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# Effect of Environmental Change on Secondary Metabolite Production in Lichen-Forming Fungi

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## 1. Introduction

The production and regulation of secondary metabolites in non-lichenized fungi, mainly ascomycetes, has been reviewed by a number of authors with an emerging understanding of the biosynthesis and the pathways involved in regulation (Keller et al., 2005; Yu & Keller 2005; and others). However, lichenized fungi make up almost half of all known ascomycetes (Kirk et al., 2001) and are known to produce over 800 secondary metabolites, most of which are unique to lichenized fungi. Many of these compounds have bioactive properties (Huneck, 1999) and some studies have shown or suggested that secondary metabolite production is influenced by changes in culture conditions, which might be regarded as environmental changes. Intense investigation of the changes in production of these unique bioactive secondary metabolites from lichen fungi have been hampered by problems associated with isolating and growing cultures of lichen fungi. Lichens have been studied for more than two centuries as morphological entities but experimental lichenology has remained a nearly unexplored scientific field for many decades because of the slow growing nature of lichens. Thomas (1939 in Stocker-Worgotter, 2001) reported the first successful resynthesis of *Cladonia pyxidata*. Since the 1970's, one major goal of experimental lichenology has been the improvement and optimization of culture conditions of lichen fungi. Culture techniques for lichen fungi have improved in recent years allowing for further research on these challenging organisms. Therefore, with greater access to cultures of lichen symbionts and progression of knowledge of non-lichenized fungi, studies are just beginning to accumulate on genes involved in production of secondary metabolites from lichen fungi; and the effects of the environment on the expression of these genes by observations in ecological studies, and through experimentation by manipulating culture conditions.

Fungal secondary metabolism is covered by extensive body of literature (see Bennett & Ciegler, 1983). Secondary metabolism is not required for survival and its products are dispensable whereas primary metabolism is essential for survival with anabolic and catabolic activities to maintain life. Secondary metabolites are chemically diverse but are produced from a few key intermediates of primary metabolism, and are generally categorized by the intermediates from which they are produced. Bennett and Ciegler (1983) summarize six categories of secondary metabolites derived from different primary intermediates. Although fungal secondary metabolites are extensive, they are generally

produced by one of just a few major pathways (Moore, 1998). The mevalonic acid pathway produces terpenes, sterols, and carotenoids. The malonate (or acetyl-polymalonyl; Elix & Stocker-Worgotter, 2008) pathway produces polyketides. Other metabolites are produced by the shikimate-chorismate (or shikimic acid; Elix & Stocker-Worgotter, 2008) pathway. This chapter focuses mainly on the polyketides produced by the acetyl-polymalonyl pathway. Polyketides constitute structurally diverse molecules produced by the successive condensation of small carboxylic acids, typically co-enzyme A activated malonate by a mechanism similar to fatty acid biosynthesis (Hopwood & Sherman, 1990). The diversity of polyketide structure produced from this pathway reflects the diversity of their biological activities.

For more thorough reviews of the structure and regulation of secondary metabolites in fungi the reader is referred to (Hopwood, 1997; Drew & Demain, 1977; Katz & Donadio, 1993; Hutchinson & Fujii, 1995; Keller & Hohn, 1997; Bennett & Ciegler, 1983). Reviews and inventories of lichen metabolites are summarized by Culberson C. F. & Elix (1989), Elix & Stocker-Worgotter (2008); Culberson C. F. (1969); Culberson C. F. (1970); Culberson C. F., et al. (1977b); Stocker-Worgotter (2008); and a recent classification of lichen substances (Culberson C. F. & Elix, 1989). The adaptive significance of secondary metabolites produced by lichen fungi has been speculated (Lawrey 1977) and numerous functional studies (reviewed in Huneck, 1999), but few studies have linked adaptation of lichen substances with environmental change.

This chapter provides a synopsis of secondary metabolite production in fungi with a focus on lichenized fungi. The synopsis includes a review of the effect of environmental parameters and fungal development on production and regulation of secondary metabolites by focusing on three genera of lichen-forming fungi but not exclusive to these taxa. The chapter also describes an original ecological study of secondary metabolite production for two species of lichen fungi along a geographic gradient. It concludes by summarizing these findings in light of the significance of secondary metabolic changes in terms of ecological and anthropogenic prospects.

## 2. Overview of secondary metabolite production and regulation

Current changes in climate have prompted a number of studies to predict future changes in species distributions, ecosystem changes, effect on rare species, and effects on invasive species. For example, *Lecanora populicola* has been predicted to expand its distribution with continued environmental change (Ellis et al., 2007). However, integral to the distribution of this species is the host tree, *Populus* sp., on which the lichen grows. *Biatora helvolla* was shown to follow the distribution of its host spruce tree after the glaciation in the Alps (Printzen et al., 1999). In another study, lichen diversity was influenced more by forest structure than by climate (Moning et al., 2009) and a recommendation was to allow native broad leaved trees to grow to maturity promoting rare lichen species. One of the more significant changes with global warming is the depletion of the atmospheric ozone layer allowing more UV-B radiation exposure to living organisms (Kerr & McElroy, 1993). Since many lichen compounds absorb UV-B (Solhaug et al., 2003) an increase in production of those compounds is expected with continued climate change. Bjerke et al. (2003) showed an increase in the levels of two tridepsides (methyl gyrophorate and gyrophoric acid) and unidentified trace metabolites after a five-year study of two arctic lichen species of *Peltigera*.

But there were no changes in thallus dimensions or nitrogen fixation activity. A shift in secondary metabolism to allow survival in a particular habitat may promote changes in species and therefore functional attributes of phenotype. One of the functional changes of lichen-fungi dealt with in this chapter is that of secondary metabolite production. To some extent fungal secondary metabolites reflect taxonomy, but some studies have suggested that secondary metabolites may also be influenced by environmental change. Environmental changes influence many cellular activities and also serve as triggers for a change in mode of reproduction, influencing the entire biology of the species.

Since most species have diagnostic compounds that are consistently produced because of genetic inheritance and species adaptation to particular niches, chemical diversity can be correlated with taxonomy. The chemical correlation with taxonomy is referred to as chemotaxonomy (reviewed by Hawksworth, 1976; Frisvad et al., 2008). Knowledge of species taxonomic diversity is a first clue to understanding the polyketide diversity in any habitat. *Ramalina americana* was split into two different species (*R. culbersoniorum* and *R. americana*) based on secondary metabolite and nucleotide sequence divergence (LaGreca, 1999). The *Cladonia chlorophaea* complex contains at least five chemospecies, which are named and determined by the secondary metabolite produced (Culberson C. F. et al., 1977a). Other examples exist to show variability among individuals within the same geographic area. Secondary metabolites may also vary even within chemospecies. For example, the diagnostic metabolite for *C. grayi* is grayanic acid, and for *C. merochlorophaea* is merochlorophaeic acid. However, these species may or may not produce fumarprotocetraric acid, a polyketide that is considered to be an accessory compound since it is not consistently produced among individuals within a species. One suggestion for the quantity of accessory compounds to vary is changes in the environment (Culberson C. F. et al., 1977a) affecting regulatory pathways that depend on fungal developmental and environmental cues.

## 2.1 Exploring diversity of secondary metabolites within three genera of lichen-forming fungi

Since lichens are named according to their fungal partner (Kirk et al., 2001), 13,500 known species of lichenized fungi are somewhat scattered throughout the ascomycete families and reflect one of several ecological groups of fungi. Other ecological groups of fungi include mycorrhizal fungi, plant and animal pathogenic fungi, and saprobic fungi. These ecological groups may be considered artificial groups that reflect changes in feeding habits because of environmental plasticity that are present in most taxonomic groups. The lichenized fungi are currently classified among three classes of ascomycetes, Sordariomycetes, Lecanoromycetes, and Eurotiomycetes, and approximately 20 species of basidiomycetes. The majority of lichen-forming fungi belong to the Lecanoromycetes (Tehler & Wedin, 2008). Three genera within the Lecanoromycetes include *Cladonia*, a large ground-dwelling genus; *Ramalina*, epiphytes on rocks and trees; and *Xanthoparmelia*, an almost exclusive rock-dwelling genus. The substratum on which fungi grow allows for a diversity of nutrients to be available to the fungus (Brodo, 1973). The three genera grow on different substrata, have large thalli, have broad global distributions, and therefore provide a good contrast for examining secondary metabolite diversity.

The genus *Cladonia* is a large genus within the family Cladoniaceae comprised of more than 400 species (Ahti, 2000) and contains more than 60 described secondary metabolites with 30 of those being major metabolites in high concentration (Ahti, 2000) and the remaining being

minor accessory compounds in lower concentration. Secondary metabolites produced by members of the genus have been extensively studied with some variability in polyketide diversity (Huovinen & Ahti, 1986a, 1986b, 1988; Huovinen et al., 1989a, 1989b, 1990). Members of the genus are mostly ground dwelling on soil or moss and sometimes occur on thin soil over rock. Other species are found on decaying wood or tree bases. All species have a primary crustose or squamulose thallus in direct contact with the substratum and a vertical fruticose thallus (podetium) often culminating in the sexually produced fruit body (apothecium) at its apex (Fig. 1A). The fungi in this genus associate with Eukaryotic unicellular green algae in the genus *Asterochloris*.

The genus *Ramalina* is comprised of 46 species in North America (Esslinger, 2011) and is often considered to be highly variable in its polyketide production. The genus is characterized by producing B-orcinol depsides and depsidones. Usnic acid is the most common cortical compound in the genus. The *R. farinacea* complex produces a variety of metabolites that are all biosequentially related (Culberson W. L., 1966) with similar variability in the *R. siliquosa* complex (Culberson C. F. et al., 1992, 1993). Members of the *R. americana* species complex alone contain more than 55 metabolites (Culberson C. F. et al., 1990, 2000). Culberson C. F. et al. (1990) described comprehensively the biogenetic relationships and geographic correlations of the chemical variation within *R. americana*. While some species within the genus grow on rocks or cliffs, other species prefer the bark of trees, and some of the generalists may be found on both rock and tree bark. The genus contains fruticose species that are attached to their substratum by a single or several holdfasts giving the thallus a tufted or sometimes pendant appearance (Fig. 1B). The degree of contact between substratum and thallus is less than that for either *Cladonia* or *Xanthoparmelia*. Species of *Ramalina* associate with eukaryotic unicellular green algae in the genus *Trebouxia*.

*Xanthoparmelia* is a large genus distributed globally with more than 406 species (Hale 1990) but in present times is thought to exceed 800 species (Crespo et al., 2007). It is also polyketide diverse containing more than 38 major compounds and 53 accessory compounds (Hale, 1990). Salazinic, stictic, fumarprotocetraric, and norstictic acids are the most common medullary metabolites and usnic acid is the main cortical compound in the genus. Species in this genus are large foliose lichens that grow on non-calcareous rock and sometimes on mineral soils as the substratum. The thallus is attached to the substratum by large numbers of rhizines, which are clusters of fungal hyphae that extend from the underside of the thallus and penetrate the substratum (Fig. 1C). With many rhizines on each thallus the degree of contact with the substratum is greater than that with *Ramalina* but less than that with *Cladonia*. *Xanthoparmelia* species associate with eukaryotic unicellular green algae in the genus *Trebouxia*.

The heteromerous thallus in each of the three genera contains highly organized layers of tissue and each layer has a specific function (see Fig. 1 inserts; Budel & Scheidegger, 2008). Because of the cylindrical nature of the thallus, fruticose lichens have outer, middle, and sometimes inner layers of thallus tissue extending upright (podetium; *Cladonia*) or outward (pendant or tufted; *Ramalina*) from the substratum, whereas foliose thalli have upper, middle and lower layers of tissue because of the flattened, leaf-like nature of the thallus against the substratum (*Xanthoparmelia*). The outer/upper layer may be comprised of a cortex (except some *Cladonia* spp.) with thick walled conglutinated fungal hyphae densely adhered to one another. This layer sometimes contains pigments or other secondary metabolites that have a number of hypothesized protective functions. The middle layer of

tissue is comprised of the medulla, which is a layer of loosely woven fungal hyphae often with air spaces. Secondary metabolites that confer an external hydrophobic property, and a continuous or patchy layer of algal cells are present in the upper or outer layer of the medulla. The lower or inner layer of tissue varies tremendously depending on the taxonomy and habitat of members of the genus. The genus *Cladonia* contains an inner hollow tube with a margin of conglutinated fungal hyphae similar to a cortex. This hollow tube is diagnostic of the genus and it provides the upright podetial thallus with increased support to successfully release fungal spores into the air current for effective dispersal. The inner layers of the primary squamulose thallus are comprised of medullary hyphae. The inner layer of *Ramalina* is a continuation of medullary hyphae with no differentiated inner tissue. The lower layer of *Xanthoparmelia* species consists of a thin lower cortex to which rhizines are attached for anchorage on rock substrata.

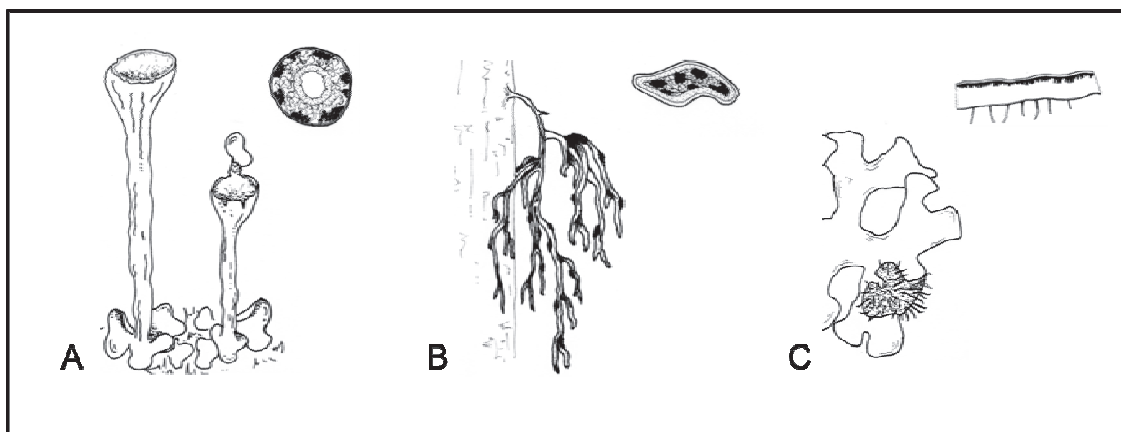


Fig. 1. Illustration of lichen growth forms for A. upright fruticose podetium and leafy squamules of *Cladonia* sp., B. pendant fruticose thallus of *Ramalina* sp. showing the single holdfast attachment to a tree, and C. foliose thallus of *Xanthoparmelia* sp. with an overturned lobe showing rhizines on the underside of the lobe. Inserts show thallus cross sections for each growth form (see text for details).

## 2.2 Regulation and production of secondary metabolites based on current knowledge of fungi

Spatial scale plays a role in interpretation of secondary metabolite production and in determination of the function of metabolites within the thallus. Concentrations of usnic acid can vary on a microscopic scale, within a thallus, by containing higher amounts in some regions of the thallus than other regions (Bjerke et al., 2005). In some species, production of a compound may not be evenly distributed, but appear to be randomly produced in specific parts of the thallus medulla. Usnic acid production was concentrated in the apothecium, pycnidium, and on the outer layer of hyphae around the algal cells of some lichens (Culberson C. F. et al., 1993; Liao et al., 2010). It is known that the cortex produces an array of compounds that are not produced by the medullary hyphae (Elix & Stocker-Worgotter, 2008). Specific functions have been studied and assigned to the compounds produced more commonly by specific tissues (see section 3.1).

Secondary compound production also varies among individuals within the geographic distribution of a single species. The concentrations of secondary compounds such as usnic

acid can vary greatly in Arctic populations of *Flavocetraria nivalis* (Bjerke et al., 2004). Intraspecifically, the chemospecies of some lichens have been observed to sort geographically (Hale, 1956; Culberson C. F. et al., 1977a; McCune, 1987; Culberson C. F. et al., 1990). Other studies have shown that these geographic patterns are not consistent (Culberson W. L. et al., 1977). Quantitative variation may be present within genetically identical species that produce biosequentially related secondary metabolites (Culberson W. L. & Culberson C. F., 1967; Culberson W. L. et al., 1977b). Various chemotypes of *Cladonia acuminata* are reported (Piercey-Normore, 2003, 2007) as well as a number of other species with chemotypes. The presence of fumarprotocetraric acid may vary even within the same location for members of the species *Cladonia arbuscula* (Piercey-Normore, 2006, 2007) and *Arctoparmelia centrifuga* (Clayden, 1992). *Cladonia uncialis* will produce squamatic acid when it is growing in coastal North America but squamatic acid is not present in specimens growing in continental North America (pers. observations). *Ramalina siliquosa* produces bands of six chemical races on the rocky coast of Wales at different distances from the oceanic spray (Culberson W. L. & Culberson C. F., 1967). Other groups of lichens also show similar habitat specific correlations such as *Cladonia chlorophaea* complex and *Parmelia bolliana* (Culberson W. L., 1970). The production of some secondary compounds, such as rhizocarpic acid, have been shown to correspond with increases in altitude (Rubio et al., 2002). However, the absence of an altitudinal correlation with usnic acid is also reported (Bjerke et al., 2004). The genus *Thamnolia* is comprised of a single species world-wide with two chemical variants, *T. vermicularis* and *T. vermicularis* var. *subuliformis*. *T. vermicularis* contains thamnolic acid and is predominant in the Antarctic. It slowly decreases in frequency across the equator in alpine habitats to the Arctic. *T. vermicularis* var. *subuliformis* contains baemycesic and squamatic acids and has the opposite trend. It is more predominant in the Arctic and decreases in frequency toward the Antarctic region. The varieties are identical in appearance but are distinguished by their secondary chemistry. With environment and geographic distribution playing such an important role in the production of secondary compounds, one might expect secondary compound production to correspond with variability of lichen phenotype.

Fungal secondary metabolites such as polyketides are produced by large multidomain enzymes, called polyketide synthases (PKS). In fungi, polyketide synthesis is catalysed by iterative Type I PKS, which are structurally and mechanistically similar to fatty acid synthases. PKSs are multidomain proteins that catalyse multiple carboxylic acid condensations (Keller et al., 2005). The fungal PKSs consist of a linear succession of domains of ketosynthetase (KS), acyl transferase (AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR), acyl carrier protein (ACP) and thioesterase (TE) (reviewed in Keller et al., 2005). The simplest fungal PKS includes the KS, AT and ACP domains, which are the minimal set of domains required for carboxylic acid condensation (Hopwood, 1997). Some fungal PKSs include KR, DH and ER domains in addition to the minimal domains, which catalyse the reduction of carbonyl groups after each cycle of condensation (Proctor et al., 2007). Fungal polyketides usually undergo modifications (reductions, oxygenations, esterifications, etc.) after they are formed. This modification is catalysed by enzymes in addition to the PKS (Proctor et al., 2007). The genes encoding the PKS and modifying enzymes are often located adjacent to each other in gene clusters. The genes in a cluster are co-regulated with transcription of all the genes being repressed or activated simultaneously

(Keller & Shwab, 2008). The polyketides produced are reduced to different degrees by the reducing domains, which are further modified by enzymes resulting in a highly diverse collection of molecules in both structure and function.

Studies of genetic regulation of fungal secondary metabolism are at an early stage (Fox & Howlett, 2008) and in lichen fungi there are few publications directly on gene expression (Brunauer et al., 2009; Chooi et al., 2008). Secondary metabolism has been studied separately with a focus on metabolite variation within and between species (Culberson W. L., 1969; Hawksworth, 1976), evolutionary hypotheses proposed for biosynthetic pathway evolution (Culberson W. L. & Culberson C. F., 1970), and phylogenomic analysis of polyketide synthase genes (Schmitt & Lumbsch, 2009; Kroken et al., 2003). The increasing number of phylogenomic analyses show that a single fungal genome may contain more than one PKS gene and each species of fungi can produce more than one polyketide or polyketide family (Proctor et al., 2007; Boustie & Grube, 2005). Each gene paralog may encode a particular polyketide product. Multiple paralogs of PKS genes have been detected (Table 1) in members of the lichen families Parmeliaceae (Opanowicz et al., 2006) and the Cladoniaceae (Armaleo et al., 2011). Six paralogs of the KS domain of PKS genes have been detected so far in the Parmeliaceae and a high number of paralogous PKS genes are expected to be present in the genomes of the Parmeliaceae because they are rich in diverse phenolic compounds. *Cladonia grayi* has been shown to contain up to 12 paralogs even though it is known to produce only two polyketides.

Paralogs may have arisen by gene duplication, mobile elements, gene fusion, or other mechanisms reviewed by Long et al. (2003). Alternative explanations for multiple, apparently non-functional, genes include horizontal gene transfer from bacteria to fungi (Schmitt & Lumbsch, 2009), horizontal gene transfer between different fungi (Khaldi et al., 2008), or adaptations triggering gains and losses through evolution (Blanco et al., 2006). Numbers of paralogs reported for lichen fungi in Table 1 are low and appear to correspond with the number of polyketides. However, these numbers are expected to be higher than reported because of recent knowledge of the numbers of paralogs present from genome sequencing projects in *Aspergillus* (Gilsenan et al., 2009), *Cladonia grayi* (Armaleo et al., 2011), and more than 200 projects in progress or completed for other ascomycetes (<http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi>). It has been reported that the number of secondary metabolite genes far exceeds the number of known compounds in an organism (Sanchez et al., 2008). For example in *Aspergillus nidulans* as many as 27 polyketide synthase genes have been identified whereas only seven secondary metabolites are known for this species and 16 paralogs are reported for *C. grayi* when only two polyketides are known to be produced by this species. Genome sequencing has also revealed unique gene clusters among various organisms, probably because an organism may have evolved to produce different secondary metabolites to best suit its biological and ecological requirements (Sanchez et al., 2008). The primer series used in Table 1 (for this study) amplified two paralogs in *Flavocetraria cucullata* and a single gene in *Alectoria ochroleuca* (Table 1). An earlier study by Opanowicz et al., (2006) reported three paralogs in both *Flavocetraria cucullata* and two paralogs in *Alectoria ochroleuca*. Variation in the number of paralogs may exist within and between populations, but more likely in this study variation may exist because of the limitation of primers available, where a larger number of paralogs might be expected to be present in all genomes.



Species	No. compounds reported	Source for no. of compounds	No. putative PKS paralogs reported	Source for no. of paralogs
<i>Alectoria ochroleuca</i>	2	Culberson C F. (1970)	2	Opanowicz et al. (2006)
<i>Alectoria ochroleuca</i>	2	This study	1	This study
<i>Aspergillus fumigatus</i>	Unknown	Not applicable	14	Nierman et al. (2005)
<i>Aspergillus nidulins</i>	7	Sanchez et al. (2008)	27	Sanchez et al. (2008)
<i>Aspergillus terreus</i>	Unknown	Not applicable	30	Nierman et al. (2005)
<i>Cetraria islandica</i>	3	Culberson C F. (1970)	3	Opanowicz et al. (2006)
<i>Cetraria islandica</i>	3	This study	3	This study
<i>Cladonia grayi</i>	2	Culberson C F. (1970)	12	Armaleo et al. (2011)
<i>Flavocetraria cucullata</i>	3	Culberson C F. (1970)	3	Opanowicz et al. (2006)
<i>Flavocetraria cucullata</i>	2	This study	2	This study
<i>Flavocetraria nivalis</i>	1	Culberson C F. (1970)	1	Opanowicz et al. (2006)
<i>Flavocetraria nivalis</i>	1	This study	1	This study
<i>Fusarium graminearum</i>	4	Hoffmeister & Keller (2007)	15	Hoffmeister & Keller (2007)
<i>Gibberella moniliformis</i>	Unknown	Not applicable	15	Schmitt et al. (2008)
<i>Hypogymnia physodes</i>	4	Culberson C F. (1970)	1	Opanowicz et al. (2006)
<i>Neurospora crassa</i>	Unknown	Not applicable	7	Galagan et al. (2003)
<i>Ramalina intermedia</i>	4	Bowler & Rundel (1974)	3	This study
<i>Ramalina farinacea</i>	7	Worgotter et al. (2004)	3	This study
<i>Tukermannopsis chlorophylla</i>	2	Culberson C F. (1970)	1	Opanowicz et al. (2006)
<i>Tukermannopsis chlorophylla</i>	1	This study	1	This study
<i>Usnea filipendula</i>	2	This study	1	This study
<i>Xanthoparmelia conspersa</i>	8	Culberson et al. (1981)	2	Opanowicz et al. (2006)
<i>Xanthoria elegans</i>	3	This study	1	This study
<i>Xanthoria elegans</i>	3	This study	1	Brunauer et al. (2009)

Table 1. Diversity of secondary metabolites and PKS paralogs expected for lichenized fungi and comparison with selected non-lichenized fungi from this study and summarized from the literature.

### 2.3 Hypothesized roles of secondary metabolite production

A fungus undergoes maximum growth when all required nutrients are available in optimal quantities and proportions. If one nutrient becomes altered, then primary metabolism is affected and fungal growth is slowed. The intermediates of primary metabolism that are no longer needed in the quantity in which they are produced, may be shifted to another pathway. It is thought that the intermediates may be used in the secondary metabolic pathways (Moore, 1998) serving as an alternative sink for the extra products of primary metabolism while allowing nutrient uptake mechanisms to continue to operate. The continued operation of primary metabolism allows continued growth but without the close integration of processes results in non-specific secondary end products maintaining effective growth (Bu'Lock, 1961 in Moore, 1998). This leaves the impression that secondary metabolism has no specific role or advantage in the fungus. However, secondary metabolism may give the fungus a selective advantage. It has been reported in many publications that secondary metabolites have a variety of functions (see below). Secondary metabolism is often triggered at a stage of fungal growth and development when one or more nutrients become limiting and growth slows down (Moore, 1998). It is thought

that when mycelial growth slows, carbohydrates are not used in growth processes and they become constant. As these carbohydrates are metabolized, secondary metabolites are produced and accumulate. The production of secondary metabolites may not serve specific functions but they may confer a selective advantage with multiple inadvertent ecological functions. Secondary metabolites may serve mainly as products of an unbalanced primary metabolism resulting from slowed growth, including metabolites that are no longer needed for growth.

Lichens and their natural products have been used for centuries in traditional medicines and are still of considerable interest as alternative treatments (Miao et al., 2001). Most natural products in lichens are small aromatic polyketides synthesized by the fungal partner in the symbiosis (Elix & Stocker-Worgotter, 2008). Polyketides are produced by a wide range of bacteria, fungi, and many plants. The finding of polyketides in forest soils, where they are exposed to harsh environmental conditions with other competing organisms, has led to the suggestion that those polyketides with antagonistic properties may structure the microbial communities in the soil (Kellner & Zak, 2009). Polyketide-producing organisms that do not live in soil may derive benefit from these compounds, which allow them to survive in discrete ecological niches by reacting to environmental variables such as light or drought, or protecting themselves from predators and parasites (Huneck, 1999). Secondary metabolites have also been hypothesized to play a role in herbivory defence, antibiotics, or as metal chelators for nutrient acquisition (Gauslaa, 2005; Lawrey, 1986, Huneck, 1999). Recently it was hypothesized that polyketides play a role in protection against oxidative stress in fungi (Luo et al., 2009; Reverberi et al., 2010) and that some metabolites such as fumarprotocetraric acid, perlatolic, and thamnolic acids contribute to the ability of lichens to tolerate acid rain events and consequences (Hauck, 2008; Hauck et al., 2009).

One explanation for high levels and numbers of secondary metabolites in lichen fungi is the slow growth of the lichen. It is known that lysergic acids are produced in the slow growing over-wintering structures (ergot) of the non-lichenized fungus *Claviceps purpurea*. The ergot in *C. purpurea* represents the slow growing overwintering stage of the fungus following the fast growing mycelial stage during the summer season where infection of the host occurs. However, lichens have no fast growing stage in comparison with *C. purpurea* and there appears to be no limitation to production of polyketides. The detoxification of primary metabolites is another hypothesis that has been proposed to explain the production of secondary metabolites. If growth of the fungus slows down, but metabolism is still very active, toxic products of primary metabolism may accumulate. The transformation of these into secondary metabolites may be one method to prevent toxic accumulation of byproducts. This hypothesis may be integrated within the first hypothesis on slow growth rates to explain the production of secondary compounds by fungi.

Regardless of the reason for secondary metabolite production (biprodukt, detoxification of primary metabolism, or leftover products after growth slows) they often elicit a function that is advantageous to survival of the lichen within its ecological niche. The advantage(s) may in part be understood by the location of the compounds within the thallus such as atranorin and usnic acid occurring more frequently in the cortical hyphae than the medullary hyphae and having a function related to photoprotection. These chemical characters are thought to be adaptive features because of their perceived ecological role. The presence or absence of polyketides has also been shown to be gained and lost multiple times in the evolution of the Parmeliaceae (Blanco et al., 2006). If the compounds allow

adaptations of lichens to their habitats and are expressed when triggered by a combination of ecological conditions (Armaleo et al., 2008), the repeated gain and loss through evolution is a result of environmentally induced expression rather than the evolutionary gain and loss of functional genes.

### **3. Observations on how specific environmental parameters influence changes in secondary metabolite production**

Production and regulation of secondary metabolites in fungi is complex with numerous environmental and developmental stimulants (Fox & Howlett, 2008) that may directly influence polyketide synthase transcription or may influence one another indirectly initiating complex signal transduction cascades. This multifaceted system makes it difficult to separate the effects of environmental parameters, developmental stages, and other factors, from one another. This section attempts to separate and describe studies involving these parameters and their effects on PKS gene expression, but concludes by integrating the significance of all parameters together.

#### **3.1 Effects of abiotic parameters: Temperature, light, pH, and humidity or drought**

Studies are beginning to accumulate that have linked environmental and culture conditions such as dehydration or aerial hyphal growth with production of secondary metabolites (Culberson C. F. & Armaleo, 1992) or exposure to strong light and drought (Stocker-Worgotter, 2001). Culberson C. F. & Armaleo (1992) showed that grayanic acid was not produced by cultured *Cladonia grayi* until aerial hyphae began to develop in the cultures. Stocker-Worgotter (2001) showed that baeomysesic and squamatic acids were not produced by *Thamnolia vermicularis* var. *subuliformis* until the culture media began to dehydrate and they were exposed to high light conditions under relatively low temperatures (15°C). These conditions reflect the conditions in the natural habitat of *Thamnolia* spp. where thalli typically grow in polar or alpine habitats exposed to cooler temperatures, under high light conditions, and dehydrating winds, that affect thallus evaporation and water content (Larson, 1979). These observations suggest that environmental parameters may trigger the production of certain compounds in some species. Numerous studies have shown a correlation between light levels and production of usnic acid (Armaleo et al., 2008; McEvoy et al., 2007a; Rundel, 1969; Bjerke et al., 2002; McEvoy et al., 2006) or other compounds (Armaleo et al., 2008; Bjerke et al., 2002; McEvoy et al., 2007b) within thalli. The amount of atranorin in the cortex of *Parmotrema hypotropum* was shown to correlate positively with the amount of yearly light reaching the thallus (Armaleo et al., 2008). In the same study norstictic acid on the medullary hyphae showed a negative correlation with yearly light levels. The authors suggested that the higher quantities of medullary compound with lower light levels may be an adaptive link between the need for production of these hydrophobic compounds when water potential increases within the thallus (from low light levels) to allow efficient carbon dioxide diffusion to the algae. As light levels decrease the water potential in the thallus increases and therefore the need for hydrophobic compounds also increases. Based on the difference in polyketide production between the medulla and the cortex with different environmental triggers for different metabolites, Armaleo et al. (2008) proposed that two different pathways with two different sets of genes were responsible for production of these compounds. This is a plausible explanation since a larger number of

paralogs are present compared with the number of polyketides actually produced by many species (Table 1). On the other hand, other studies did not report a relationship between light and polyketide production (Fahselt, 1981; Hamada, 1991; Bjerke et al., 2004).

Growth media and available nutrients may influence the secondary metabolites produced by lichen fungi. The presence of gene clusters for production of a potentially larger variety of polyketides than is produced within each species, is supported by the work of Brunauer et al. (2007). Cultured lichen fungi have been shown to produce secondary metabolites that are not present in the naturally collected lichen. The authors offered two explanations for this 1) the lichen fungus may adapt to the conditions in the artificial media triggering induction of an alternate pathway, and 2) enzyme activity may be shifted by availability of certain trace elements, carbohydrates, or unusual pH of the medium. These external factors may affect expression of genes involved in regulation of secondary metabolites or on the genes directly involved in metabolite production. For example, the transcription factor, *VeA* (velvet family of proteins) is regulated by light levels and has been reported to repress penicillin biosynthesis (Sprote & Brakhage, 2007). The velvet complex subunits coordinate cell development and secondary metabolism in fungi (Bayram & Braus, 2011). These proteins are reported to be conserved among several species of fungi including *Aspergillus* spp., *Neurospora crassa*, *Acremonium chrysogenum*, and *Fusarium verticilloides* (Bayram et al., 2008; Dreyer et al., 2007; Kumar et al., 2010).

The effect of pH on gene expression in fungi is reviewed by Penalva & Arst (2002). Regulation of gene expression by pH, is thought to be mediated by a transcription factor (*pacC*). Higher pH, resulting in alkaline conditions that mimic *PacC* mutations, causes an increased production of penicillin in *Aspergillus nidulins* and in *Penicillium chrysogenum*. Carbon source also influences penicillin production where some sources will repress the effects of an alkaline pH on penicillin production (Suarez & Penalva, 1996). On the other hand, acidic growth conditions promote production of aflatoxins in *Aspergillus parasiticus* and *A. nidulins* (Keller et al., 1997). If pH regulation is an important determinant in plant pathogenicity (Penalva & Arst, 2002) and in sclerotial development in *Schlerotinia sclerotiorum* (Rollins et al., 2001), then it might also be expected to influence the controlled parasitic interaction (Ahmadjian & Jacobs, 1981) between lichen fungi and algae and the production of polyketides in fungi linking observations on environmental parameters and developmental changes in culture. For example, Stocker-Worgotter (2001) showed that species within the genera *Umbilicaria* and *Lassalia* produce their diagnostic secondary metabolites only when grown on an acidic medium (potato-dextrose-agar). Species of *Umbilicaria* and *Lassalia* (*U. mammulata*, *L. papulosa*) typically grow on acidic granite rocks and have not been reported on any other substratum, suggesting that the pH of the substratum may also influence PKS gene expression in these species. However, other factors specific to the rock habitat may also influence PKS gene expression such as mineral composition of the rock or the presence of other organisms. The significance of the substratum to lichen fungi is reviewed by Brodo (1973). The bark of different tree species and the diversity of rock types can have different pHs, nutrients, and water holding capacity making them suitable for some species but not for other species. Lichens growing under other conditions have also shown changes in production of secondary metabolites. The quantity of depsides was highest in *Ramalina siliquosa* cultures when the pH was 6.5 and incubation temperature was 15C (Hamada, 1989). Hamada (1982) also showed that the depsidone, salazinic acid, was highest in *R. siliquosa* when the annual mean temperature was approximately 17C.

Microorganisms capable of growing over a wide range of pH have gene expression under control of the pH of their growth medium (Penalva & Arst, 2002). It has been found that the signals generated in response to environmental conditions are relayed through proteins including CreA for carbon, AreA for nitrogen and PacC for pH signaling. These proteins may have positive or negative effects on metabolite production. With regard to two *Cladonia* species, *C. pocillum* and *C. pyxidata*, it has been suggested that pH is the driving environmental factor responsible for the morphological difference between the two species (Gilbert, 1977; Kotelko & Piercey-Normore, 2010). Secondary metabolite production varies among members of the *Cladonia chlorophaea* complex, which have been found to share virtually identical morphologies but different secondary metabolites (Culberson C. F. et al., 1988; Culberson W. L., 1986). *Cladonia grayi* and *C. merochlorophaea* grow at lower pH than *C. chlorophaea sensu stricto* or other members of the complex. If pH is regulating production of polyketides that are diagnostic among these chemospecies, then the species complex represents the range of versatility the species has acquired to adapt to changing environmental conditions.

### 3.2 Carbon source may influence the secondary metabolite pathway

The lichen association involves a fungal partner and an autotrophic partner, a green alga or cyanobacterium. The carbon source provided by the photobiont has been shown to have an impact on the secondary metabolism of the mycobiont. The more common of these green algal photobionts are in the genera *Trebouxia*, *Myrmecia* and *Coccomyxa*. These algae are thought to produce the sugar ribitol, and *Trentepohlia* produces erythritol (Honegger, 2009). This sugar alcohol is transferred to the mycobiont where it is metabolized into mannitol. This is an irreversible reaction where mannitol becomes unavailable to the fungal partner. Secondary compounds produced by *Xanthoria elegans* were strongly induced by the presence of mannitol with negligible effects by ribitol (Brunauer et al., 2007). An early study of nutritional implications in *Pseudevernia furfuracea* examined the production of polyketides after applying different carbon sources to natural thalli incubated in a moist water-filled chamber (Garcia-Junceda et al., 1987). Production of atranorin is not enhanced by glucose but it is enhanced by remobilization of storage carbohydrates to produce acetate as the starting intermediate. Production of lecanoric acid is enhanced by glucose and may be a result of the catabolism of mannitol or glucose. The production of atranorin was favoured when catabolism of mannitol or glucose was repressed by a synthetic inhibitor. Hamada et al. (1996) measured the yield of secondary metabolites from nine species of lichen fungi and compared media supplemented with 0.4% and 10% sucrose. All species showed an increase in metabolite production in the 10% sucrose media. It follows that if ecological conditions are varied (as in the microenvironment of a lichen thallus) and/or algal physiology is varied (Hoyo et al., 2011), then a combination of different polyketides may be produced within a single thallus by the availability of different types of starting units.

It has been reported that the availability and type of carbon and nitrogen source affect polyketide production (Keller et al., 2002). As the sole carbon source, sugars like glucose, sucrose or sorbitol, have been found to support high aflatoxin production along with increased fungal growth and sporulation. On the other hand, peptones and more complex sugars such as galactose, xylose, lactose and mannose do not support aflatoxin production. Studies on *Aspergillus* species have shown different effects of nitrogen sources in growth medium on aflatoxin and sterigmatocystin production (Keller et al., 2002). Keller et al. (1997) reported an increased amount of sterigmatocystin and aflatoxin production in ammonia-based medium and a decreased amount in nitrate-based medium.

The ability of lichens to adapt to changes in light levels, depends on the stability of thylakoid membranes, which protect them from attack by reactive oxygen species (Berkelmans & van Oppen, 2006). Therefore, the choice of algal partner would depend largely on the habitat conditions in which the developing lichen thallus is found. If the choice of alga depends on habitat conditions, and different algae produce different starting units, then the polyketide production would also depend on the habitat conditions and the alga. For lichen thalli that are thought to contain multiple algae simultaneously (Piercey-Normore, 2006; Hoyo et al., 2011), the predominant alga would provide the majority of starting carbohydrates, with a specific combination of carbohydrates available for different biosynthetic pathways.

### 3.3 Environmental cues affecting secondary metabolite production

The development of non-lichenized fungi and secondary metabolite production appears to be coordinated (reviewed in Schwab & Keller, 2008; Bennett & Ciegler, 1983). Morphogenesis of the macrolichens (fruticose and foliose) is highly complex compared with crustose lichens and the vegetative phase of many non-lichenized fungi. The macrolichen thallus is comprised of differentiated “tissues” arranged in layers (see section 2) that often produce different metabolites (see Honegger (2008) for a review of morphogenesis in lichens). Thallus development in lichens has been examined using microscopy (Honegger, 1990; 1993; Joneson & Lutzoni, 2009) and recently a study has described a number of genes that correlate with symbiont recognition and early thallus development (Joneson et al., 2011). Observations of cultures of lichen-forming fungi have suggested that thallus development may be involved in production of secondary metabolites. For example, a major compound umbilicic acid produced by *Umbilicaria mammulata* was produced by cultures of *U. mammulata* only after lobe-like structures were formed in dehydrating medium. Similarly, cultures of *Cladonia crinita* produced its major substance, fumarprotocetraric acid and its satellite substances only after podetial structures were formed (Stocker-Worgotter, 2001). Species of *Ramalina* produced secondary metabolites only after layers of mycelia became differentiated (Stocker-Worgotter, 2001). As further research is conducted on development in lichens it is expected that more links between development and production of secondary metabolites will become evident.

Regulation of fungal secondary metabolism to some extent is thought to depend on the chromosomal organization of biosynthetic genes. A global transcription factor, which is encoded by genes that are unlinked with biosynthetic gene clusters, may also control the production of secondary metabolism (Fox & Howlett, 2008). Genes encoding global transcription factors regulate multiple physiological processes and are thought to respond to pH, temperature, and nutrients. Signal cascades that regulate fungal morphogenesis are necessary for fungi to sense environmental change and adapt to those changes. These signaling cascades have been studied more intensely with reference to fungi that are human pathogens (Shapiro et al., 2011). Environmental cues may initiate a shift between morphological growth forms that is necessary for survival of the fungus but causes disease in the host. Studies on mycotoxin production and regulation of the genes responsible for mycotoxin production in species of *Aspergillus* have shown that the gene, *veA*, regulates production of three aflatoxin biosynthetic genes and another toxin in *A. flavus* (Duran et al., 2007). *veA* (velvet A) has also been shown to regulate penicillin production in *A. nidulans* (Kato et al., 2003). The same gene, *veA*, has also been reported to be involved with regulation of aflatoxin production in *A. parasiticus*, suggesting that the regulatory mechanism may be

conserved among species of *Aspergillus* (Duran et al., 2007). Another gene, *laeA*, has also been shown to regulate expression of biosynthetic gene clusters in species of *Aspergillus* (Bok & Keller, 2004; Keller et al., 2005; Fox & Howlett, 2008). In addition, it has been shown that *laeA* negatively affects the regulation of *veA* (Kale et al., 2008). The loss of *laeA* results in gene silencing (Bok et al., 2006b; Perrin et al., 2007).

#### **4. Variation in secondary metabolite production may change along the geographic distribution of a species – An empirical study**

##### **4.1 Background to the study**

The most widely studied secondary metabolite produced by lichen-forming fungi is usnic acid, a cortical compound that absorbs UV light. Seasonal and geographic variation has been shown to occur in populations of the usnic acid producing lichens *Flavocetraria nivalis* and *Nephroma arcticum* in Arctic and Antarctic regions (Bjerke et al., 2004, 2005; McEvoy et al., 2007). These are regions that are highly exposed to strong UV light, desiccating winds, and harsh temperature changes. Other secondary metabolites examined on large geographic scales include alectoronic acid, a-collatolic acid, and atranorin produced by *Tephromela atra*, a crustose lichen that grows on tree bark. That study showed a significant variation between localities (Hesbacher et al., 1996) but no relationship with tissue age, grazing, or reproductive strategy. In a study on the *Cladonia chlorophaea* complex the levels of fumarprotocetraric acid increased from coastal North Carolina to the Appalachian mountains in the interior of the state (Culberson C. F. et al., 1977a). The authors interpreted this geographical gradient of higher levels of fumarprotocetraric acid in mountain populations, as providing protection against harsher environmental conditions in the mountains than in the coastal area. If environment influences secondary metabolite production, then changes should be observed along a gradient of environmental conditions over a species distribution.

Although Hesbacher et al. (1996) showed that thallus age has no effect on secondary compound concentrations for atranorin and alectoronic acid, Golojuch & Lawrey (1988) showed that concentrations of vulpinic and pinastric acids are higher in younger lichens. Bjerke et al. (2002) showed that the most exposed sections of the thallus (such as the tips of *C. mitis*) accumulate greater concentrations of secondary compounds than less exposed sections of the thallus. However, it is not known if the metabolites are actively produced in the exposed and younger tips, or if the metabolites are lost in the older parts of the thallus as the thallus ages and the fungal tissue degrades, giving the appearance that the tips have more metabolites. High concentrations of secondary metabolites were reported in the sexual and asexual reproductive bodies rather than the somatic (vegetative) lichen tissue (Liao et al., 2010; Culberson C. F. et al., 1993). Geographic and intrathalline variation suggest a functional role for these metabolites that has been described in a theory called optimal defence theory (ODT). The theory states that plants and fungi will allocate secondary compounds where they are most beneficial to the organism (Hyvärinen et al., 2000), implying an active production of secondary metabolites, which is contrary to the current theories of secondary metabolite production (see section 2.3). The inconsistency in findings to explain geographic trends and the intrathalline variation in secondary metabolite production may be addressed by increasing sample size and geographic distance to capture the population variation and prevent saturation of larger scale geographic variation. Relationships between metabolite production and geographic location should be evident in a north – south direction because of differences in climate. It would also be expected that the

production of intrathalline metabolites would be coordinated because of their hypothesized function regarding environmental changes.

The objectives of this study were 1) to test the relationship between the quantity of secondary metabolite produced and geographic location over latitudinal range, and 2) to test the relationship between metabolites produced within a thallus to determine whether production of one compound is dependent on production of another compound.

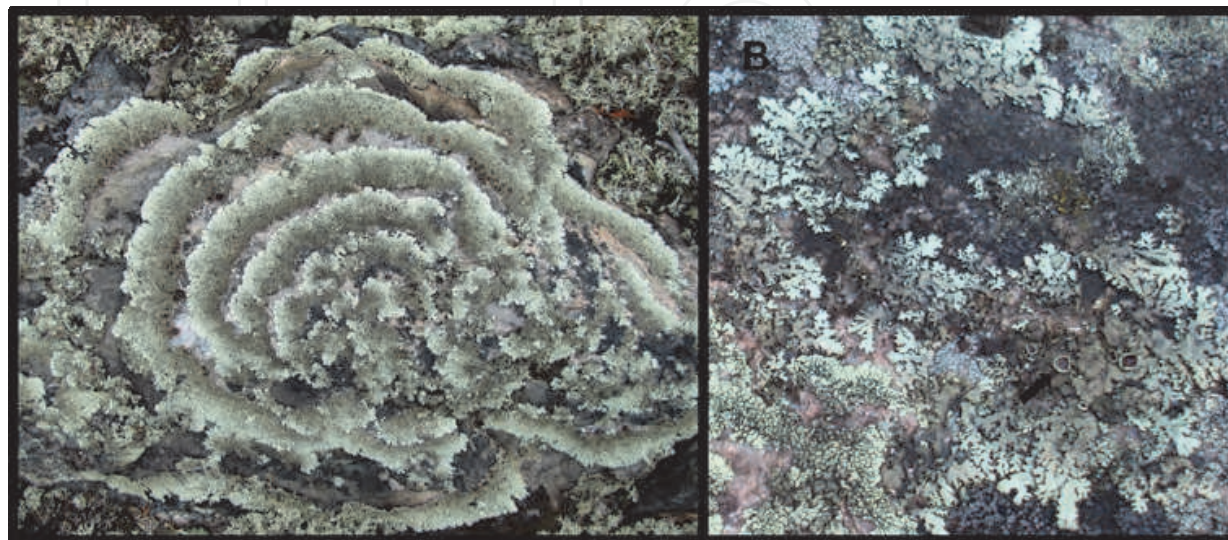


Fig. 2. Shield lichens inhabit exposed rock of the Precambrian shield in North America showing A. *Arctoparmelia centrifuga*, a yellow-green foliose thallus with concentric rings of growth, and B. *Xanthoparmelia viriduloumbrina*, yellow-green foliose thallus with brown apothecia (arrow) and wide lobes. Photo of *A. centrifuga* by T. Booth.

## 4.2 Methods

### 4.2.1 Species and sampling strategy

Two species were chosen for this experiment, *Arctoparmelia centrifuga* and *Xanthoparmelia viriduloumbrina* (Fig. 2). Both lichen species are saxicolous, foliose lichens that grow on the Precambrian shield in North America belonging to the family, Parmeliaceae (Ascomycotina). Originally part of the *Xanthoparmelia* genus, *Arctoparmelia* was reclassified as a separate genus (Hale, 1986) and currently both genera are in the Parmeliaceae. *Arctoparmelia centrifuga* is a yellow-green foliose lichen that grows in concentric rings (Fig. 2A). The center of the ringed pattern discolours with age, the source of its specific epithet ('retreat from centre'). The thallus lacks a lower cortex, appearing white underneath, and is found growing on exposed rock. The major compounds produced by *A. centrifuga* include atranorin, usnic acid, alectoronic acid, and an unidentified aliphatic acid (Culberson C. F., 1969). *Xanthoparmelia viriduloumbrina* is a yellow-green foliose lichen with straplike lobes. The underside is brown, with brown rhizines. Maculae, which are absent from this species (Lendemer, 2005), are discolourations on the thallus surface caused by the absence of the photobiont beneath the cortex. The lichen grows on exposed rocks and a morphologically similar species *X. stenophylla* has a pH tolerance ranging between 4.1 and 7.0 (Hauck & Jürgens, 2008). The secondary compounds produced by *X. viriduloumbrina* include usnic acid, salazinic acid, consalazinic acid and an accessory compound, lobaric acid (Hale, 1990). Both species, *X. viriduloumbrina* and *A. centrifuga*, reproduce sexually and the algal partner is *Trebouxia*.



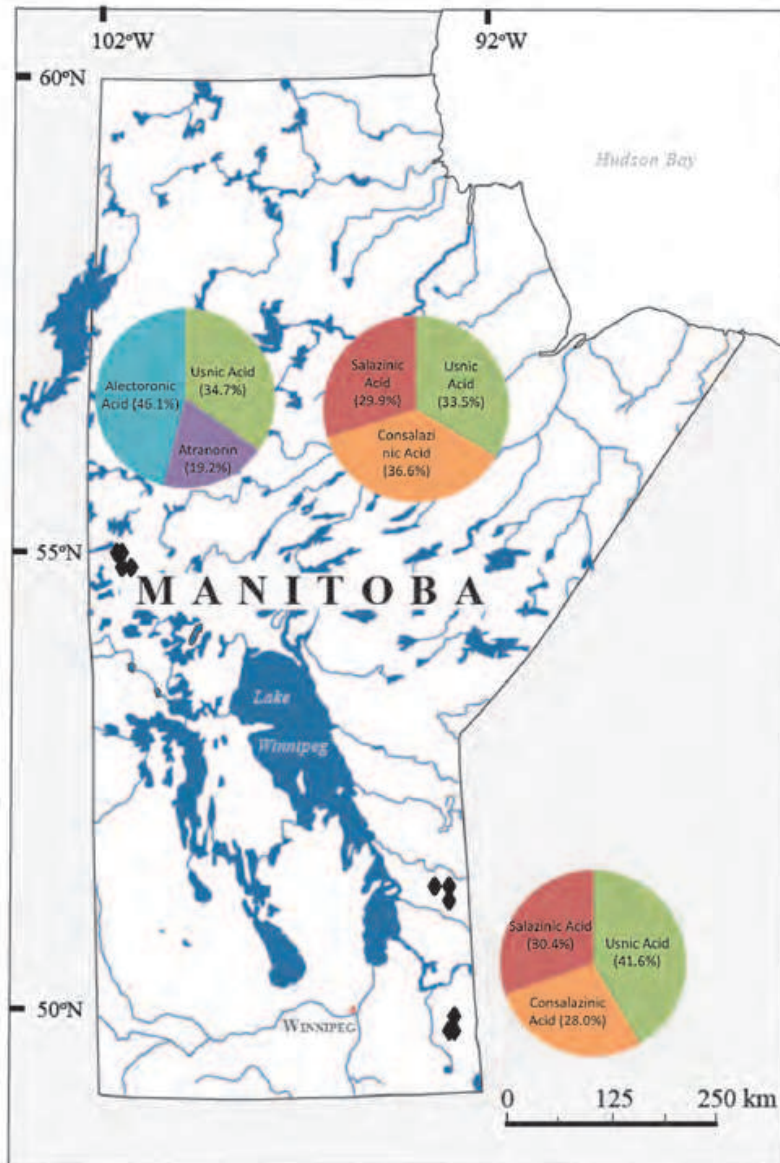


Fig. 3. Map of Manitoba, Canada, showing latitude (left) and longitude (top), location of collection sites (black diamonds), and proportion of secondary metabolites from *X. viridulombrina* (usnic, salazinic and consalazinic acids) in northern and southern sites, and proportion of secondary metabolites from *A. centrifuga* (usnic, alectoronic acids, and atranorin) in northern sites. (Map was provided by R. Lastra).

Sampling for both species occurred along a northwest-southeast transect covering a distance of approximately 700km along the Precambrian shield in the province of Manitoba (Fig. 3). The Precambrian Shield extends northwest-southeast along on the eastern shore of Lake Winnipeg. Twenty-nine transects measuring 40m in length and evenly spaced 1m x 1m quadrats were placed every 10m for sample collection. Vouchers were collected and deposited in the University of Manitoba Herbarium (WIN-C). Ninety-five samples of *A. centrifuga* were collected and 109 samples of *Xanthoparmelia viridulombrina* were collected in the summer of 2010.

#### 4.2.2 Quantitative Thin Layer Chromatography

Portions of young thallus lobes weighing 5mg DW (Mettler PM460 DeltaRange) were placed in 1.5 mL Eppendorf tubes. Extraction of secondary compounds was done following Culberson C. F. (1972) with 3.3mL acetone washes and three incubations for 5, 5, and 10 minutes. Acetone extracted samples were processed using thin layer chromatography (TLC; Orange et al., 2001; Culberson C. F., 1972, 1974). The protocol was standardized by placing 46uL on each spot of the silica-coated glass TLC plate (Fisher Scientific, Ottawa, Ontario, Canada) and placed in solvent A (toluene 185 mL: dioxane 45 mL: glacial acetic acid 5mL) for migration of the solvent to the top of the plate. After drying, pictures were taken of each plate for short-wave (254 nm) and long-wave (365 nm) ultraviolet light. These photos were used to quantify the secondary compound. The plates were then sprayed with 10% sulphuric acid and baked in an 80C oven until colours developed (10 minutes). Secondary metabolites were determined by comparison with known characteristics (Culberson C. F., unpub; Orange et al., 2001), by using a standard for Rf comparison, and an usnic acid commercial standard (ChromaDex, Santa Ana, CA).

Secondary compounds were quantified using Digimizer (Version 4.0.0. MedCalc Software, Mariakerke, Belgium, 2005-2011). Photos of TLC plates taken under short and long wave UV light were used. Three compounds for each species were quantified. Two measures were used to arrive at compound quantity (in pixels). The first was the area of the spot. The second measure was brightness or average intensity under UV light. This was the average pixel value on a scale between 0 (black) and 1 (white). The purpose of the brightness quantity was to account for the thickness of the silica plate. At 250 µm thick, greater saturation of the extract could occur in an area on the plate. The two values of spot area and brightness were multiplied together to get a total pixel value for the individual compound. Usnic acid, atranorin, salazinic acid and consalazinic acid were all quantified under short-wave ultraviolet light and were analyzed by inverting the quenched spots on the plate to allow the pixel area to be determined. Pixels in the dark quenched spots cannot be determined. Aleatoronic acid was quantified by its fluorescence under long-wave ultraviolet light (365nm). No inversion was necessary because brightness values were already positive.

#### 4.2.3 Data analysis

Univariate statistics were done using JMP® (Version 8.0.1 SAS Institute Inc., Cary, NC, 2009). Quantities of secondary compounds were log transformed and plotted against the independent variable, latitude for northern sites, southern sites, and all sites for *X. viriduloumbrina*; and for northern sites only for *A. centrifuga*. Spearman's correlation was used to measure the relationship between compound quantities and latitude. Four correlations were calculated, one for *A. centrifuga* and three for *X. viriduloumbrina*. Pairwise regression analyses between compounds for each species were done. P values were recorded for the significant relationships. Pie charts were created to show the proportion of secondary compounds in northern and southern sites for each species based on the average log transformed pixel quantity for each secondary compound.

#### 4.3 Results

*Xanthoparmelia viriduloumbrina* was collected in all locations of both northern and southern sites. *A. centrifuga* was collected only in northern sites because the species was

absent from the southern sites. *Xanthoparmelia viriduloumbrina* consistently produced three compounds (usnic, consalazinic and salazinic acids) and occasionally one accessory compound, lobaric acid with up to two unknown compounds. *A. centrifuga* consistently produced three compounds (usnic and alectoronic acids and atranorin) and up to four unknown compounds (Fig. 4).

The proportion of secondary compounds within *X. viriduloumbrina* was relatively similar between the three collection sites (Fig. 2). The pie-charts showed the cortical compound usnic acid was the most abundant compound overall and within the southern site. The medullary compound consalazinic acid had the highest proportion in the northern site. Alectoronic acid was the largest proportion of the three compounds for *A. centrifuga*.

Secondary metabolites produced by *Xanthoparmelia viriduloumbrina* showed four significant correlations with latitude. Spearman's correlations were conducted for *Xanthoparmelia viriduloumbrina* on each secondary compound, usnic acid, consalazinic acid and salazinic acid, across the entire study area (n=109; 5 degrees latitude). Salazinic acid decreased significantly from the southern to the northern collection sites (Spearman's rho = -0.3330 and p = 0.0004) (Fig. 5A). There were no significant trends for usnic acid (p = 0.1321) or consalazinic acid (p = 0.5720) for all collections sites.

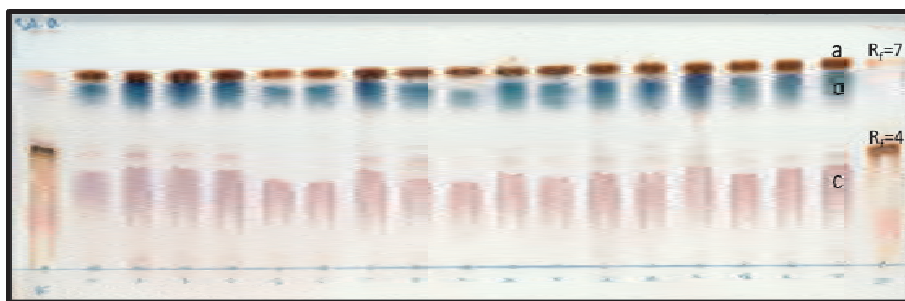


Fig. 4. Image of a developed TLC plate showing 17 polyketide profiles for *Arctoparmelia centrifuga*. Each profile contains a yellow-brown spot at Rf class of 7 (a) determined to be atranorin, a blue-green spot at Rf class of 6 (b) determined to be usnic acid, and a peach spot at Rf class of 3 (c) determined to be alectoronic acid. Profiles shown on the far left and right are the reference profiles for Rf classes 4 and 7.

Similarly, Spearman's correlation analyses in the northern collection sites (n=35; 2 degrees latitude) produced two significant results. Salazinic and consalazinic acids increased significantly in *Xanthoparmelia viriduloumbrina* from southern to northern sites even within a 2 degree latitude (salazinic acid; Spearman's rho = 0.7124 and p = 0.0001) (consalazinic acid; Spearman's rho = 0.3523 and p = 0.0379) (Fig. 5B and C). Usnic acid produced no significant trend (p = 0.3364). Spearman's correlations were also conducted for *Xanthoparmelia viriduloumbrina* in the southern collection sites (n=74; 2 degrees latitude) where salazinic acid decreased significantly from southern to northern sites (Spearman's rho = -0.3371 and p = 0.0033) (Fig. 5D). Usnic acid and consalazinic acid showed no significant correlation (rho = 0.2627; p = 0.1770 respectively) in the southern sites. However, metabolites produced by *A. centrifuga* showed no significant correlations with latitude. Analyses with *A. centrifuga* could only be conducted for northern sites because the species was absent from the southern sites.

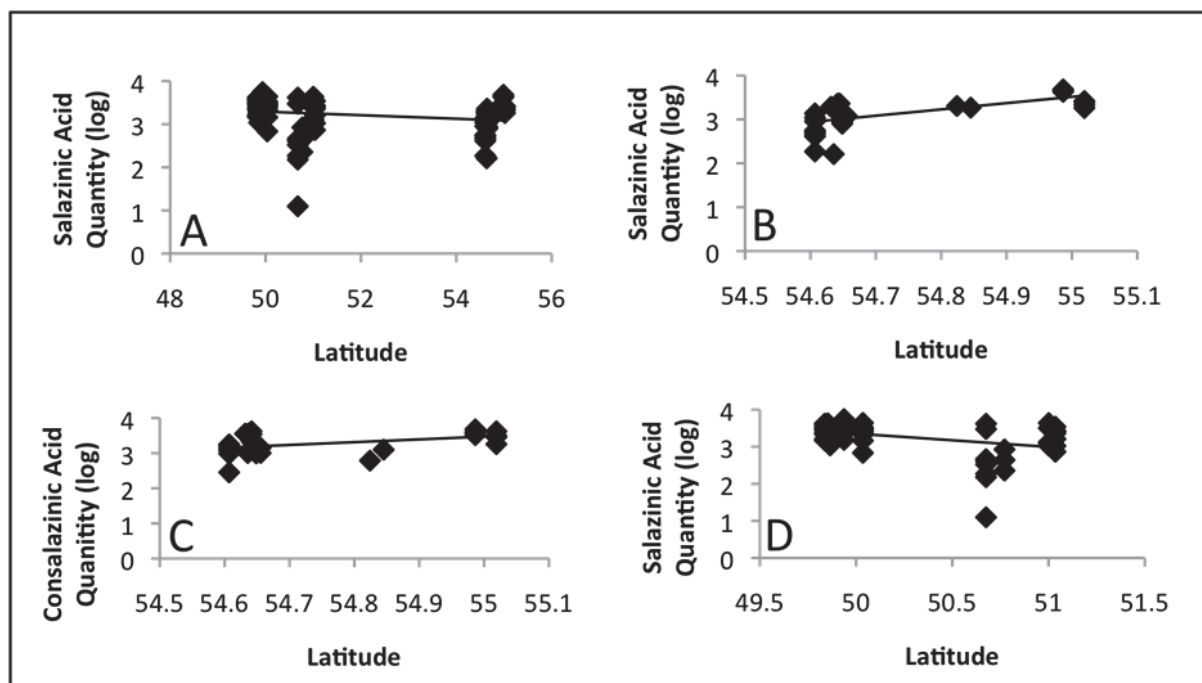


Fig. 5. Relationship between log transformed quantified secondary metabolites produced by *Xanthoparmelia viriduloumbrina* and latitude for A. salazinic acid from specimens collected from all sites; B. salazinic acid from specimens collected only in northern sites; C. consalazinic acid from specimens collected only in northern sites; and D. salazinic acid from specimens collected only in southern sites.

Pairwise regression analyses were conducted between the three metabolites produced by each species to determine whether the production of one compound is related to the production of another compound. Within *Xanthoparmelia viriduloumbrina* regressions between secondary compounds were significant between all three combinations. The relationship between usnic acid and consalazinic acid, between consalazinic acid and salazinic acid, and between usnic acid and salazinic acid were all significant at  $p=0.0001$  (Fig. 6A, B, and C). The regression analyses between secondary compounds produced by *Arctoparmelia centrifuga* showed one significant relationship. Changes in the quantity of usnic acid and atranorin were significant at  $p=0.0001$  (Fig. 6D). Other combinations showed no significant relationship.

#### 4.4 Discussion

##### 4.4.1 Shield lichens adapt to different habitats

The significant decrease in the quantity of salazinic acid from southern to northern latitudes (Fig. 5) are great enough to suggest that *X. viriduloumbrina* is responding to environmental changes. Hamada (1982) reported that dark rock colours, higher temperatures, and southern exposures result in larger quantities of salazinic acid in thalli of *R. siliquosa*. The average mean temperature in the northern sites for 2006 was 1.7°C lower than that in the southern sites (National Climate Data and Information Archive, 2011). If the overall difference in salazinic acid across all sites reflects a large scale response to temperature, then the significant increase in levels of salazinic acid within the northern sites, suggests a response to more localized environmental parameters as the mean annual temperature would not differ as significantly as it would across all sites, in such a small area. Salazinic acid has also

been shown to change with other environmental parameters. The production of salazinic acid is dependent on osmotic pressure and may increase with increased sucrose and low nitrogen levels (Hamanda & Miyagawa, 1995; Behera & Makhija, 2001). The increased production of salazinic acid in low nitrogen and high sucrose culture conditions with *Bulbothrix setschwanensis* (Behera & Makhija, 2001) supports the finding that salazinic acid is produced only in cultures with the algal partner of *B. setschwanensis* present (Behera et al., 2000). The quantity of salazinic acid decreased initially under ozone stress and then increased in what was thought to be stress induced defence (MacGillvray & Helleur, 2001). One explanation is that the compound has antioxidant properties (Amo de Paz et al., 2010) having potential use in treatment of Alzheimer's and Parkinson's diseases (Amo de Paz et al., 2010), and a modified structure of the molecule may be cytotoxic to some tumor cells (Micheletti et al., 2009). The similar trend in consalazinic acid could be explained by the increasing quantity of salazinic acid. The relationship between consalazinic acid and salazinic acid has been known for a long time since they are quite similar chemically and consalazinic acid is considered a co-metabolite of salazinic acid (O'Donovan et al., 1980).

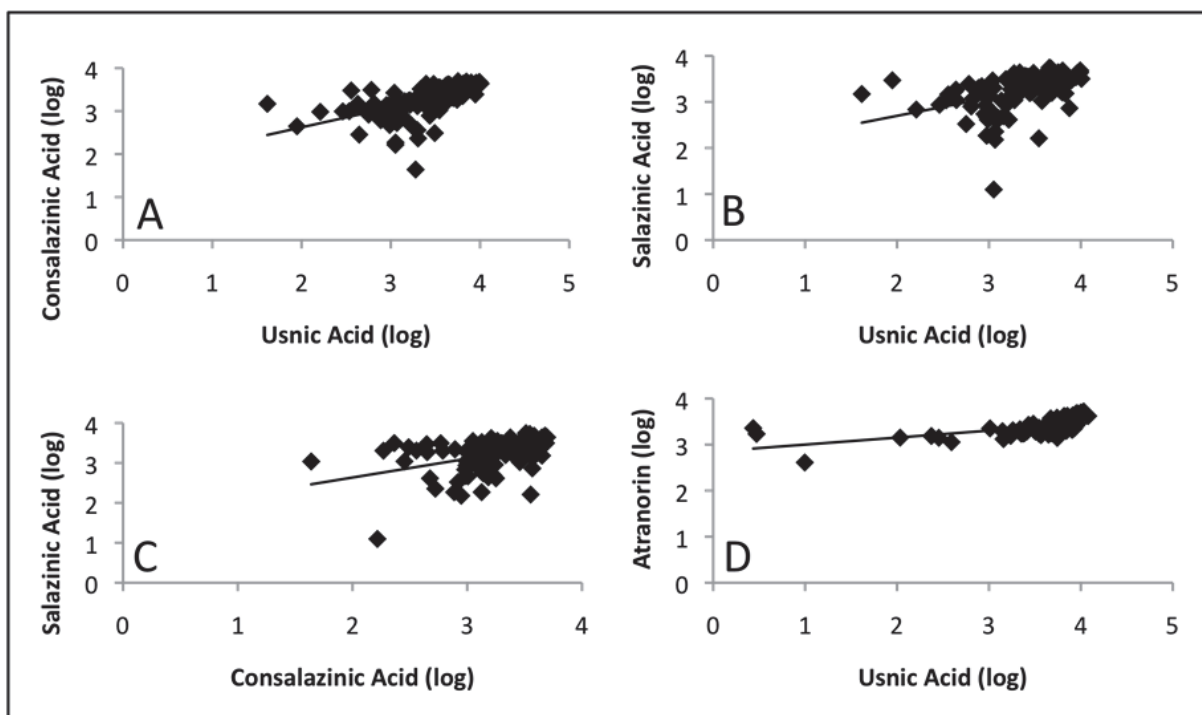


Fig. 6. Pairwise regression analysis of log transformed quantities of secondary metabolites produced by each species showing significant linear relationships between A. consalazinic and usnic acid in *X. viriduloumbrina* ( $y=1.69+0.46x$ ); B. salazinic and usnic acids in *X. viriduloumbrina* ( $y=1.91+0.39x$ ) C. salazinic and consalazinic acids in *X. viriduloumbrina* ( $y=1.69+0.47x$ ); and D. artranorin and usnic acid in *A. centrifuga* ( $y=2.84+0.15x$ ). All other comparisons were not significant.

#### 4.4.2 Absence of expected relationships suggest localized adaptation

The absence of a relationship between the cortical secondary metabolites and geographic location was unexpected since the literature contains numerous examples of changes in usnic acid or atranorin with light levels. However, the major photoprotective function that

has been assigned to usnic acid and atranorin was not accounted for in this study. The 5 degree latitude difference in this study resulted in a temperature and daylength difference. But the change in UV light levels was not likely to be sufficient to produce changes in cortical compounds as was evident for McEvoy et al. (2006) and Bjerke et al. (2002), where increased light gradients were measured from forested locations to exposed alpine locations. In this study the habitat was relatively constant with open jack pine bedrock of the Precambrian shield regardless of whether the location was in the northern or southern regions. The literature on usnic acid is large and includes environmental science as well as medical applications (Cocchietto et al., 2002; Ingólfssdóttir, 2002) suggesting that the functions of usnic acid are numerous and diverse.

Similarly, the bioactive function assigned to the medullary metabolite, alectoronic acid, is not related to habitat. Alectoronic acid concentration was highest in heavily grazed thalli and lowest in thalli with the lowest level of grazing damage by snails (Hesbacher et al., 1996) but the differences were not significant. These differences were however, correlated with geographic distance within 10 km. Alectoronic acid is also known to have antimicrobial properties (Gollapudi et al., 1994) suggesting that levels of alectoronic acid may change in response to the presence of other living organisms or damage they inflict on the lichen thallus. Changes in production of alectoronic acid are not dependent on thallus age and like many secondary compounds, will exhibit intrathalline variation (Hesbacher et al., 1996). Localized production of usnic and alectoronic acids may occur depending on light levels or microbial/herbivore activity that was not measured in this study.

#### **4.4.3 Environmental change influences production of metabolites in a coordinated fashion**

Since the proportion of metabolites for each of the northern and southern regions was similar (Fig. 3), some of them showed a significant relationship with one another (Fig. 6). Environmental changes may be coordinating the production of the metabolites. The coordinated production of usnic acid with salazinic acid is consistent with the results of Valencia-Islas et al. (2007) and Amo de Paz et al. (2010), who show that usnic acid and salazinic acid share similar effects due to air pollution and antioxidant behavior. The significant relationship between usnic acid and consalazinic acid is also expected. If consalazinic is a co-metabolite of salazinic acid (O'Donovan et al., 1980), and usnic increases significantly with salazinic (Fig. 6B), then it follows that consalazinic would also increase with usnic.

The coordinated production of two cortical compounds, usnic acid and atranorin, is also a significant relationship. These metabolites are not biogenically related and therefore the coordinated production cannot be explained as pathway intermediates. However, the extensive literature describing their photoprotective properties and pollution sensitivities suggest that similar environmental features may influence both metabolites. Valencia-Islas et al., (2007) reported that concentrations of atranorin were greater than those of usnic acid, which were greater than those of salazinic acid. Salazinic acid increased at the expense of chloratranorin and atranorin suggesting the same starting carbohydrates were used for production of both compounds; hence, the pathways were in competition for the starting carbohydrates. The relationship between salazinic and consalazinic acids could be explained by the biogenic relationship. However, the relationship between usnic acid and atranorin, produced from different pathways, do not have a biogenic relationship but may be explained by environmental changes.

## 5. Significance of secondary metabolite production with respect to on-going climate change

A number of environmental predictions of future global climate conditions are predicted in the fourth assessment of the United Nations Intergovernmental Panel on Climate Change (2007). The outlook included an increase in average temperature; an increase in intensity and length of droughts; an increase in global water vapour, evaporation and precipitation rates which will cause increasing tropical precipitation and decreasing subtropic precipitation; an increase in sea levels from glacial melt; and anthropogenic carbon dioxide production will further increase atmospheric carbon dioxide levels (Meehl et al., 2007). Most of these changes will have implications on the future adaptability and secondary metabolite production of lichen species. These secondary metabolites protect against increasing environmental stresses such as light exposure, water potential changes, microbial and herbivore interactions, and other changes associated with changes in environmental conditions.

Increases in temperature may require the increase of secondary metabolites such as salazinic acid to mitigate the effects of higher temperatures on lichen biology. The relationship between temperature and production of salazinic acid is thought to be related to the effect of hydrophobic properties of the metabolite. The metabolite, being produced by medullary hyphae, would ensure a hydrophobic environment to optimize carbon dioxide transfer to the algal cells. A higher temperature increases the water potential of the thallus and more need for hydrophobic conditions to allow optimal carbon dioxide exchange between air spaces and algal cells. However, a higher thallus temperature may also promote the initiation of transferring one algal partner for another partner. Depending on the taxonomic extent of different algal partners this may invoke different carbohydrate starting units or trigger a different biosynthetic pathway for secondary metabolite production. The predicted increases in average annual temperature in northern geographic areas may also promote temperate species of lichens to move further north into previously uninhabitable environments. Simultaneously, this may cause a more northerly movement of lichens that are adapted to or can tolerate cooler environments. The effects on epiphytic lichens will also be significant based on the availability of host tree species and how well the host trees adapt to climate change. Cool temperature plant species that do not adapt well to warmer temperatures may become fewer in number in northern regions. Fewer plant species may reduce the availability of suitable habitat for lichens specialized to growing on the bark of specific tree species. Species of lichens that are generalists, colonizing a number of different tree species or other substrata, will be better adapted to environmental changes than specialist species, because previously lost tree hosts may be replaced by succeeding species of plant host.

Droughts will further affect the plant community. Plants that are not drought resistant may become fewer in number and replaced by drought resistant species. Extreme drought may cause further loss of plants and increase soil erosion. Such a situation would create the opportunity for terricolous lichen expansion but perhaps on a scale too slow to prevent significant losses. Under the scenario of increased degree and frequency of drought, it might be expected that there will be an increased production of mineral chelating compounds and hydrophilic compounds; or institution of physiological mechanisms to retain water within the thallus. These physiological changes might be expected because rain would become less reliable as a source of water and nutrients.

Increasing carbon dioxide and atmospheric nitrogen levels may negatively affect lichen species overall. Being poikilohydric organisms, their passive absorption of air, water and substrate nutrients will be impacted by increased acidity due to pollution. Past research has shown that ozone and carbon dioxide kill the photobiont, which ultimately kills the lichen. Some secondary metabolites have the ability to mitigate these effects and some lichens are better adapted to polluted environments than others. Increases in pollution will entail increases in secondary compound quantities that neutralize the negative effects of acidity with the lichen. Usnic acid is a compound found within lichens inhabiting acidic environments. Higher acidity from pollution will negatively affect these species because of usnic acid's limited ability to control acidity. However, basic substrates have the ability to buffer against acidification, which is the result of most types of pollution. This could mean that those lichens will be better able to adapt to increased acid levels than usnic acid containing lichens. On the other hand, lichens growing on basic substrata could be at risk from acidification of limestone causing deterioration of the substratum or a change in the pH to a pH that is intolerable by the lichen.

Pollution is also thought to be responsible for the increased levels of ultraviolet light caused by the loss of atmospheric ozone. Cortical compounds and other compounds within the thallus that offer protection to the sexual and asexual reproductive structures and photobionts, may ensure that those lichen species will have some protection from increase ultraviolet light. Species lacking those photoprotective compounds may endure degradation of photobionts and an increased frequency of mutations due to ultraviolet light exposure. Environmental stress may stimulate the production of cortical compounds in species that normally do not produce them; in species that do not produce them frequently; and in increased quantities for the species that already produce them.

If biochemical diversity decreases in response to climate change (Hauck, 2011), fewer secondary metabolites will be available for herbivore defense and, therefore, more grazing on lichen thalli will occur. Metabolites that would normally be lost to the soil, where they have an effect on growth of plants and microbes, may become reduced in type and concentration of metabolite. The lower concentration of the metabolites in the soil will have a reduced effect on growth of plants and microbes. This reduced impact will allow more microbes and plants to grow among mats of lichens and perhaps outcompete lichen growth sooner than would be expected. With fewer compounds there might also be less protection from ultraviolet light and a diminished ability for lichens to adapt to environmental changes that require secondary metabolites. However, fungi are plastic and may adapt in other ways or produce an array of different types of compounds with similar effects. This scenario of the production of other ecologically valuable metabolites may be plausible since so many gene paralogs have been reported (Table 1) that have no known associated function.

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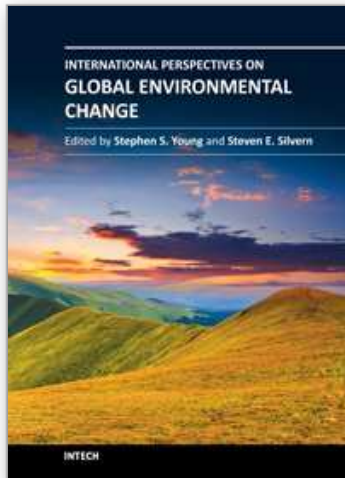


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