungal activity. Usnic acid alone did not inhibit or reduce RGR in either of the plant pathogens tested, whereas the total extract of *C. amaurocraea* containing usnic acid showed synergistic activity.

Vulpinic acid had the greatest effect among the three compounds studied, but we cannot exclude the possibility that our TLC preparation included traces of other compounds in addition to vulpinic acid. The apparent low bioactivity for *V. pinastri* total extracts on *O. novo-ulmi* ssp. *americana* may result from the low concentrations of vulpinic acid.

Our findings on the two plant pathogens justify further work that might include a larger number of isolates and species. The identification of bioactive compounds against *S. sclerotiorum* and *O. novo-ulmi* ssp. *americana* offers an opportunity to develop alternative strategies to control these fungi.

Acknowledgments The authors thank S. Athukorala for help with fungal culturing, N. Kenkel for statistical advice, and T. Booth and K. Fontaine for checking the English. Lichens were collected with Parks Canada (permit number WAP-2008-67). This project was funded by National Science and Engineering Research Council of Canada (NSERC). Experiments comply with current Canadian laws.

References

Agrios GN (2005) Plant Pathology, 5th edn. Elsevier Academic Press, Burlington, MA

Brasier CM (2000) Intercontinental spread and continuing evolution of the Dutch elm disease pathogen. In: Dunn CP (ed) The elms: breeding, conservation, and disease management. Kluwer Academic, Boston

Brodo IM, Sharnoff S, Sharnoff S (2001) Lichens of North America. Yale University Press, New Haven, 795 pp

Budge SP, Whipps JM (2001) Potential for integrated control of *Sclerotinia sclerotiorum* in glasshouse lettuce using *Coniothyrium minitans* and reduced fungicide application. Phytopathology 91:221–227

Culberson CF, Armaleo D (1992) Induction of a complete secondary product pathway in a cultured lichen fungus. Exp Mycol 16:52–63

Culberson CF, Culberson WL, Johnson A (1977) Second supplement to "Chemical and botanical guide to lichen products". American Bryological and Lichenologial Society, Missouri Botanical Garden, St. Louis

Culberson CF, Culberson WL, Johnson A (1985) Does the symbiont alga determine chemotype in lichens? Mycologia 77:657–660

Elix JA, Stocker-Wörgötter E (2008) Biochemistry and secondary metabolites. In: Nash TH III (ed) Lichen biology, 2nd edn. Cambridge University Press, Cambridge

Francolini I, Norris P, Piozzi A, Donelli G, Stoodley P (2004) Usnic acid, a natural antimicrobial agent able to inhibit bacterial biofilm formation on polymer surfaces. Antimicrob Agents Chemother 48:4360–4365

Halama P, van Haluwin C (2004) Antifungal activity of lichen extracts and lichenic acids. BioControl 49:95–107

Hawksworth DL (1976) Lichen chemotaxonomy. In: Brown DH, Hawksworth DL, Bailey RH (eds) Lichenology: progress and problems. Academic Press, London

Huneck J (1999) The significance of lichens and their metabolites. Naturwissenschaften 86:559–570

Huneck J, Yoshimura I (1996) Identification of lichen substances. Springer, Berlin

Hunt R (1978) Plant growth analysis. In: The Institute of Biology's Studies in Biology, no. 96. Arnold, London

König G, Wright A (1999) ^{1}H and ^{13}C-NMR and biological activity investigations of four lichen-derived compounds. Phytochem Anal 10:279–284

Land CJ, Lundström H (1998) Inhibition of fungal growth by water extracts from the lichen *Nephroma arcticum*. Lichenologist 30:259–262

Lauterwein M, Oethinger M, Belsner K, Peters T, Marre R (1995) In vitro activities of the lichen secondary metabolites vulpinic acid, (+)-usnic acid, and (−)-usnic acid against aerobic and anaerobic microorganisms. Antimicrob Agents Chemother 39:2541–2543

Lawrey J (1986) Biological role of lichen substances. Bryologist 89:111–122

Masuya H, Brasier C, Ichihara Y, Kubono T, Kanzaki N (2010) First report of the Dutch elm disease pathogens *Ophiostoma ulmi* and *O. novo-ulmi* in Japan. Plant Pathol 59:805

Miao V, Coeffet M, Brown D, Sinnemann S, Donaldson G, Davies J (2001) Genetic approaches to harvesting lichen products. Trends Biotechnol 19:349–355

Orange A, James PW, White FJ (2001) Microchemical methods for the identification of lichens. British Lichen Society, London

Rao DN, LeBlanc F (1965) A possible role of atranorin in the lichen thallus. Bryologist 68:284–289

Saharan GS, Mehta N (2008) Sclerotinia diseases of crop plants: biology, ecology and disease management. Springer, Heidelberg

Sokal RR, Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research, 3rd edn. Freeman, New York

Soylu S, Yigitbas H, Soyle E, Kurt S (2007) Antifungal effects of essential oils from oregano and fennel on *Sclerotinia sclerotiorum*. J Appl Microbiol 103:1021–1030

Whiton JC, Lawrey JD (1984) Inhibition of crustose lichen spore germination by lichen acids. Bryologist 87:42–43