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Review article

Lichen photobionts and metal toxicity

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Abstract

Symbiotic algae or cyanobacterial partners associated with fungi in a lichen are considered the symbionts most sensitive to abiotic stress. Stress caused by high concentrations of metals produce increased levels of highly reactive oxygen species that result in lipid peroxidation as well as damage to proteins and nucleic acids. Excess metals impact primarily on the photobiont as they decrease the concentration and integrity of chlorophyll and the quantum yield of photosynthesis reduce the output of molecular oxygen. Cells experience plasmolysis, electrolyte leakage, swelling of cristae in mitochondria and thylakoids in chloroplasts, and decrease viability. Although the primary fungal symbiont, the mycobiont, provides a degree of defense by binding metal ions in anionic cell wall sites, photobionts may nevertheless be exposed to super-optimal metal concentrations. However, within photobiont cells chelators and chaperones containing sulfhydryl groups bind and detoxify metals and decrease the impact of metals. Increased cysteine concentrations have been documented in photobionts subjected to metal stress. Proline also forms stable complexes with dangerous free radicals that could otherwise produce damage in the cytosol, and photobionts show increased levels of free proline in response to high concentrations of metals. Heat shock proteins chaperone metal ions to cellular destinations where they will be harmless, and increased concentrations of hsps have been found in photobionts exposed to excess cadmium. Defense mechanisms involving primary and secondary metabolites of the mycobiont have so far been little investigated in lichen photobionts but these may also provide a degree of photobiont protection. Recent literature suggests complex interactions of photobionts with metals that depend upon the metal, its concentration, and the strain of photobiont.

Keywords: Algae, metals, metal toxicity, metal tolerance, lichens, photobionts

1. Introduction

In an increasingly industrialized world many organisms are experiencing increased exposure to widely distributed metals, and militarization is increasingly responsible for large scale dissemination of radionuclides and other metals. Lichens are vulnerable, like other species, but exhibit a range of tolerance. At one end of the spectrum some species are extraordinarily sensitive to atmospheric pollution while at the other end many species show extreme tolerance to environmental extremes. They are of singular interest because they often grow where conditions are too stringent for other organisms and also because they have developed a reputation as indicators of environmental malaise. Interactions among symbiotic associates within a thallus may partially explain the spectacular success of lichens in unusual environments such as those rich in metals.

Fungi, as heterotrophs, acquire fixed carbon through saprobism, facultative and obligatory parasitism or participation in mutualistic symbioses such as mycorrhizae and lichens. Although the thallus is extremely complicated and probably more like an ecosystem or community than a typical organism (Fahselt, 2008), lichens are classically considered to involve one primary fungal partner along with at least one interdependent autotrophic associate.

Many lichen symbioses involve a fungus (mycobiont) and autotrophic partner (photobiont) which may be an alga or cyanobacterium or possibly both. Symbiosis may be an apt way to describe relatively complex lichen taxa with internal stratification, but in other lichens, e.g. those with less organized thalli such as some crustose lichens, the mycobiont may best be viewed as parasitic on algae or cyanobacteria, with the arrangement termed "controlled parasitism" (Ahmadjian, 1993). Although most lichen species are crustose, the relationship among their bionts is

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still poorly understood. This is partly due to the firm attachment of these lichens to their substrata and the consequent difficulty of obtaining sufficient biomass for conventional ecophysiological investigations. The ecological success of lichenization is substantial, as about 20% of all known fungal species are lichenized. Lichens are dominant in many terrestrial environments including cold polar regions (Larson, 1987; Kappen, 1993).

Molecular techniques have shown that lichens are a polyphyletic and taxonomically diverse group of organisms and it has been confirmed that they have evolved many times in the past 200 million years (Honegger, 1993; Gargas et al., 1995; Lutzoni et al., 2001). The number of lichen species has been recently estimated at between 13,500 and 20,000 with 98% of mycobionts species of ascomycetes (Feuerer and Hawksworth, 2007). Taxonomically, lichens are treated as special forms of fungi, "lichenized fungi", and are in fact named after mycobionts. Mycobionts are obligate symbionts and usually not found free-living under natural conditions. If they are obligate symbionts they cannot, by definition, be grown independently. Although they can be grown independently under controlled conditions they do not form thallus-like structures in axenic culture (Ahmadjian, 1993; Stocker-Wörgötter and Türk, 1994; Stocker-Wörgötter, 2001).

2. Lichen Photobionts – Algae and Cyanobacteria in a Symbiotic Environment

There are approximately 25 genera of algae and 15 genera of cyanobacteria so far reported as lichen photobionts (Tschermak-Woess, 1988). The most widely-distributed green algal photobionts (phycobionts) include *Trebouxia* (*sensu lato*, including *Asterochloris*), *Trentepohlia*, *Coccomyxa*, *Myrmecia* and *Dictyochloropsis* (Ahmadjian, 1993), and the commonest cyanobacterial partners (cyanobionts) are *Nostoc*, *Scytonema*, *Stigonema*, *Gloeocapsa* and *Calothrix*, in order of their frequency.

In contrast to mycobionts, many of the cyanobacteria and filamentous green algae of the order Trentepohliales associating with fungi in lichens may be found in nature as free-living organisms (Ahmadjian, 1993). Nevertheless, other photobionts, e.g., species of *Trebouxia* (*sensu lato*, including *Asterochloris*) occurring in 20% of all lichens (Kroken and Taylor, 2000), are only occasionally found in the free-living state (Ahmadjian, 1993). Photobionts from lichen thalli may be grown in culture after isolation from lichen thalli (Bubrick, 1988; Ahmadjian, 1993) and some strains are available from culture collections (e.g. UTEX, SAG, CCAP, ATCP, ASIB).

Whereas many fungi absorb nutrients through penetration of a medium and intracellular mycelium, lichen-forming fungi have developed an ecologically interesting nutritional innovation (Sanders, 2001) involving hyphae closely associated with intact algal and/or cyanobacterial cells and forming a structure analogous to the leaf of plants. Photobionts in a lichen are surrounded by mycobiont hyphae which constitute 90% of the total thallus biomass, or more depending on the lichen species (Ahmadjian 1993), an environment that represents a large departure from the life style of free-living algae and cyanobacteria.

All photobionts provide carbohydrates from photosynthesis and many of the cyanobionts provide ammonia from nitrogen fixation to the mycobiont. Carbon is liberated as polyols (polyhydric alcohols), with ribitol the commonest transfer substance produced by species of *Trebouxia* and *Chlorella*. Sorbitol is released by *Pleurococcus* photobionts and erythritol by filamentous species of *Trentepohlia*. Cyanobacterial photobionts supply carbon as glucose. Typically most photosynthates, possibly more than 90%, are released from the photobiont and are taken up and metabolized by the mycobiont (Ahmadjian, 2004).

3. Uptake of Metals by Lichens

Along with provision of a growing environment for photobionts and protection for them against intense irradiation and herbivory (Dayan and Romagni, 2002; Edwards et al., 2004; Gauslaa, 2005; Pöykkö et al., 2005), the mycobiont also supplies the photobiont with required minerals. The lichen thallus is effective in absorbing minerals from the atmosphere, from wet and dry atmospheric deposition all of which can include metal-rich atmospheric particulates up to 100 μm in diam. (Richardson, 1995), as well as particles from the substrate (Brown and Beckett, 1985), which can remain within thalli unaltered for extended periods of time and yet are not necessarily detrimental (Bargagli and Mikhailova, 2002). Distribution of minerals within lichen thalli is not homogenous, and depends on morphological properties (Mrak et al., 2007). The toxicity of metals is obviously determined by chemical and physical parameters such as abundance of particles, concentration and chemical forms of metals, pH and temperature, but this review will demonstrate that many other factors are also implicated.

4. Photobionts and Metals

Metal homeostasis in photobionts

Cyanobacteria and algae, like other organisms, have evolved diverse strategies to maintain metal homeostasis. Photobionts must exclude metals not required for metabolism and retain those which are necessary, while ensuring that essential ions are in optimal intracellular concentrations (Cobbett and Goldsbrough, 2002).

Responses of photobionts to metal excess

Growth inhibition. Growth rates decrease under metal stress and are thus commonly used as a standard indication of toxicity. Rates are easily measured and closely reflect incapacitation of cells. Photobiont growth due to metal excess was demonstrated many years ago when Nash (1975) observed that zinc and cadmium, at 5×10^{-5} M, inhibited the growth of *Trebouxia gelatinosa* and *T. erici* photobionts. Later Bačkor et al. (1998) showed growth inhibition by copper, cadmium and mercury on *T. irregularis* cultures, with relative overall toxicity at 300 μM concentrations in *Trebouxia* media: Hg > Cd > Cu. With the photobiont *T. erici*, excess metals produced growth inhibition in this order: Hg > Cd > Co > Zn > Cu (Bačkor and Váczi, 2002). However, excess copper, up to 3 mM, did not reduce growth of the copper-tolerant strain, only of the wild type. At 4 mM copper, the growth of both wild and tolerant strains was impaired, although the copper tolerant strain was least affected. It must be noted that although such high copper concentrations are ecologically unrealistic, most metals in culture media, such as in the *Trebouxia* medium, precipitate as solids with phosphates, sulfates, hydroxides and EDTA (Bačkor and Dzubaj, 2004), so free concentrations are actually much lower than would appear from expressions of molarity.

Cytological effects. Tarhanen (1998) observed that Cu and Ni plasmolysed cells of *Trebouxia* in the lichen *Bryoria fuscescens.* Under acidic conditions (pH 3), mitochondrial cristae swelled and chloroplast thylakoid membranes degenerated. Hexavalent chromium supplied to the lichen *Xanthoria parietina* was mainly localized in the mycobiont (Sanità di Toppi et al., 2004), but nevertheless caused detachment of the plasmalemma from cell walls of the photobiont *Trebouxia.* Subsequently it was determined that cadmium was stored in lichen thalli of *X. parietina* as electron-opaque, cadmium-containing precipitates in chloroplasts of the photobiont, plasmalemma and cell walls (Sanità di Toppi et al., 2005a). Furthermore, precipitates were localized at the photobiont-mycobiont interface as well as in mycobiont cell walls, plasmalemma and concentric bodies in the cytosol. Cadmium, like chromium, caused detachment of the plasmalemma from cell walls as well as swollen thylakoids.

Enzymatic activity. Superoxide dismutase (EC 1.15.1.1; SOD), catalase (EC 1.11.1.6; CAT), guaiacol peroxidase (EC 1.11.1.7; GPX), NADPH/dependent glutathione disulfide reductase (EC 1.6.4.2; GR) and ascorbate peroxidase (EC 1.11.1.11; APX) are considered as main antioxidant enzymes contributing to the control of ROS (Pinto et al., 2003). The activity of these enzymes in relation to metal excess has recently been assessed in a few studies of lichens (Sanità di Toppi et al., 2004; Cuny et al., 2004; Sanità di Toppi et al., 2005b; Monnet et al., 2006), but their role in photobionts is so far unclear.

Photosynthesis and respiration. Respiration and photosynthesis are both sensitive to metals and due to the sensitivity of lichen photobionts to metal excess, parameters related to these processes are frequently used as an indicator of metal stress on lichens. Assessing assimilation pigments is a sensitive and inexpensive approach to gauging the influence of metals on the photosynthetic apparatus of lichens, as excess toxic metals negatively impact on chlorophyll biosynthesis. Chlorophyll *a* content decreases with increased external doses of copper and mercury in axenic photobiont cultures (Bačkor and Dzubaj, 2004), a consequence of increased levels of chlorophyll *b*. Oxidation of the methyl group on ring II, converting chlorophyll *a* to chlorophyll *b* (Chettri et al., 1998) could explain a decreasing chlorophyll *a/b* ratio while total chlorophyll remains constant. In the case of mercury application, however, both chlorophyll *a* and chlorophyll *b* are decreased (Bačkor and Dzubaj, 2004). Other metals (Cd, Ni, Zn and Co) have less effect on assimilation pigments at least in short term experiments (Bačkor and Zetíková, 2003; Bačkor and Dzubaj, 2004).

Total carotenoid content is also sensitive to metals such as copper, and reduced concentrations provide a further indication of short term metal debilitation (Bačkor et al., 2003). Another parameter used in modern lichenological studies to assess stress is the integrity of chlorophyll *a*. The ratio of optical densities at 435 nm and 415 nm (OD 435/OD 415), referred to as the phaeophytinization quotient (PQ), reflects the ratio of chlorophyll *a* to phaeophytin *a*. In intact lichens and healthy photobiont cells, PQ values are c. 1.4 (Ronen and Galun, 1984), but with excess metals such as copper and mercury this parameter decreases to values of 1 or less (Garty et al., 1992).

Determination of the potential quantum yield of electron transfer through photosystem II (PSII), chlorophyll *a* fluorescence, is another experimental tool for gauging stress responses. This method is widely-used because it is non-destructive, equipment and operating costs are reasonable, and hundreds of samples may be analyzed in a short time as well as repeatedly over time. Results are usually expressed as F_V/F_M, calculated as the maximal fluorescence (F_M) less the minimal fluorescence (F_0), all divided by F_M of dark adapted plants: $(F_M - F_0)/F_M = F_V/F_M$. Normal values of F_V/F_M in lichens are c. 0.6–0.7, while lower values indicate damage to PSII. Intracellular copper content greater than 4.0 μmol g^{-1} decreases chlorophyll *a* fluorescence in the intact lichen *Ramalina fastigiata* (Branquinho et al., 1997). The ratio F_V/F_M decreased also in lichen *R. lacera* following short-term exposures to $CuSO_4$ (Garty et al., 2007). Excess copper and cadmium decreased the ratio F_V/F_M in axenic cultures of the photobiont *Trebouxia erici* (Bačkor et al., 2003; 2007a).

Photosynthetic oxygen evolution rate (OER) in *T. erici* suspensions after short-term exposure to increased concentrations of copper and cadmium showed, with

increasing irradiance, a curvilinear increase reaching a maximum of 20 pmol (O_2) μg (chl *a*)$^{-1}$ s^{-1} at photosynthetic photon flux density c. 300 μmol m^{-2} s^{-1} (Bačkor et al., 2007a). The OER was reduced by 10 μM of copper to c. 7 pmol (O_2) μg (chl *a*)$^{-1}$ s^{-1} and to below control levels by 10 μM cadmium.

Nutritional levels of metals in lichen algae

Photobionts isolated from lichens have identical nutrient requirements to non-lichenized cells of similar species and are grown on standard nutrient media.

Cyanobacterial photobionts may be cultivated on KM, ASM-1 and BG-11 media, and eukaryotic photobionts on *Trebouxia* liquid or agar media and Bold´s Basal Medium (BBM) (Bubrick, 1988; Ahmadjian, 1993).

To date, effects of metal deficiency have not been demonstrated in lichen photobionts, but in plants the symptoms include abnormal growth and development and altered metabolic processes. Deficiency of essential metals causes cholorosis and decreases productivity (Mehra and Farago, 1994).

Metals required for algal and cyanobacterial metabolism, include macroelements (K, Mg) and micro-elements (Co, Cu, Fe, Mn, Mo, Ni, V, Zn). These must be obtained from the environment and may be toxic at high concentrations, while other metals (e.g. Cd, Hg, Pb) are toxic even in low concentrations (Pawlik-Skowrońska and Skowroński, 2001). Photobiont cells require trace amounts of some metals, e.g. Fe, Zn, Mo, Co, Mn and Cu, in nutrient media (Bubrick, 1988; Ahmadjian, 1993).

Cobalt functions as a cofactor and activator for enzymes and is also essential for atmospheric nitrogen fixation in cyanobionts (Mehra and Farago, 1994). Copper plays a significant role in physiological processes in plants, such as photosynthesis, respiration, metabolism of nitrogen, and synthesis of protein and cell walls, because many enzymes require copper. It is also required as a cofactor in the thylakoid lumen electron transport protein plastocyanin (Eriksson et al., 2004). Iron is important for chlorophyll synthesis (Mehra and Farago, 1994) and is a cofactor for supercomplexes in the photosynthetic electron transfer chain. Manganese is critical to PSII function, as the manganese cluster in the donor side of PSII catalyzes the water-splitting reaction. Molybdenum is essential for most biological systems (Mehra and Farago, 1994) as it is required by key enzymes catalyzing reactions in carbon, sulfur and nitrogen metabolism. Nickel is important to urease and hydrogenase catalysed reactions (Muyssen et al., 2004), while in some algal species vanadium is necessary for haloperoxidases (Almedeida et al., 2001). Zinc is required for activation of peptidases, dehydrogenases, proteinases and phospho-hydrolases (Mehra and Farago, 1994).

Lichen photobionts exposed to excess metals

Lichens are frequently exposed to excess metals with atmospheric pollution the main source (Seaward and Richardson, 1989), but industrial substrata, e.g., metal-rich mine tailings, may be significant local sources of metals (Purvis and Halls, 1996; Bačkor and Fahselt, 2004a;b; Pawlik-Skowrońska et al., 2006). In rare cases substrates containing high concentrations of toxic metals may support a rich flora of lichen species. High metal substrata may support specific lichen associations (Purvis and Halls, 1996).

Photobionts in relation to metal tolerance of lichens

When grown aposymbiotically, axenic cultures of photobionts seem to be more sensitive to excess metals, e.g. copper, than mycobionts (Bačkor et al., 2006a; 2007a), but photobionts show differential sensitivity. That is, photosynthesis of lichens containing cyanobacteria is more sensitive to zinc, cadmium and copper than of lichens with eukaryotic photobionts (e.g. *Trebouxia*) (Brown and Beckett, 1983). The cyanobacterial *Nostoc* photobiont is more sensitive to manganese than eukaryotic *Dictyochloropsis* photobiont (Paul and Hauck, 2006), but the sensitivity of eukaryotic photobionts also varies. For example, lipid metabolism is more affected by copper and lead in *Coccomyxa,* than in *Trebouxia* (Guschina and Harwood, 2006).

In one lichen community on iron-rich rocks all nine lichen species contained the same photobiont, *Trebouxia simplex* (as *T. jamesii*) (Beck, 1999). However, in centuries-old copper mines in Central Slovakia ITS rDNA sequences indicates involvement of several photobionts (Bačkor, unpublished results). Participation of these in tolerant lichens thriving on mine spoils from the Middle Ages suggests parallel selection has taken place in a number of lineages within a few hundred years.

That selection of tolerance occurs in nature is supported by successful production of tolerance under laboratory conditions. The development of tolerance of lichens to metals that is seen in nature has been simulated in the laboratory by gradually increasing, over a three-year period, the copper concentration of the medium a copper-tolerant photobiont strain was obtained from wild-type *Trebouxia erici* (UTEX 911) (Bačkor and Váczi, 2002). When exposed to excess copper the tolerant genotype exhibited uptake, growth rates, pigment content, membrane integrity, dehydrogenase activity, photosystem II activity, synthesis of free proline and non-protein thiols that were not significantly different from control lichens growing on normal substrata (Bačkor et al., 2003; 2004; 2007b).

Detoxification and tolerance of photobionts to metals

Symbiotic aspects of detoxification and tolerance of metals in lichens. Besides providing nutritional benefits, interactions between symbionts enable many lichens to colonize habitats where separate bionts are unable to survive. In addition, lichens are able to resist environmental stresses, such as intense radiation, desiccation, and extremes of temperature and pressure (Vera et al., 2004). Although vulnerable to atmospheric pollutants, because cuticle and stomatal systems are lacking, lichens have developed mechanisms that permit reconciliation, not only with acid rain and other airborne contaminants such as metals, but also with excess metals in substrata.

Many lichens tolerate high concentrations of metals in atmosphere or substrata, while others have developed a specific requirement for metal rich environments. In fact, disjunctive distributions may reflect the availability of suitable sites (Nash, 1989). The lichen *Lecanora cascadensis* is used as marker of high copper content in substrate (Czehura, 1977), and recently many other species have been recognized as associating with copper-rich substrata. Some of the most typical taxa include *Lecidea inops* ("copper lecidea"), *Lecanora chalcophila*, *L. subaurea*, *Acarospora montana*, *A. rugulosa*, *A. sinopica* and *Rhizocarpon oederi* (Purvis and Halls, 1996). Lichens can contain considerable amounts of copper, reaching 5% dry weight (Purvis, 1984) or as much as 16% in *Acarospora rugulosa* Körb. (Chisholm et al., 1987). Iron content has been reported up to 9% dry weight (Seaward, 1973).

Symbiotic interactions in lichens probably facilitate survival of photobionts in metal-rich environments. Although the photobiont itself has tolerance mechanisms for dealing with metal stress, the mycobiont also provides a degree of protection to the photosynthetic partner(s). Mycobiont cell walls, typically constituting more than 90% of the lichen biomass, exhibit anionic binding sites (Collins and Farrar, 1978) capable of binding cations, including metals. Mycobiont production of organic acids such as oxalic, citric and malic acids (Pawlik-Skowrońska et al., 2006) and secondary metabolites (Purvis, 1984; Purvis et al., 1987) may also help to chelate metals from reaching photobiont cells. Metal uptake in photobionts may thus be limited because metal cations do not always have access to photobiont cells.

Richardson and Nieboer (1983) found that approximately 20% of the nickel supplied to lichens was localized in the algal zone. Binding of lead cations in lichens was restricted to fungal hyphae, while the photobiont contained much less (Garty, 2001). Uptake of chromium and cadmium, as demonstrated by TEM coupled with X-ray microanalysis, is also mainly in mycobiont hyphae (Sanità di Toppi et al., 2004; 2005a).

Exclusion of metals by photobiont cell walls. Although metals in lichens are often immobilized in some way by the mycobiont (Goyal and Seaward, 1982), there may nevertheless be considerable amounts associated with photobiont cells. As in the case of mycobionts, photobiont cell walls may also bind metals in negatively charged anionic sites involving carboxyl, phosphoryl, amine and hydroxyl groups (Collins and Farrar, 1978). Metal uptake is usually accompanied by the release of protons in a rapid and probably passive physicochemical process (Nieboer et al., 1976; Brown 1987), with a maximal quantity of metal absorbed from experimental solutions within a few minutes. Concentration of mobile cations in cation-active layer of lichens depends on their concentration in the lichen environment and pH (Kłos et al., 2005; 2006). The cation-binding capacity of photobiont wall materials is probably limited, although in some experiments the walls of the axenic cultures of lichen photobiont *Trebouxia erici* bind approximately 70% of the total cell copper (Bačkor et al., 2003).

Experimental replacement of bound metals by other metals allows determination of the quantities of metals bound to extracellular exchange sites and calculation of amounts of intracellular metals (total content – extracellular content = intracellular content). Nickel chloride ($NiCl_2$) and disodium ethylenediamine tetraacetic acid (Na_2EDTA) have been the most frequently employed displacement reagents to date (e.g., Brown and Beckett 1983; 1985; Beckett and Brown, 1984; Brown, 1987).

However, lead nitrate ($Pb(NO_3)_2$) is also effective in removing extracellularly-bound metals (Garty, 2001), and in the moss *Fontinalis antipyretica,* two consecutive immersions in a 50 mM solution were sufficient to remove all metals from cell walls without destruction of plasmalemma (Vásquez et al., 1999). The results of these studies quantitatively demonstrated the regulatory effects of the extracellular ionic environment provided by cell walls on intracellular metal uptake by lichen symbionts.

Metal interactions with the plasmalemma of photobiont cells. The plasma membrane may be regarded as the first "living" structure which is targeted by metal toxicity (Hall, 2002). Metal uptake is much slower in intracellular than extracellular binding sites, with maximal internal uptake of copper from a liquid medium by the lichen *Dermatocarpon luridum* observed after 3–6 h (Monnet et al., 2005). As is the case with intact lichens, intracellular uptake of photobionts is typically much less than extracellular. Intracellular copper in axenic cultures of the lichen photobiont *Trebouxia erici*, for example, in long-term experiments was c. 30% of the total copper (Bačkor et al., 2003).

Plasma membrane function may be rapidly altered by metals; for example, long-term exposure to excess copper induces leakage of potassium from *Trebouxia erici* photobiont cultures and, following loss of potassium, intracellular copper content increased (Bačkor et al., 2003). Copper increases the number of plasmolyzed cells in

T. erici cultures (Bačkor and Váczi, 2002).

Integrity of the plasma membrane may also be assessed using trypan blue. This dye is excluded from cells by intact membranes, while cells with damaged membranes take up the coloring agent and staining can be used as an indicator of membrane functioning following exposure to toxic chemicals such as metals. Long-term exposure to excess copper produces a significant decrease in viability due to membrane damage in *T. erici* photobiont cells in axenic culture (Bud'ová et al., 2006).

Metals are often involved in oxidative stress resulting from the production of reactive oxygen species (ROS) as these damage biomolecules such as lipids, proteins and nucleic acids (Vavilin et al., 1998). In both plants and lichen photobionts, lipid peroxidation is usually quantified using malondialdehyde (MDA) which, when reacted with thiobarbituric acid, forms colored products or so-called thiobarbituric acid reactive substances (TBARS). Thus in algae, as in other plants, the TBARS content is positively correlated with the degree of membrane lipid peroxidation and with increased ion permeability (Vavilin et al., 1998). Based on TBARS content, cells of the lichen photobiont *T. erici* were differentially sensitive to copper and cadmium as well as dependent upon concentrations (Bačkor et al., 2007a). Copper, a redox reactive metal, caused a concentration-dependent increase in TBARS production indicating membrane damage during the short-term exposure; oxidation of copper leads to the formation of the superoxide anion, $\cdot O^{2-}$, and subsequently hydrogen peroxide, H_2O_2, and via Fenton-type reactions the highly reactive hydroxyl radical (Stohs and Bagchi, 1995). In similar experiments cadmium on the other hand had no stimulatory effect on TBARS production in *T. erici* at any tested concentration (Bačkor et al., 2007a), although its intracellular uptake was similar to that of copper.

Although little is known about sensitivity to metals in most photobionts, differences in metal sensitivity have been observed between some species; for instance, as noted previously, lipid metabolism of *Coccomyxa* photobionts was more greatly affected than that of *Trebouxia* by copper and lead (Guschina and Harwood, 2006). Differential responses of plasma membranes to redox reactive copper were even found within one photobiont species, *T. erici*. For example, a copper-tolerant strain of *T. erici*, when exposed to excess long-term copper, demonstrated much less membrane damage than wild type; the tolerant type exhibited less electrolyte leakage from cells, less intracellular potassium leakage and a lower degree of MDA production (Bačkor et al., 2003; Bačkor et al., 2007a).

Intracellular chelation and sequestration of metals in lichen photobionts. To preserve normal metabolic functioning, excess metals must be excluded from the cytosol and may be removed through efflux or compartmentalization. In plants metal ions complex with cell chelators in the cytosol, and complexes are then transported to the vacuole which is the main repository for metals and other toxic substances (Clemens, 2001), thus assisting in detoxification of metals (Clemens, 2001). Metal ions bound by both chelators and chaperones contribute to metal homeostasis in living cells.

The amino acid cysteine contains a sulfhydryl (thiol group, -SH) which is the site of metal binding. Cysteine-containing peptides such as glutathione (GSH) and phytochelatins (PCs) are responsible for metal sequestration in living cells. A cadmium-tolerant strain of the alga *Chlamydomonas reinhardtii* has significantly higher levels of cysteine than the cadmium-sensitive strain (Hu et al., 2001) and similarly a copper-tolerant strain of the lichen photobiont *Trebouxia erici* synthesizes significantly higher cysteine levels than wild *T. erici* even in a control culture medium (Bačkor et al., 2007b). A cysteine pool may thus be critical to photobiont defense against metal toxicity.

The tripeptide glutathione (L-gamma-glutamyl-L-cysteinylglycine) occurs in a reduced monomeric form (GSH), as well as an oxidized dimeric form (GSSG). In living cells, GSH is the most prevalent and functionally important of these forms, due to its potential role in providing protection against active oxygen species including toxic metals (May et al., 1998). The enzyme glutathione reductase which converts GSSG to GSH is inducible by oxidative stress. On the other hand, excessive toxic metals may considerably decrease GSH content by metal-induced oxidation to GSSG, possibly due to inactivation of glutathione reductase. The ratio of GSH to GSSG in the cells may thus be used as an indicator of toxicity in cells (Pawlik-Skowrońska et al., 2002). Typical levels of GSH, expressed as nmol SH/g dry weight, in *Trebouxia* range from c. 2000 to 3000 (Bačkor et al., 2007b). In a whole lichen, GSH content ranges from 500 (*L. polytopa*) to c. 3000 nmol SH/g dw (*Xanthoria parietina*), values similar to those of aposymbiotically grown mycobionts from *Cladonia cristatella*, *Physcia adscendens*, *Physconia grisea* and *Xanthoria parietina* (Pawlik-Skowrońska et al., 2002; Pawlik-Skowrońska et al., 2006; Bačkor et al., 2006a). However, at copper concentrations of 10 μM, the GSH content in aposymbiotically grown mycobiont cultures of *Cladonia cristatella* increases (Bačkor et al., 2006a), but it decreases in *Trebouxia erici* aposymbiotically cultivated under identical experimental conditions (Bačkor et al., 2007b). This is consistent with the greater sensitivity to excess metals of the lichen photobiont than the mycobiont.

In the presence of some metals, phytochelatin synthase (EC 2.3.2.15) catalyzes the conversion of glutathione to phytochelatins (PCs), low molecular weight thiol peptides with the typical structure $(\gamma Glu\text{-}Cys)_n$ – Gly, where n = 2–11 (Grill et al., 1985). Lichen thalli produce phytochelatins in response to cadmium, lead and zinc (Pawlik-Skowrońska et al., 2002). In addition, Bačkor et al. (2007b) found increased synthesis of phytochelatins in the lichen

photobiont *T. erici* in response to excess cadmium or copper. However, cadmium was a more potent activator of phytochelatin synthesis under identical experimental conditions and even able to induce synthesis of phytochelatins with longer (more stable) chains, up to PC_5. Synthesis from glutathione was found to be a metal concentration dependent process. So far, phytochelatin levels in lichens and their bionts have been determined in only a limited number of studies, but phytochelatins are produced in lichens to a lesser extent than GSH (Pawlik-Skowrońska et al., 2002; Pawlik-Skowrońska et al., 2006). Only eukaryotic photobionts synthesize phytochelatins and no phytochelatin synthesis was observed in aposymbiotically grown mycobionts of lichens (Pawlik-Skowrońska et al., 2002; Pawlik-Skowrońska et al., 2006; Bačkor et al., 2006a).

Metallothioneins (MTs) are low molecular weight (6–7 kDa), cysteine rich proteins found in plants, animals, fungi and some prokaryotes. MTs can bind metals. Binding is through the thiol group of cysteine, which represents nearly 30% of its amino acid residues. Cysteine richness in MTs differs between higher plants and animals (Yang et al., 2005). To our knowledge, metallothioneins have not yet been reported in lichen photobionts; however, as they occur in cyanobacteria, it is probably only a matter of time until they are also confirmed in cyanobionts.

Another initial response to biotic as well as abiotic stresses, at least in plant cells, is an increase in free proline concentrations. Accumulations of this amino acid may permit osmotic adjustment and provide protection for enzymes, biological membranes and polyribosomes by forming stable complexes with free radicals that could otherwise prove toxic. Proline may also play a role in maintaining $NAD(P)^+/NAD(P)H$ ratios during stress at values similar to those characteristic of normal growing conditions (Hare and Cress, 1997). Free proline accumulates in response to copper stress in axenic cultures of wild and copper-tolerant strains of the lichen photobiont *Trebouxia erici*. As a result of short-term exposure, a copper-tolerant strain exhibits significantly more intracellular proline than a wild type. Increased proline in photobiont culture media alleviates the toxic effects of copper in both strains, but particularly in the tolerant strain (Bačkor et al., 2004). Proline inhibition of metal-induced loss of potassium in the copper-tolerant strain is similar to that in the free-living alga *Chlorella vulgaris* (Mehta and Gaur, 1999).

Due to reactivity of metal ions with sulphur, nitrogen and oxygen, the amino acid histidine and organic acids such as citrate, malate and oxalate have been implicated in a number of different biological phenomena, and one of these is metal tolerance (Clemens, 2001). Although organic acids are found in lichens (Pawlik-Skowrońska et al., 2006), their involvement in metal tolerance has only been demonstrated in relation to the intact symbiotic unit and never examined in axenic cultures of photobionts. Histidine levels may also be potentially involved with sensitivity to metals, because histidine concentration is correlated with the degree of nickel tolerance in yeasts (Pearce and Sherman, 1999) and higher plants such as *Alyssum*, *Brassica* and *Thlaspi* (Sharma and Dietz, 2006). However, there is little evidence that histidine plays a role in lichens although it could explain differential sensitivity to metals amongst photobionts.

Other low molecular weight molecules serving as antioxidants potentially capable of nullifying the effects of metal stress have also been discovered in algae, with carotenoids, ascorbate, flavonoids and tocopherols the most studied (Pinto et al., 2003). These substances may remove ROS before crucial metabolic processes are disrupted and also play a role in detoxification of metals in photobiont cells.

Metals and extremes of other environmental factors such as temperature and oxygen trigger changes in the transcript levels of numerous genes encoding proteins. Heat shock proteins (hsp) are a group of chaperones which specifically deliver metal ions to cell organelles and metal-requiring proteins; hsps are highly conserved and involved in maintenance of protein homeostasis within cells (Hershko, 1998; Hightower, 1991). Hsps are grouped into families according to their molecular weight and are synthesized in response to stresses (Sanità di Toppi and Gabrielli, 1999; Bierkens, 2000). The hsp70 family is one of the most abundant (Hartl, 1996), and because the amount of hsp70 increases with stress, concentration is used as an environmental biomarker (Bierkens, 2000).

Bačkor et al. (2006b) studied the expression of hsp70 in axenic cultures of *T. erici* during short-term exposure to excess cadmium and copper and found that copper-treated cells maintained a relatively constant amount of hsp70 over all tested concentrations, up to 10 mM, but cadmium caused an increase in hsp70 expressions, especially at the lowest concentration (1.0 μM). However, stress protein expression in lichens and their bionts has been studied little.

5. Conclusions

Most studies of metal toxicity in lichen photobionts in the last decade involved excess copper and cadmium, but now the effects of other metals, e.g., manganese, lead, zinc, mercury and chromium, have also been studied. Both binding of metals on cell wall sites and intracellular sequestration in vacuoles have been demonstrated in lichen photobionts, but photobionts are nevertheless impacted by metals because neither of these avoidance mechanisms provides total protection against high concentrations. Damage to highly sensitive photobionts revolves largely around generation of the superoxide radical, and other potentially destructive highly reactive oxygen species

capable of damaging DNA, lipids and proteins. Membranes are particularly vulnerable and prone to leakage under metal stress, and the photosynthetic apparatus is highly susceptible.

Superoxide dismutase is increased in response to excess metal exposure, as are other enzymes that metabolize reactive oxygen species. Free-living cyanobacteria produce metallothioneins that bind metals, and cyanobionts in lichens may also generate these, although this has not been demonstrated to date. Furthermore, if tolerance mechanisms of lichen photobionts are similar to those of plants or other free-living photosynthesizers, histidine may be involved in protection against superoptimal amounts of metals. Heat stress proteins, generated in response to various kinds of stresses, may chaperone metals to photobiont organelles, and there is also a possibility that histidine, ascorbate, flavonoids and organic acids play a role in detoxification as they do in other organisms.

While the full range of photobiont response mechanisms has obviously not yet been determined, responses appear to be complex. Furthermore the effects of suberabundance of metals hinge upon the tolerance of a particular photobiont to particular metals.

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