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Photosynthesis in Lichen: Light Reactions and Protective Mechanisms

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1. Introduction

Lichens are symbiotic associations (holobionts) established between fungi (mycobionts) and certain groups of cyanobacteria or unicellular green algae (photobionts). This symbiotic association has been essential in establishing the colonization of terrestrial and consequently dry habitats. About 44 genera of algae and cyanobacteria have been reported as lichen photobionts. Due to the uncertain taxonomy of many of these photobionts, these numbers were considered as approximations only. Ahmadjian (1993) estimates that only 25 genera were typical lichen photobionts. The most common cyanobionts are *Nostoc*, *Scytonema*, *Stigonema*, *Gloeocapsa*, and *Calothrix*, in order of frequency (Büdel, 1992). Green algal photobionts include *Asterochloris*, *Trebouxia*, *Trentepohlia*, *Coccomyxa*, and *Dictyochloropsis* (Gärtner, 1992). These authors assessed that more than 50% of all lichen species are associated with *Trebouxia* and *Asterochloris* species. However, this is just estimation since in only 2% of the described lichen species the photobiont genus is reported (Tschermak-Woess, 1989), mostly by the difficulties to isolate and then characterize the algae from the lichen thalli. Lichens are well known for their slow growth and longevity. Their radial growth is measured in millimetres per year (Hale, 1973), while individual lichens live for hundreds or even thousands of years. It is assumed that in lichens the photobiont population is under mycobiont control. Lichenologists have proposed some control mechanisms such as, cell division inhibitors (Honegger, 1987), phytohormones (Backor & Hudak, 1999) or nutrients competition (Crittenden et al., 1994; Schofield et al., 2003).

Similar to plants, all lichens photosynthesise. They need light to provide energy to make their own matter. More specifically, the algae in the lichen produce carbohydrates and the fungi take those carbohydrates to grow and reproduce. The amount of light intensity needed for optimal lichen growth varies widely among species. The optimum light intensity range of most algal photobionts in axenic cultures is very low, between 16-27 $\mu\text{mol m}^{-2} \text{s}^{-1}$. If the response of cultured photobionts to light is similar to that of the natural forms (lichen), then there must be additional mechanisms protecting the algae in the lichen that are not developed under culture conditions. Pigments and crystal of secondary metabolites in the

upper cortex are supposed to decrease the intensity of light reaching the photobionts especially under desiccated conditions by absorbing certain wavelengths and by reflecting light (Heber et al., 2007; Scott, 1969; Veerman et al., 2007). Apparently, the balance between energy conservation and energy dissipation is tilted towards dissipation in many poikilohydric autotrophs, whereas, in higher plants, energy conservation assumes dominance over energy dissipation. It thus appears that sensitivity to excess light is higher in the mosses and the lichens than in higher plants (Heber, 2008).

Lichens are found among poikilohydric organisms, those that cannot actively regulate their water content, but are capable of surviving long periods in a desiccated state (Kappen & Valladares, 2007). In the dry state many lichens exhibit an enhanced resistance to other stress. For instance, heat resistance up to 70-75 °C in species from sheltered microhabitats and up to 90-100 °C in species from exposed microhabitats (Lange, 1953). Desiccation tolerance was described in nematodes and in rotifers observed by van Leeuwenhoek in 1702, and has since been discovered in four other phyla of animals, algae, fungi, bacteria, in ca. 350 species of flowering plants and ferns and in most bryophytes and seeds of flowering plants (Alpert, 2006; Proctor & Tuba, 2002). Among them, algae, lichen and bryophytes can be considered fully desiccation-tolerant plants because can survive very rapid drying events (less than 1 h) and recover respiration and photosynthesis within a few minutes (Oliver & Wood, 1997; Proctor & Smirnoff, 2000). Most lichen-forming fungi and their photobionts are fully adapted to daily wetting and drying cycles, but die off under continuously moist conditions (Dietz & Hartung, 1999; Farrar, 1976a, 1976b). It is well known that photosynthesis in homoiohydric plants is very sensitive to water stress conditions (Heber et al., 2001), especially under high irradiance. Under these conditions, reactive oxygen species (ROS) generation associated to photosynthetic electron transport is enhanced. The question arises of how lichen algae can maintain the function of their photosynthetic machinery under continuous desiccation-rehydration processes. We will review in this chapter the possible mechanisms which should allow maintaining of photosynthesis performance under the life style of poikilohydric organisms.

2. Methods for isolating lichen photobionts

One of the main problems to study the mechanisms of photosynthesis in lichens under well-controlled conditions is to develop an appropriate method for isolating the lichen photobionts. Many chlorolichens contains more than one photobiont. For instance, *Ramalina farinacea* includes two different *Trebouxia* photobionts (TR1 and TR9) and isolation of these algae allowed to characterise physiological differences between both of them (Casano et al., 2010; del Hoyo et al., 2011). There are different methods in function of the objective of investigation. We can distinguish between those which allow and not allow obtaining axenic cultures.

Axenic cultures are useful to study the taxonomy, biochemical, molecular or physiological behaviour of microscopic algae outside the symbiosis. There are lots of methods, but the most popular isolation method was developed by Ahmadjian (1967a, 1967b) and consists of cutting the lichen photobiont layer into thin slices, then grinding it between two glass slides and finally spreading the homogenate on a solid agar medium. There are several variations to this method, but the main common problem to all of them is the long time required after isolation to obtain clones.

On the other hand, non-axenic cultures can be used for studying algal metabolites or enzymatic activities of the lichenized photobionts. These methods consist in homogenization of the lichen thalli, followed by separation of the photobiont from the mycobiont and fragments of thalli using differential centrifugation (Richardson, 1971), gradient centrifugation on CsCl_2/KI (Ascaso, 1980) or on Percoll® gradients (Calatayud et al., 2001), and/or filtering (Weissman et al., 2005).

Here we resume the fast and simple methods developed in our laboratories (Gasulla et al., 2010): a low-scale isolation method (micromethod) and a large scale one (macromethod).

The micromethod for isolation of lichen photobionts starts from 15–25 mg dry weight (DW) of lichen material that is washed first in tap water, and then slowly stirred in sterile distilled water in a bucket for 5 min. The fragments of thalli are homogenised in an sterile eppendorf tube with a pellet pestle and resuspended in sterile 1 ml of isotonic buffer (0.3 M sorbitol in 50 mM HEPES pH 7.5). After filtration through sterile muslin, the filtrate is centrifuged at $490\times g$ in a bench-top microcentrifuge (Micro 20, Hettich, Germany) for 5 min. The pellet is resuspended in 200 μl of sterile isotonic buffer and then loaded on 1.5 ml, of sterile 80 % Percoll ® in isotonic buffer. After centrifugation at $10000\times g$ for 10 min a clear green layer must be present near the top of the eppendorf tube and some grey particles and pellet at the bottom of the tube (Fig. 1). The green layer is recovered (ca. 400 μl), avoiding to take any drop of the upper interphase. Then, the green layer is diluted 2-fold with sterile distilled water and centrifuged at $1000\times g$ for 10 min. The supernatant is discarded; the pellet is resuspended in 2 ml of sterile distilled water and a drop of Tween 20 is added. The resulting suspension is sonicated at 40 KHz (Elma Transsonic Digital 470 T, 140% ultrasound power) for 1 min and again centrifuged at $490\times g$ for 5 min. This treatment is repeated five times. The final pellet containing the isolated algal cells is resuspended in 1 ml of sterile distilled water. This micromethod can be scaled up to a macromethod, which allows preparation of large amounts of photobiont cells.

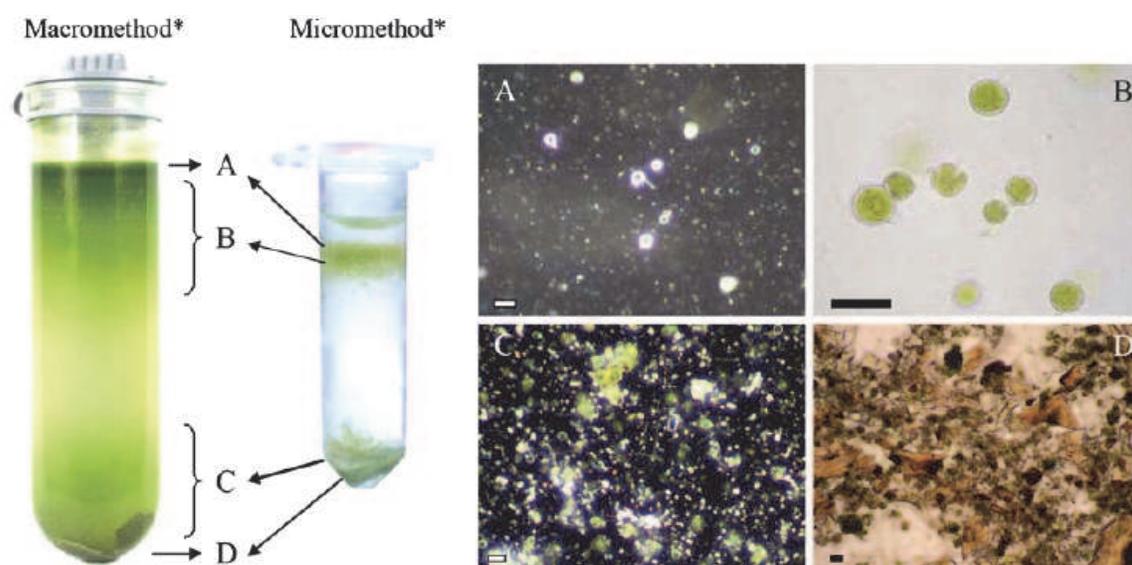


Fig. 1. Separation of *Ramalina farinacea* fractions after centrifugation of the extract of thalli at $10,000\times g$ for 10 min on 40 ml (macromethod), or 1.5 ml (micromethod) of 80 % Percoll ® in isotonic buffer. A–D, each optical micrograph refers to the corresponding Percoll layer; A and C phase contrast microscopy. Scale = 15 μm . * Not real size. Photograph from Gasulla et al., (2010).

In the macromethod, one to two g DW of lichen thallus are homogenised with a mortar and pestle in 20 ml of sterile isotonic buffer. The steps following are similar to the micromethod, but the volume for resuspension of the first pellet is 1 ml. The second centrifugation step is carried out on 40 ml of sterile 80% Percoll in isotonic buffer using a fixed-angle rotor (221.22 V01/V02, Hermle, Germany). After this centrifugation step in the macromethod, four layers are visible: a) a 2–3 ml dark green supernatant at the top of the tube on the Percoll layer; b) a large and diffuse light green layer in the upper part of the Percoll gradient; c) a thick layer at the bottom of the tube and d) a grey pellet (Fig. 1). Five millilitres of the “b” layer are recovered and the subsequent isolation steps for this layer are identical to those described for the micromethod.

The algal suspensions isolated with any one of both methods, are diluted 100 folds with sterile distilled water and 50 μl of this suspension are spread on sterile 1.5% agar 3xN (meaning three times more nitrogen content in the form of NaNO_3) Bold's Basal Media (3NBBM) (Bischoff & Bold, 1963) in each of five Petri dishes using the streak method and sterile technique. The isolated algae are cultured under 15 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (PPFD) with a 12 h photoperiod at 17°C. The number of algal colonies growing on each plate is counted after 45 days. Several colonies must be selected under the stereo-microscope and subcultured onto Petri dishes containing 1.5% agar 3NBBM medium supplemented with glucose (20 g l^{-1}) and casein (10 g l^{-1}) (Ahmadjian, 1967a) using a sterile toothpick.

3. Effects of water content on the carbon budget of lichens

The thallus water content is mainly determined by the water availability of the environment. When desiccated, their water status is frequently in the range of 10–20 % in respect to their fresh weight (Rundel, 1988). This state would be lethal for most of the vascular plants and organisms, however the vast majority of lichens are desiccation-tolerant and can survive in a suspended animation until water becomes available again, then they revive and resume normal metabolism (Kappen & Valladares, 2007). Upon rehydration they recover normal photosynthetic rates within a short time span, 15–60 min or less (Fos et al., 1999; Jensen et al., 1999; Tuba et al., 1996). Therefore, lichens may be the predominant life-form in extreme environments like cold and hot deserts. In lichens, photosynthetic activity of the photobiont partner is restricted to a short time when thalli are at least partly hydrated and solar radiation is available at temperatures within the range suitable for photosynthesis. Frequent drying and wetting cycles and the correlated in- and re-activation of photosynthesis is a pattern observed in most terrestrial habitats and produced by the nocturnal dewfall or fog (del Prado & Sancho, 2007; Kershaw, 1985; Lange, 1970; Lange et al., 2006). Typically, dewfall occurred in the night when temperatures had declined substantially from their daytime maximum value. Lichens readily absorb water from dewfall, and this water activates dark respiration (CO_2 exchange below the zero line) through the remaining night time hours. Sunrise activates net photosynthesis (CO_2 exchange above the zero line) but the peak was not reached after 1–2 h when the water content started to decrease. The net photosynthesis rate of lichens depends in large part on the water content of their thalli (Green et al., 1985; Lange & Matthes, 1981). In many lichens, when the thallus is fully saturated with water, diffusion of CO_2 to the phycobiont is hindered and maximum rates of CO_2 assimilation do not occur (Lange & Tenhunen, 1981). Furthermore, at maximum water saturation in continuous light, the photobiont eventually dies because all of its products are translocated to the fungus (Harris & Kershaw, 1971). It is only when the thallus dries to a 65–

90 % of the maximum water content that peak photosynthesis occurs. Thereafter, with increasing temperatures and light intensities, both water content and net photosynthesis decline. Desiccation occurs reasonably slowly, over hours rather than minutes (Kappen, 1974). Lichen photobionts are able to maintain maximum photosynthetic activity until the water content reach the 20 % (Gasulla et al., 2009), thus, during this period lichens photosynthesize at rates that are sufficient to allow a net positive carbon gain over the year. Thalline growth rates depend on the frequency and length of this period per day and year (Lange & Matthes, 1981). On the other hand, although carbon fixation is inhibited during desiccation, electron flow through the photosystems continues, and excitation energy can be transferred from photo-excited chlorophyll pigments to $^3\text{O}_2$, forming singlet oxygen ($^1\text{O}_2$), while superoxide and hydrogen superoxide can be produced at photosystem II and photosystem I by the Mehler reaction (Halliwell, 2006; Kranner & Lutzoni, 1999; Peltier et al., 2010). Likewise, rehydration of lichens produces a burst of ROS during the first minutes and then decrease (Minibayeva & Beckett, 2001; Weissman et al., 2005). Thus, although lichens have adapted their carbon assimilation necessities to their living conditions, they will need specific mechanisms to avoid the development of oxidative damage during the desiccation and rehydration processes. We can follow two levels of protection mechanisms at the photobiont cellular level. First, processes directed to the dissipation of excess light energy as heat, which can be considered as oxidative stress avoidance mechanisms. Second, enzymatic or non-enzymatic antioxidant systems that can constitute oxidative stress tolerance mechanisms.

4. Production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during desiccation/rehydration

Aerobic organisms generate ROS as a side-product product of metabolism. In healthy cells occurs at a controlled rate, but many abiotic and biotic stress conditions lead to cellular redox imbalance and accumulation of ROS (Foyer & Noctor, 2003; Halliwell & Gutteridge, 1999; Mittler, 2002; Sharma & Dietz, 2009; Smirnoff, 1993) that causes molecular and cellular damage. Free radicals are atoms or molecules with an unpaired electron, which is easily donated, thus, most free radicals are very reactive (Elstner & Osswald, 1994; Halliwell & Gutteridge, 1999). Oxygen is a highly oxidizing molecule that forms free radicals and participates in other oxidative chemical reactions (Abele, 2002; Finkel & Holbrook, 2000). Oxygen radicals include singlet oxygen ($^1\text{O}_2$), superoxide ($\text{O}_2^{\bullet-}$), the hydroxyl radical (OH^{\bullet}) (Elstner & Osswald, 1994; Finkel & Holbrook, 2000; Halliwell & Gutteridge, 1999). Together with hydrogen peroxide (H_2O_2) that is not a free radical but is also highly reactive. ROS accumulation is the most likely source of damage to nucleic acids, proteins and lipids that can, as a final result, conduct to cell death (Zapata et al., 2005).

Every free radical formed in a living organism can initiate a series of chain reactions that will continue until they are eliminated (Halliwell, 2006). Free radicals disappear from the organism only by reactions with other free radicals or, more important, due to the actions of the antioxidant system that will be treated in section 6 of this review. Any imbalance in the redox state, which altered equilibrium in the direction of pro-oxidant molecules production, may result in univalent reduction of molecular oxygen to the potentially dangerous radical anion superoxide (Foyer & Noctor, 2003). Its formation is an unavoidable consequence of aerobic respiration (Møller, 2001) and photosynthesis (Halliwell, 2006), but imbalances may also occur under changing environmental conditions such as desiccation-rehydration cycles experienced by lichens, causing oxidative damage in cells of the photobiont and mycobiont.

Several reactions in chloroplast and mitochondria generate the free superoxide radical ($O_2^{\bullet-}$) which can in turn react with hydrogen peroxide (H_2O_2) to produce singlet oxygen (1O_2) and the hydroxyl radical (OH^{\bullet}) (Casano et al., 1999; Elstner, 1982). Moreover, in the presence of Fe or Cu (II), OH^{\bullet} radicals are formed as quickly that can attack and damage almost every molecule found in living cells as lipids, amino acids and even nucleic acids by direct attack or activation of endonucleases (Kranner & Birtic, 2005; Yruela et al., 1996). They can, for example, hydroxylate purine and pyrimidine bases in DNA (Aruoma et al., 1989), thus enhancing mutation rates. Oxidative damage to proteins changes their configuration, mostly by oxidizing the free thiol residues of cysteine to produce thiyl radicals. These can form disulphide bonds with other thiyl radicals, causing intra- or inter-molecular cross-links. After oxidative modification, proteins become sensitive to proteolysis and/or may be inactivated, or may show reduced activity (Kranner & Birtic, 2005). Singlet oxygen and OH^{\bullet} can also initiate peroxidation chain reactions in lipids. Lipid peroxides decompose to give volatile hydrocarbons and aldehydes (Esterbauer et al., 1991; Valenzuela, 1991). Accumulation of malondialdehyde, an indicator of lipid peroxidation, has been observed in lichens during dehydration (Kranner & Lutzoni, 1999). The latter can act as secondary toxic messengers that disseminate initial free radical events (Esterbauer et al., 1991). On the other hand, hydrogen peroxide is a ubiquitous constituent of plant cells under a fine homeostatic control which prevents its accumulation (Foyer & Noctor, 2003; Ros Barceló, 1998).

In most organisms, desiccation is associated with production of ROS and associated deleterious effects (Weissman et al., 2005). ROS may modify the properties of the thylakoids, thereby changing the yield of Chl *a* fluorescence, leading to photoinhibition (Ort, 2001). Under normal growth conditions, the production of ROS in cells is low ($240 \mu M s^{-1} O_2^-$ and a steady-state level of $0.5 \mu M H_2O_2$ in chloroplasts, Polle, 2001). Many stresses such as drought stress and desiccation disrupt the cellular homeostasis of cells and enhance the production of ROS ($240-720 \mu M s^{-1} O_2^-$ and a steady-state level of $5-15 \mu M H_2O_2$, Polle, 2001). The production of ROS during desiccation results from pathways such as photorespiration, from the photosynthetic apparatus and from mitochondrial respiration (Mittler et al., 2002). Whether lichens have photorespiration process is not clear and likely it depends on the species (Ahmadjian, 1993). We hypothesize (from genomic data), that probably, in algae, the glycolate oxidase reaction in peroxisomes is prevented by the presence of the alternative enzyme glycolate dehydrogenase, which does not produce hydrogen peroxide.

As we have said, lichens can tolerate dehydration, but what happen in the chloroplast when water is unavailable as reductant? When desiccated in the light, chlorophyll molecules continue to be excited, but the energy not used in carbon fixation will cause formation of singlet oxygen (Kranner et al., 2005). In these cases, oxygen can be the electron acceptor forming superoxide and here can begin the generation of ROS (Heber et al., 2001). Desiccation stress in lichens shows features similar to reversible photoinhibition (Chakir & Jensen, 1999; Jensen & Feige, 1991) because ROS production is enhanced during dessication process (Weissman et al., 2005). In the same manner, excess of illumination causes either excessive reduction on the acceptor side or oxidation on the donor side (Anderson & Barber, 1996) producing ROS in the thylakoids. Therefore, under both high illumination and dehydration, reactive oxygen species are a major cause of damage in photosynthetic organisms (Demmig-Adams & Adams, 2000; Halliwell, 1984).

On the other hand, less studied in algae and lichen but potentially important is Nitric Oxide (NO). NO is a relatively stable paramagnetic free radical molecule involved in many physiological processes in a very broad range of organisms. These functions include signal transduction, cell death, transport, basic metabolism, ROS production and degradation (Almagro et al., 2009; Curtois et al., 2008; Ferrer & Ros Barcelo, 1999; Palmieri et al., 2008). It is now clear that NO[•] and, in general, most of the Reactive Nitrogen Species (RNS) (NO[•], NO⁺, NO⁻, NO^{•2}, and ONOO⁻), are major signalling molecules in plants (Durner & Klessig, 1999) which can be synthesized during stress responses at the same time as H₂O₂ (Almagro et al., 2009). Feelisch & Martin (1995) suggested a role for NO in both the early evolution of aerobic cells and in symbiotic relationships involving NO efficacy in neutralizing ROS. In addition, NO is involved in the abiotic stress response of green algae such as *Chlorella pyrenoidosa* Pringsheim, by reducing the damage produced by photo-oxidative stress (Chen et al., 2003).

The first work that focused on NO production in lichens was published, by Weissman et al. (2005), who carried out a microscopy study of *Ramalina lacera* (With.) J.R. Laundon. These authors described the occurrence of intracellular oxidative stress during rehydration together with the release of NO by the mycobiont, but not by the photobiont. We have recently reported evidence that NO is involved in oxidative stress in lichens exposed to the oxidative agent cumene hydroperoxide (Catalá et al., 2010) Our group has studied the role of NO during rehydration of the lichen *Ramalina farinacea* (L.) Ach., its isolated photobiont partner *Trebouxia* sp. and *Asterochloris erici* (formerly *Trebouxia erici*). The results showed that lichen NO plays an important role in the regulation of lipid peroxidation and photobiont photo-oxidative stress during rehydration. Its role is similar in plants and animals where NO is known to modulate the toxic potential of ROS and to limit lipid peroxidation, acting as a chain-breaking antioxidant to scavenge peroxy radicals (Darley-Usmar et al., 2000; Kroncke et al., 1997; Miranda et al., 2000). Our data showed that rehydration is accompanied by ROS and NO generation and thus confirmed the results of Weissman et al. (2005). Moreover, the inhibition of NO action altered the photosynthetic activity of the photobionts suggesting that NO is involved in PSII stabilization and could be related with the limited role of classical antioxidant systems during desiccation-rehydration cycles in *Asterochloris* photobionts. These results point to the importance of NO in the early stages of lichen rehydration. However, there are needed further studies on NO function, or on the occurrence of NO in other lichens.

5. Mechanisms of light energy dissipation in lichen algae

Plants have developed mechanisms to prevent the formation of ROS by dissipating the excess of energy as heat. This phenomenon comprises several processes, which applying a nomenclature derived from Chl *a* fluorescence theory are globally known as non-photochemical quenching (denoted as NPQ or q_N). The light energy when reach the photosystems is transformed in heat, fluorescence or photochemistry. These processes compete among them and then make possible estimate the proportion of light energy employed in photosynthetic electron transport or dissipated as heat from direct measurement of the variation of fluorescence emission. The electron transfer from the reaction center chlorophyll of PSII (P680) to the primary quinone acceptor of PSII (Q_A) produces losses in fluorescence emission in a process known as photochemical quenching. Photochemical quenching (q_P) reaches maximal values when all the quinone pool is oxidized and electron

transport is not impaired (open reaction centers) and minimal values if all the quinone pool is reduced (closed reaction centers). The value of q_p usually maintains a non-linear relationship with the actual proportion of reduced quinone (Kramer et al., 2004). The rate of heat loss is correlated with the non-photochemical quenching of fluorescence; this is, with the loss of fluorescence emission that is independent of photochemical events and results in heat dissipation of light energy (Baker, 2008). In consequence, the maximum fluorescence emission (termed F_m) will be registered when all reaction centers are closed (what is obtained by a saturating flash of light) and mechanisms of thermal energy dissipation are inactivated (what is assumed to happen within a dark period of about 20 min for most vascular plants). After new exposure to light, the mechanisms of heat dissipation become active and after a new saturating flash the value of the fluorescence peak (F_m') will be lower. Hence, the expression $NPQ = (F_m/F_m') - 1$ (Bilger & Björkman, 1990) describes the extent of energy dissipation. When a leaf is kept in the dark, Q_A becomes maximally oxidized and the heat dissipation mechanisms are relaxed. Exposure of a dark-adapted leaf to a weak modulated measuring beam (photosynthetically active photon flux density -PPFD- of *ca.* $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) results in the minimal level of fluorescence, called F_o (Baker, 2008). If taken into account the maximal variable fluorescence after dark adaptation ($F_m - F_o$) and after a period in the light ($F_m' - F_o'$), an alternative expression (Schreiber et al., 1986) for non-photochemical quenching can be calculated like $q_N = 1 - (F_m' - F_o') / (F_m - F_o)$.

According to Krause & Weis (1991), non-photochemical quenching can be related to three different events: high trans-thylakoidal pH gradient (q_E), to state I-state II transitions (q_T) and to photosystem II photoinhibition (q_I). The "energy-dependent" quenching (q_E), originated by the formation of a proton gradient across the thylakoidal membranes, is the main mechanism implied in the development of NPQ (Krause & Weis, 1991; Müller et al., 2001). This q_E is characterized by its rapid relaxation kinetics, occurring within 3 min of darkness (Li et al., 2002; Munekage et al., 2002). Under excessive light, an elevated proton concentration in the thylakoid luminal space activates violaxanthin de-epoxidase, generating antheraxanthin and then zeaxanthin. The increase of violaxanthin deepoxidation (DPS) leads to an increase of thermal energy dissipation that is correlated with the NPQ parameter of chlorophyll fluorescence (Demmig-Adams et al., 1996). The generation of NPQ requires also the participation of a protein associated to PSII, the protein Psbs of the light harvesting complex antenna, which can change its conformation at lower pH and increase its affinity for zeaxanthin. De-epoxidated xanthophylls then binds to the subunit S where could accept the excitation energy transferred from chlorophyll and thereby act as a direct quencher in NPQ (Holt et al., 2004; Krause & Jahns 2004; Niyogi et al., 2004; Ruban et al., 2002; Spinall-O'Dea et al., 2002). An alternative explanation is that PsbS alone can cause the quenching and zeaxanthin acts as an allosteric activator, but not as the primary cause of the process (Crouchman et al., 2006; Horton et al. 2005).

The transthylakoidal proton gradient is generated by water splitting in the thylakoid lumen, but also by the Q cycle of quinones around cytochrome b_6/f complex. The latter allows that the proton gradient and NPQ generation (like ATP production) can be maintained by cyclic electron flow and probably by a thylakoidal NADH dehydrogenase complex (Guéra et al., 2005). In some mosses and chlorolichens has been described the possibility of the activation of energy dissipation by CO_2 dependent protonation. Carbon dioxide, which, at a pK of 6.31, acts as a very weak protonating agent, is capable of promoting NPQ in a light independent way, provided some zeaxanthin is present (Bukhov et al., 2001; Heber et al., 2000; Heber, 2008; Kopecky et al., 2005).

In vascular plants NPQ changes in response to diurnal variations in the light environment. Large changes in the composition of the xanthophyll cycle are observed over the course of the day. This consisted of increases and subsequent decreases in the zeaxanthin and antheraxanthin content of the leaves that paralleled the changes in incident PFD rather closely (Adams & Demmig-Adams, 1992). There are also acclimations to the plant light regime. Sun plants or sun acclimated leaves possess a higher capacity for the use of light in photosynthesis and also for rapid increases in xanthophyll cycle-dependent energy dissipation. Sun-grown leaves typically exhibit a larger total pool size of the xanthophyll cycle components as well as a greater ability to convert this pool to antheraxanthin and zeaxanthin rapidly under high light (Demmig-Adams & Adams, 1993, 1996). Finally, seasonal changes in NPQ have been described for several evergreen species (Adams et al., 2001; Zarter et al., 2006a, 2006b).

Most green algal photobiont cultures require low light intensities of 10-30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Ahmadjian, 1967a; Friedl & Büdel, 2008). One reason which could justify the necessity of low intensities for lichen algae culture is that compared to surficial light measurements, the light reaching the photobiont is reduced by 54-79% when dry, and 24-54% when fully hydrated (Büdel & Lange, 1994; Dietz et al., 2000; Ertl, 1951; Green et al., 2008). During desiccation a decrease in fluorescence emission is observed in lichens (Veerman et al., 2007). This decrease is likely associated with their phototolerance and could be caused by structural changes in the thallus that induce changes in light-scattering and shading properties or by changes in shape and aggregation of algae (de los Rios et al., 2007; Scheidegger et al., 1995; Veerman et al., 2007). The thallus also offers some protection against photodamage through the use of light-absorbing pigments (Gauslaa & Solhaug, 1999; Holder et al., 2000). All this kind of features decreases the exposure of the photosynthetic apparatus of the photobiont to light and can be described as sunshade mechanisms (Veerman et al., 2007). However, shading or the production of sun-protectant pigments reduce but cannot prevent photooxidation. Photosynthetic pigments absorb light, whether the organisms are hydrated or desiccated, but energy conservation by carbon assimilation is possible only in the presence of water. Photosynthetic reaction centers can remain intact during desiccation in lichens and then photoreactions threaten to cause severe photooxidative damage (Heber & Lüttge, 2011).

Piccotto & Tetriach (2010) found some similarities of lichens with the acclimation of vascular plants to the light regime, as the photobiont activity of lichen should be significantly modulated by the growth light regime of the thallus. According to these authors, lichen chlorobionts show the same general variation patterns described in "sun" and "shade" leaves, with optimisation of light absorption and harvesting capacity in chlorobionts of thalli grown in shaded habitat, and increased photosynthetic quantum conversion and lower Chl *a* fluorescence emission in chlorobionts of thalli grown under direct solar irradiation. However, this comparison should be complicated by the influence in lichens of extrinsic factors, as nitrogen availability, or intrinsic factors, as light transmittance through the peripheral mycobiont layer. These "sun" and "shade" patterns are maintained by the chlorobionts independently of the symbiosis. Our group isolated the two species of phycobionts that are always coexisting in the lichen *Ramalina farinacea*, and we observed that one species, so called TR9, was better adapted to high irradiances than the other one, so called TR1 (Casano et al., 2010). The proportion of the two phycobionts in the lichen changes depending on the local conditions, allowing the lichen behaves either as a "sun" or

as “shade” species. The ecophysiological plasticity of this symbiosis allows the lichen proliferates in a wide variety of habitats.

In lichens, the exposure to high light in the hydrated state produces photoinhibition in chlorolichens and cyanolichens, an effect much more pronounced in cyanolichens, which do not reverse photosynthetic depression after a recovery period as chlorolichens do. Photoinhibition can be largely diminished if both kinds of lichens are in the desiccated state or become desiccated during the period of high light exposure (Demmig-Adams et al., 1990a). Demmig-Adams et al. (1990b) described significant increases of zeaxanthin content after two hours of exposure to high light of six chlorolichens previously adapted to a low light regime. This effect was not observed in cyanolichens. Following treatment of the thalli with an inhibitor of the violaxanthin de-epoxidase, dithiothreitol, the response of green algal lichens to light became very similar to that of the blue-green algal lichens. Thus, Demmig-Adams et al. (1990b) proposed that the higher light stress in blue-green algae lichens is related to the absence of an effective accumulation of zeaxanthin (lack of the xanthophyll cycle) in cyanobacteria. On the other hand, Heber & Shuvalov (2005) and Heber (2008) report differences in the recovery under different light treatments between mosses collected in autumn-winter or in spring-summer, indicating seasonal acclimation of poikilohydric organisms to the light regime, but they found that in this case it was independent of zeaxanthin accumulation. In lichens the xanthophyll cycle activation cannot either be assumed as a general response to light dissipation under stress conditions because some lichen species, like *Pseudevernia furfuracea* or the isolated lichen photobiont *Trebouxia excentrica*, do not increase the xanthophyll DPS upon dehydration or rehydration (Kranner et al., 2003, 2005).

Relationship between NPQ and desiccation tolerance in lichens has been studied during the last years. For instance, Calatayud et al. (1997) found that in the thallus of *Parmelia quercina*, NPQ increases during dehydration, which seems to be related with the conversion from violaxanthin to zeaxanthin observed during the desiccation. These effects were also observed in *Ramalina maciformis* (Zorn et al., 2001). Fernández Marín et al. (2010) described in the lichen *Lobaria pulmonaria* that violaxanthin deepoxidase needs a time to be activated during desiccation and then de-epoxidation of violaxanthin to zeaxanthin occurs only when the tissue has lost most of its water and dehydration is slow. Kranner et al. (2005) described that activation of the xanthophyll cycle in *Cladonia vulcani* is dependent of the symbiosis because an effective accumulation of zeaxanthin can be observed in the lichen during desiccation and rehydration processes, but not in its isolated photobiont. In accordance, during a study carried out in the isolated photobiont *Asterochloris erici* (Gasulla et al., 2009) we did not found significant differences in the deepoxidation state of xanthophylls after rapid or slow desiccation treatments when compared with controls. On the other hand, a significant increase in the deepoxidation state was registered after exposure of the isolated *A. erici* to high light intensities. Kosugi et al. (2009) found that the responses to air drying and hypertonic treatments of *Ramalina yasudae* and its isolated *Trebouxia sp.* photobiont are different in three ways: 1) PSII activity is completely inhibited in the desiccated lichen but not in the isolated photobiont; 2) the dehydration induced quenching of PSII fluorescence was lower in the lichen than in the photobiont; and 3) the isolated *Trebouxia* was more sensitive to photoinhibition than the *R. yasudae* thalli. The authors proposed that a lichen substance or mechanism lost during the photobiont isolation could be implied in light

dissipation. In any case, all these results indicate that the activity of the algae is modulated by the symbiotic association with fungi, but also that other factors, as the speed of desiccation can influence on the photosynthetic activity after rehydration.

Alternative mechanisms of energy dissipation have been described to take place in mosses and lichens, where new quenching centres are functional during desiccation (Heber et al., 2006a, 2006b, 2007; Heber, 2008). In a work based on changes on the emission spectra of chlorophylls, Bilger et al. (1989) proposed that with green algal symbionts desiccation induces a functional interruption of energy transfer between the light harvesting Chl *a/b* pigment complex and PSII and that this can be largely restored by rehydration with humidified air. Heber et al. (2006a, 2006b) described that the recovery of fluorescence levels after drying was better in dark dried mosses than in sun-dried mosses, an effect that the authors can not ascribe to zeaxanthin and classical NPQ protection. Heber & Shuvalov (2005) found the existence in briophytes and lichens of an alternative quencher of chlorophyll fluorescence characterised by a long wavelength (720 nm) emission. Helped by results obtained in desiccated spinach leaves, Heber (2008) proposed that electrons can be redirected in the dried state from pheophytin to a secondary chlorophyll placed very close to P₆₈₀, implying the formation of the radical pair P₆₈₀⁺Chl⁻. A further and slower recombination between Chl⁻ and a carotene molecule positively charged should complete a photoprotective cycle in desiccated leaves. The authors argued that the similarity of fluorescence emission spectra of desiccated leaves with those obtained in a desiccated fern and a desiccated moss should justify the extrapolation of this model to poikilohydric plants. A stronger support for the hypothesis of alternative sinks for energy or electrons during desiccation was obtained by Veerman et al. (2007) with steady-state, low temperature, and time-resolved chlorophyll fluorescence spectroscopy. These authors presented a model in which a pigment molecule with a peak emission at 420 nm should act as a sink for energy accumulated on P680 in the lichen *Parmelia sulcata* under desiccation. Heber et al. (2007) afford more evidences for the existence of these alternative mechanisms, as they found in dehydrated mosses and lichens a quenching mechanism independent of light activation. This mechanism is probably dependent of conformational changes in a protein-pigment complex as is inhibited by glutaraldehyde or heat treatments. This mechanism is also dependent of the speed of desiccation, as fast drying is less effective in decreasing chlorophyll fluorescence than slow drying. Gasulla et al. (2009) found that the basal fluorescence (F_0) values in desiccated *Asterochloris erici* were significantly higher after rapid dehydration, than after slow dehydration, suggesting higher levels of light energy dissipation in slow-dried algae. Higher values of PSII electron transport were recovered after rehydration of slow-dried *A. erici* compared to rapid-dried algae. The authors suggest that there is probably a minimal period required to develop strategies which will facilitate transition to the desiccated state in chlorobionts. In this process, the xanthophyll cycle and classical antioxidant mechanisms play a very limited role. More recently, Komura et al. (2010) have found proof that the quencher of chlorophyll fluorescence under desiccation conditions is not a chlorophyll molecule and suggest a new kind of quenching in PSII antenna or aggregation in PSII. The mechanisms implicated in protection under desiccation should be dependent of the desiccation rate, independent of light and probably associated to conformational changes in a chlorophyll-protein complex (Heber et al., 2007; Heber, 2008; Heber et al., 2010; Heber & Lüttge, 2011). Finally, Heber et al. (2011) propose that photoprotection is achieved by the drainage of light energy out of the reaction centers.

6. Antioxidant systems in lichen algae

Photobionts, as aerobic organisms, have to prevent and control oxidative stress damages through a complex antioxidant system capable to maintain controlled the levels of ROS, what is known as redox homeostasis. This defence system has co-evolved with aerobic metabolism to counteract oxidative damage from ROS (Gülçin et al., 2002). It has been studied in detail in plants, and includes enzyme activities of the so-called ascorbate-glutathione cycle (Asada, 1994; Foyer & Halliwell, 1976; Mittler, 2002), superoxide dismutase (SOD; Bowler et al., 1992), peroxidases (POX; Esteban-Carrasco et al., 2000, 2001; Ros Barceló et al., 2007; Zapata et al., 1998), catalase (CAT; Mittler, 2002) along with redox metabolites, like ascorbic acid, and glutathione (GSH; Noctor & Foyer, 1998). In the ascorbate-glutathione cycle, the two major antioxidant molecules, ascorbate and glutathione, play an important role as reductants, and they are involved in the scavenging of H_2O_2 produced by SOD (Kranner & Birtic, 2005; Lascano et al., 1999). Indeed, Superoxide dismutases catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and prevent the further and dangerous conversion into OH^{\bullet} (Casano et al., 1997). Peroxidases catalyse the oxidation of a wide range of substrates at the expense of H_2O_2 (Ros Barceló et al., 2007; Zapata et al., 2005). Finally, catalases break down H_2O_2 very rapidly producing H_2O and O_2 , but are much less effective than peroxidases at removing H_2O_2 because of their lower affinity (high K_m) to H_2O_2 (Kranner & Birtic, 2005).

Concerning to antioxidant metabolites, glutathione and ascorbate are the main antioxidants present in organisms. Glutathione are involved in scavenging of the highly reactive OH^{\bullet} by a cycle that includes the glutathione reductase enzyme (GR) to recover the biologically active glutathione molecule (Noctor & Foyer, 1998). On the other hand, ascorbate reacts rapidly with OH^{\bullet} , $O_2^{\bullet-}$ and 1O_2 (Halliwell & Gutteridge, 1999), forming monodehydroascorbate (MDA) and then dehydroascorbate (DHA). Regeneration of Asc may occur via a Mehler peroxidase reaction sequence or through the Asc-GSH cycle (Foyer & Halliwell, 1976).

In desiccation tolerant organisms, as lichens, the desiccated state is characterised by little intracellular water and almost no metabolic activity. Many deleterious effects are associated to this state, such as, irreversible damage to lipids, proteins and nucleic acids through Maillard reactions and ROS (Kranner et al., 2002). During their lifetime, lichens undergo continuous cycles of dehydration-rehydration and therefore they have to must be able to (i) limit the damage to a repairable level, (ii) maintain physiological integrity in the dried state, and (iii) mobilise mechanisms upon rehydration that repair damage suffered during desiccation and subsequent rehydration (Bewley, 1979; Oliver & Bewley, 1997; Oliver et al., 2000). In a first conclusion, desiccation tolerance and prolonged longevity in the desiccated state seems to depend on the ability to prevent light damage (as discussed in the previous section) and activate the biochemical mechanism to scavenge free radicals produced during dehydration-rehydration cycles, using the two described pathways: antioxidant molecules such as glutathione or ascorbate and antioxidant enzymes capable of scavenging free radicals (Kranner & Birtic, 2005). Particularly, ROS-antioxidant interactions has been described in lichens and it is well known that an enhancement of antioxidant status occurs in the symbiotic partnership being more resistant to environmental stress than either partner alone (Kranner et al., 2005). However, within the few studies carried out with lichens, there is not a clear relationship between desiccation tolerance and antioxidant levels. Cellular activities of the antioxidant enzymes ascorbate peroxidase, catalase, and superoxide

dismutase as well as the auxiliary enzyme glutathione reductase and the pentose-phosphate pathway key enzyme glucose-6-phosphate dehydrogenase were shown to increase, decrease, or remain unchanged in response to desiccation and rehydration, depending on the species and the experimental conditions (Weissman et al., 2005). For instance, Mayaba & Beckett (2001) observed that activities of SOD, CAT, and ascorbate peroxidase (AP) were similar during wetting and drying cycles in *Peltigera polydactyla*, *Ramalina celastri* and *Teloschistes capensis*, which grow in moist, xeric and extremely xeric habitats, respectively. Kranner (2002) neither observed a correlation between GR activity and the different degrees of desiccation-tolerance of three lichens, *Lobaria pulmonaria*, *Peltigera polydactyla* and *Pseudevernia furfuracea*. Weissman et al. (2005) even reported that after rehydration *Ramalina lacera* lost almost all CAT activity and SOD decreases by 50-70%. In our laboratories we have observed that during both desiccation period and recovery of *Asterochloris erici*, the levels of superoxide dismutase and peroxidase decreased under both slow and rapid dehydration. Thus, in *A. erici* a longer dehydration time does not lead to a higher accumulation or preservation of classical antioxidants during dehydration/desiccation (Gasulla et al., 2009). Therefore, as a general conclusion, enzymatic antioxidants are perhaps more likely to be involved in removing ROS produced during normal metabolism or by other stresses rather than during rehydration following severe desiccation (Kranner et al., 2008).

In many organisms, the major water-soluble low-molecular weight antioxidants are the tripeptide GSH (glutathione, γ -glutamyl-cysteinyl-glycine) and ascorbate (Noctor & Foyer, 1998). Ascorbate is known to be a non-enzymatic antioxidant of major importance to the assimilatory and photoprotective processes, its function being central to the defence system. Ascorbate acts as an antioxidant by removing hydrogen peroxide generated during photosynthetic processes in a group of reactions termed the "Mehler peroxidase reaction sequence" (Asada, 1994). Also, ascorbic acid may act as a direct electron donor in photosynthetic and mitochondrial electron transport (Miyake & Asada, 1992) in the ascorbate-glutathione cycle (Foyer & Halliwell, 1976). In addition, it is a cofactor for violaxanthin de-epoxidase in chloroplasts. In lichens, it has been reported that the ascorbate plays an important antioxidant role against oxidative stress, such as excessive light or atmospheric pollution (Calatayud et al., 1999). In desiccation-tolerant higher plants, ascorbate forms the first line of defense against oxidative damage (Kranner et al., 2002). However, Kranner et al. (2005) did not find any relationship between ascorbate and the desiccation tolerance of the lichen *Cladonia vulcanii* nor its photobiont. Furthermore, ascorbate levels were undetectable in *A. erici* (Gasulla et al., 2009).

On the other hand, GSH not only can scavenge ROS reacting with $\text{OH}\cdot$ to form $\text{GS}\cdot$; it can also react with another $\text{GS}\cdot$, forming glutathione disulphide (GSSG) (Kranner, 2002). In addition, the redox couple of glutathione (GSH-GSSG) is involved in protecting protein thiol-groups by forming protein-bound glutathione (PSSG) (Kranner & Grill, 1996). In plants, accumulation of GSSG is often correlated with increased stress. Indeed, GSSG can be recycled by the NADPH dependent enzyme GR and in desiccation-tolerant organisms GSSG accumulates during desiccation and is re-reduced to GSH during rehydration (Kranner et al., 2006). Going back to the work of Kranner (2002), it has been reported that desiccation caused oxidation of almost all GSH in the lichens *Lobaria pulmonaria*, *Peltigera polydactyla* and *Pseudevernia furfuracea*, and rehydration caused the inverse effect. However, after a long desiccation period, in *P. furfuracea* the recovery of the initial concentration of GSH was very rapid, while *P. polydactyla* did not re-establish the GSH pool initial level. It has been demonstrated that NADPH dependent enzyme GR activity is high during rehydration

process and therefore it is not a limiting factor to explain the differences between different lichen species. It is more likely that the capacity to reduce GSSG was correlated with the reactivation or synthesis “de novo” of glucose-6-phosphate dehydrogenase, an enzyme of the oxidative pentose phosphate pathway (Kranner, 2002).

7. Conclusion

Two different ways to protect the photosynthetic machinery of lichen chlorobionts were considered in this review. The first one, based in the dissipation of light energy and the second based in the presence of antioxidant activities. Dissipation of light energy at the level of the chlorobiont's PSII can be achieved by the classical mechanisms based in the xanthophyll cycle, but proof is increasing in favour of the presence of new sinks for conversion of light energy into heat. The activation of these sinks is independent of zeaxanthin accumulation and probably requires an additional pigment and conformational changes in some protein(s) associated to the reaction center or the antenna. Further research is needed to determine the chemical nature and action mechanism of this (or these) alternative energy sink(s).

The lichen is more resistant to oxidative stress than the photobiont or the mycobiont alone. The main antioxidant substance in lichens seems to be glutathione. However, there is not a clear relationship between desiccation tolerance and antioxidant levels. Glutathione, ascorbic acid and antioxidant activities such as SOD or POX can increase, decrease, or not change, depending on the desiccation tolerance of the organism as well as the mode and duration of the treatment. Probably, constitutive levels of these antioxidants are high enough to protect cells against abrupt changes in the environmental conditions, mainly light intensity and humidity.

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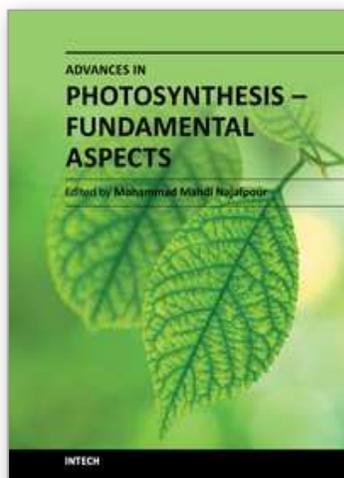
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Photosynthesis is one of the most important reactions on Earth. It is a scientific field that is the topic of many research groups. This book is aimed at providing the fundamental aspects of photosynthesis, and the results collected from different research groups. There are three sections in this book: light and photosynthesis, the path of carbon in photosynthesis, and special topics in photosynthesis. In each section important topics in the subject are discussed and (or) reviewed by experts in each book chapter.

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