DEPARTAMENT DE BOTÀNICA, BIOLOGIA VEGETAL I HORTICULTURA

INSIGHTS ON DESICCATION TOLERANCE OF THE LICHEN PHOTOBIONT TREBOUXIA SP. PL. IN BOTH THALLINE AND ISOLATED ONES.

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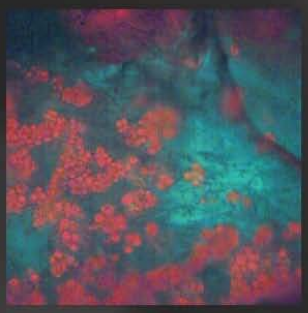
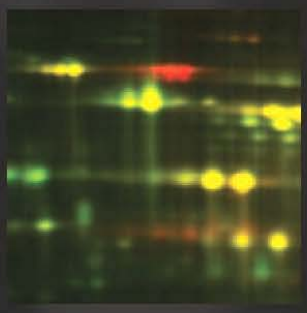
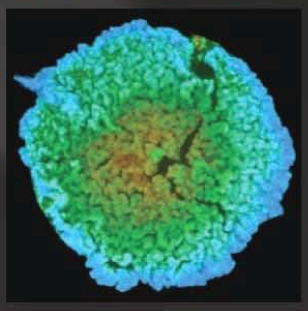


INSIGHTS ON DESICCATION TOLERANCE OF THE LICHEN PHOTOBIONT TREBOUXIA SP. PL. IN BOTH THALLINE AND ISOLATED ONES

Francisco Gasulla Vidal

TESIS DOCTORAL

UNIVERSITAT DE VALÈNCIA
FACULTAT DE CIÈNCIES BIOLÒGIQUES
DEPARTAMENT DE BOTÀNICA
INSTITUT CAVANILLES DE BIODIVERSITAT I BIOLOGIA EVOLUTIVA

INSIGHTS ON DESICCATION TOLERANCE OF THE LICHEN PHOTOBIONT *TREBOUXIA* SP. PL. IN BOTH THALLINE AND ISOLATED ONES

MEMORIA PRESENTADA POR
EL LICENCIADO
FRANCISCO GASULLA VIDAL
PARA OPTAR AL GRADO DE
DOCTOR EN CIENCIAS BIOLÓGICAS.
VALENCIA, 2009

La Dra. Eva Barreno Rodríguez, Catedrática de Botánica de la Facultad de Ciencias Biológicas de la Universitat de València, el Dr. Alfredo Guéra Antolín profesor titular del Departamento de Biología Vegetal de la Universidad de Alcalá de Henares, y la Dra. Ángeles Calatayud Chover investigadora del Departamento de Horticultura del Instituto Valenciano de Investigaciones Agrarias,

CERTIFICAN:

Que la memoria titulada "Insights on desiccation tolerance of the lichen photobiont *Trebouxia* sp. pl. in both thalline and isolated ones" que presenta Francisco Gasulla Vidal para aspirar al Grado de Doctor en Ciencias Biológicas ha sido realizada bajo nuestras direcciones.

Y para que conste a los efectos oportunos expedimos el presente certificado en Burjassot, diciembre de 2009.

Fdo. Dra. Eva Barreno Rodríguez

Fdo. Dr. Alfredo Guéra Antolín

Fdo. Dra. Ángeles Calatayud Chover

*

"A butterfly flapping its wings in Beijing
can cause a hurricane next month in New York"
J. Gleick

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Fuerza y honor

Contents

ABBREVIATIONS

3NBBM	3X N Bold's Basal Medium
A	antheraxantin
c-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAN	2,3-Diaminonaphthalene
$DCFH_2$-DA	2,7-Dichlorodihydrofluorescein diacetate
DCMU	3-(3',4'-dichlorophenyl)-1,1-dimethylurea
DPS	de-epoxidation state of the xanthophyll cycle
ETR	relative electron transport rate
F'_o	minimum fluorescence intensity in illuminated samples
F'_v/F'_m	maximum quantum yield of PSII in light-adapted samples
F'_m	maximum fluorescence intensity in illuminated samples
F_m	maximal fluorescence intensity in dark-adapted samples
F_o	minimal fluorescence intensity in dark-adapted samples
F_s	fluorescence intensity during exposure to light
F_v	variable fluorescence ($F_v = F_m - F_o$)
F_v/F_m	maximal quantum yield of PSII photochemistry
Hsp	Heat shock proteins
LEA	Late Embryogenesis Abuntant proteins
MV	methyl viologen.
NO	nitric oxide
P700	reaction center of the PSI
PAR	photosynthetically active radiation
POX	peroxidase
PSI	Photosystem I
PSII	Photosystem II
q_E	energy-dependent quenching
q_I	photoinhibitory quenching
q_M	middle phase quenching
q_N	non-photochemical quenching

q_P	photochemical quenching
r	growth rate
r.h.	relative humidity
R_i	protein spot standardized log abundance ratio
ROS	reactive oxygen species
RWC	relative water content
SOD	superoxide dismutase
T_2	population doubling time
TTC	triphenyltetrazolium chloride
V	violaxathin
Z	zeaxanthin
ϕ_{PSII}	quantum yield of PSII during exposure to light

1. GENERAL INTRODUCTION

1.1. Types of lichen photobionts

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 *Trebouxia*, *Trentepohlia*, *Coccomyxa*, and *Dictyochloropsis* (Gärtner 1992). These authors assessed that more than 50 % of all lichen species are associated with *Trebouxia* species. However, this is just an estimation since in only 2% of the described lichen species the photobiont genus is reported (Tschermak-Woess 1989).

1.2. Taxonomy and circumscription of the genus *Trebouxia* Puymaly

Famintzin & Boranetzky (1867) and Schwendener (1869) were the first who published specific descriptions and illustrations of coccal green algae resembling *Trebouxia* and named them "*Cystococcus humicola* Nägeli". Puymaly (1924) suggested the use of the name *Trebouxia arboricola* Puymaly instead of *Cystococcus humicola* for all algae isolated from *Xanthoria parietina*. For this new genus Puymaly adopted a diagnosis that had already been given by Treboux (1912). The first authors (Chodat 1913; Warén 1920) roughly described some species of *Trebouxia* or *Cystococcus* on the basis of a few morphological characters. Archibald (1975) carried out the first monographic study for the *Trebouxia* genus. In this work, he proposed the division of the genus in *Pseudotrebouxia* Archibald, which included those species with vegetative cell division, and in *Trebouxia* Puymaly, for species that do not. Gärtner (1985) and Tschermak-Woess (1989) also recognized differences in the formation of non-motile reproductive cells, but rejected Archibald's concept. According to Ettl and Gärtner (1984), all species of *Trebouxia* only undergo protoplast division to form zoospores and autospores and not vegetative cell division. Tschermak-Woess (1989) proposed that two subgroups of *Trebouxia* be recognized: the

subgenus *Trebouxia* for species that produce autospores and the subgenus *Eleutherococcus* for species that do not produce autospores. Meanwhile, Friedl (1989b; 1993), on the basis of his comparative study of pyrenoid ultrastructure, did not find evidence to support the division of *Trebouxia* into two genera and found variability even within one strain, although he recognized differences in autospore formation and distinguished cell cycles A and B, resulting in groupings rather similar to Tschermak-Woess' subgenera (Fig. 1.1). Additionally, Tschermak-Woess did not exclude the possible future elevation of the subgenera *Trebouxia* and *Eleutherococcus* as two separate genera; in this case, she suggested using the generic name *Asterochloris* for those species producing no autospores (Tschermak-Woess 1989). In 1980, she had already described a new algal genus and species, *Asterochloris phycobiontica* Tschermak-Woess, based on her observations of the phycobiont of lichen *Anzina carneonivea* (Anzi) Scheidegger (Tschermak-Woess 1980).

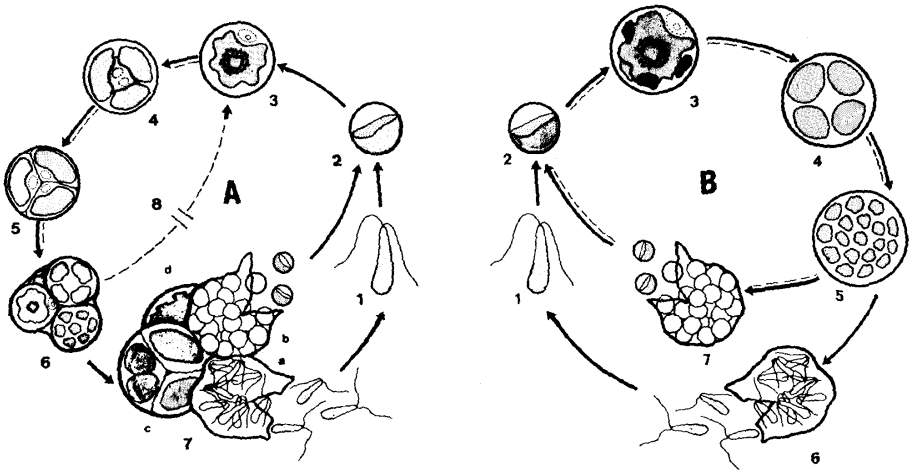


Figure 1.1.- Two types of cell cycles of *Trebouxia* photobionts. **A.** Cycle begins with a free swimming zoospore (1), that comes to rest and develops into a non-motile vegetative cell (2), which matures (3), and divides (4,5), to form autospores (6), and zoospores (7). Groups of autospores dissociate into single cells (8). a. ruptured zoosporangium releasing zoospores; b. sporangium with arrested zoospores that have formed small vegetative cells; c. autospore with beginning autospore formation; d. vegetative cell newly released form group of autospores. **B.** cycle begins with a free-swimming zoospore (1), that comes to rest and develops into a vegetative cell (2), that matures (3), and divides to form a zoosporangium (4,5). A zoosporangium can form ether free swimming zoospores (6), or arrested zoospores (7). (Drawing from T. Friedl; reprinted from Friedl, T. 1989. Inaugural dissertation, Universität Bayreuth).

The systematic position of the genus is also unclear. Following the classification in van den Hoek *et al.* (1995)*Trebouxia* is indicated as belonging to the order Pleurastrales but alternatively, it has been placed in the Microthamniales. While John & Tsarenko (John *et al.* 2002) continue to place *Trebouxia* in the large, traditional, though demonstrably artificial order, the Chlorococcales, in recognition of the current lack of consensus in applying the results of recent molecular studies.

As a consequence of this non consensus, in our work we have continued using the term *Trebouxia* for the whole genus, including those species assigned as *Asterochloris* by some authors.

1.3. *Trebouxia* description

The members of the genus *Trebouxia* are described as coccoid green algae that may form autospore packages in the form of tetrades or larger cell that are occasionally unilaterally thickened, without a gelatinous sheath. Chloroplast exists in complexes. Cell shape is spherical to elliptic. The cell walls are thin, only occasionally unilaterally thickened, without a gelatinous sheath. Chloroplasts in adult cells assume a central position and develop lobed or crenulate edges.. Chloroplasts have one or multiple pyrenoids that are either naked or, in few species, enclosed by a starch sheath. Starch is usually deposited as starch grains in the chloroplast stroma (Fig. 1.2). The nucleus is always positioned excentrically in a pronounced indention of the chloroplast. Only asexual reproduction is known via autospore formation or naked, two flagellated zoospores with a stigma spot (Ettl & Gartner 1995; Helms 2003).

1.4. Optimal and limiting environmental conditions

In homoiomerous lichens, photobionts are intermixed with the fungal partner forming one big uniform layer. Meanwhile, in heteromerous lichens, photobionts appear in the algal layer where the cells are wrapped by hyphae and in some cases penetrated by haustoria. This layer is surrounded by the cortex, formed by densely agglutinated fungal hyphae building a protective outer layer, and by the medulla, formed by loosely interwoven

fungal hyphae which allow water and gas circulation.

Lichens are well known for their slow growth and longevity. Their radial growth is measured in millimetres per year (Hale 1983), while individual lichens live for hundreds or even thousands of years. In lichens that grow in length at lobe or branch tips two different phases of the photobiont cell cycle have been recognized: Phase 1, the cells grow till a certain type of size and divide actively into endospores, which mainly occurs at the growing areas of the thalli; Phase 2, the cells enlarge becoming senescent and dying, which happens more frequently in the mature regions (Hill 1992; Schofield *et al.* 2003). 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).

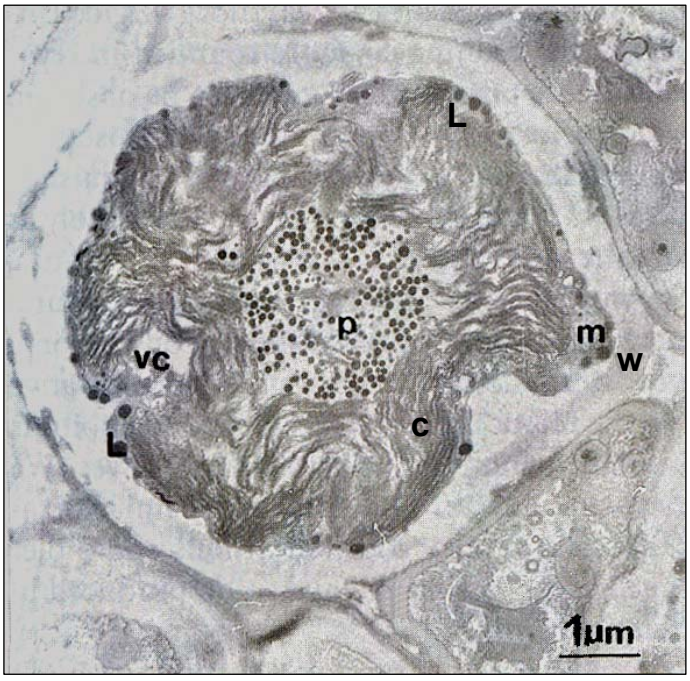


Figure 1.2.- Electron micrograph of *Trebouxia* photobiont and mycobiont cells from *Lasallia hispanica*. L, lipid bodies; m, mitochondria; p, pyrenoid; vc, vesicular complex; w, cell wall; c, chloroplast. Modified from De los Ríos & Ascaso (2002).

Similar to plants, all lichens photosynthesise. They need light to provide energy to make their own food. More specifically, algae in the lichen produce carbohydrates and the fungi take those carbohydrates to grow and reproduce. The optimal light intensity for optimal lichen growth varies widely among species. The optimum light intensity range of most algal photobionts in axenic cultures ranges between 16-27 μmol m^{-2} s^{-1}; however, some *Trebouxia* strains lose their colour when cultured at light intensities above 11 μmol m^{-2} s^{-1} (Ahmadjian 1967). Kosugi *et al.* (2009) also found that the dry isolated T*rebouxia* sp. of *Ramalina yasudae* had a much higher sensitivity to strong light than the dry lichen thalli. If the light response of cultured photobionts is similar to that of the natural forms (lichen), then there must be additional mechanisms that protect the algae in the lichen that are not present 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 (Scott 1969; Veerman *et al.* 2007; Heber *et al.* 2007).

Another important factor in the ecology of lichen is its tolerance to thermal stress. In the dry state many lichens exhibit heat resistance up to 70-75 ºC in species from sheltered microhabitats and up to 90-100 ºC in species from exposed microhabitats (Lange 1953). Likewise, if freezing (and subsequent thawing) does not occur too rapidly, many species can resume normal photosynthesis following freezing at temperatures as low as -196 ºC (liquid nitrogen) for several days (Kappen 1974), and storage in freezers (-20 to -60 ºC) for years is also tolerated (Larson 1978). However, in moist state, photosynthetic capacity may be damaged at warmer temperatures (36 – 45 ºC) and at cold temperatures (-25 ºC) (Benedict 1990; Ahmadjian 1993). Optimal growth temperatures of *Trebouxia* photobionts depend on the geographical localization of the lichen from which they are isolated. Those from temperate-zone species grow well between 18-20 ºC, while photobionts of Antarctic or alpine species have a temperature optimum near 15 ºC (Schofield *et al.* 1972; Aoki *et al.* 1998). In general, the optimum temperature range lies between 15-20ºC (Ahmadjian 1967), but there are exceptions like *Trebouxia excentrica* with an optimal temperature at 12ºC (Kranner *et al.* 2005) or 24ºC for *Trebouxia* isolated from Brazilian lichens (Cordeiro *et al.* 2005).

According to Paterson *et al.* (1983), moisture is the most important factor that influences lichen growth. The degree of thallus saturation and the fluctuation of moisture content (i.e., wetting and drying) are important components of what Scott (1969) termed a *hydroregulatory mechanism of growth control*.

1.5. Effects of water content on photosynthesis and respiration

Lichens are among poikilohydric organisms, those that cannot actively regulate their water content. Therefore, 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. Upon rehydration they recover normal photosynthetic rates within a short time span, 15-60 min (Tuba *et al.* 1996; Fos *et al.* 1999; Jensen *et al.* 1999). Most lichen-forming fungi and their photobionts are fully adapted to continuous wetting and drying cycles, but die off under continuously moist conditions (Farrar 1976a; Farrar 1976b; Dietz & Hartung 1999). Consequently, abundant epiphytic lichen growth is typically found in boreal to temperate climates, but bryophytes dominate epiphytic communities in temperate rainforests (Sillet & Antoine 2004). However, lichens may be the predominant life-form in extreme environments like cold and hot deserts.

Water uptake can be produced by the absorption of water in the liquid or in the vapour state. Green algal lichens (chlorolichens) have the ability to reactivate their photosynthetic metabolism either using water vapour or liquid water, wheras cyanobacterial lichens (cyanolichens) only do it with liquid water (Lange *et al.* 1986; Schroeter 1994). For this reason, chlorolichens with *Trebouxia* as photobiont dominate in arid, hot or cold ecosystems, but cyanolichens with internally stratified thalli (*Peltigera*, *Sticta* and *Nephroma* spp.) are abundant in moist habitats (Lange *et al.* 1986; Kappen 1988; Sillet & Antoine 2004). Historically, the relationship between thallus hydration and photosynthesis and respiration has been intensively studied. 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 (Lange 1970; Kershaw 1985; Lange *et al.* 2006; del Prado & Sancho 2007). Fig. 1.3 demonstrates studies carried out with lichen *Teloschistes capensis*, in the Negev Desert (Israel), where dewfall occurred approximately half the days of the year (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

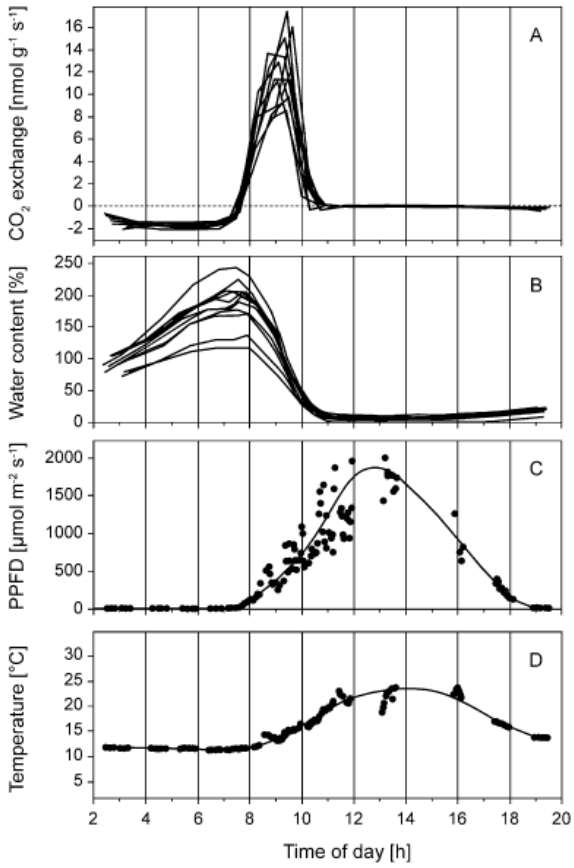


Figure 1.3.- *Teloschistes capensis*: natural dial time courses of dry-weight related CO_2 exchange of 12 samples after nocturnal moistening by fog and fog drizzle (September 10, panel A); thallus water content (panel B), incident photosynthetic active photon flux density (PPFD, panel C), and air temperature (panel D). (Image from Lange *et al.* 2006)

exchange above the zero line) but the peak was not reached until 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 (Lange & Matthes 1981; Green & Snelgar 1985). 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 of 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). During this period lichens photosynthetize at high rates that is 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).

Although lichen thalli cannot actively control the physical water relations, some variations in the structure or the inner anatomy of the thallus could be very important in the rate of water uptake and loss (Rundel 1988; Sancho & Kappen 1989; Scheidegger *et al.* 1995; Schroeter & Scheidegger 1995). Water storage capacity is higher in lichen adapted to dry habitats as a consequence of the presence of distinctive anatomical and morphological traits, like the thicker upper cortex and medulla (Fos *et al.* 1999). In this way lichens increase water holding capacity and increase the time available for photosynthetic activity. On the other hand, lichens growing in wet habitats have developed thallus adaptations to increase gas exchange and water loss. Some thalli have soredia, cyphellae, pseudocyphellae, or hyphae encrusted with secondary compounds, in order to maintain air passages even in water saturated thalli (Kershaw 1985; Green *et al.* 1985; Ahmadjian 1993; Büdel & Scheidegger 1996). Some tropical and subtropical lichens have the algal cells surrounded by only small amounts of fungal tissue, and a cortex is absent to maximize gas exchange (Snelgar *et al.* 1981; Fritz-Sheridan & Coxson 1988; Ahmadjian 1993).

1.6. Effects of desiccation on morphology and ultrastructure

Desiccated lichen thalli shrink without wilting, although they develop changes in consistency and colour. The severity of the shrinkage depends on thalline morphology. Crustose and squamose thalli, which are affixed with their whole lower surface to the substratum, shrink mainly in the vertical axis, but thalli with no tight contact to the substratum shrink in all dimensions (Honegger 2006). Photobionts with thin and hyaline walls collapse (cytorrhysis), whereas the mycobiont with thick and rigid walls cannot be sufficiently deformed during water loss and therefore cavitate (implode) (Scheidegger *et al.* 1995; Schroeter & Scheidegger 1995; Honegger *et al.* 1996; Honegger 1998). After drying, the protoplast of both photobiont and mycobiont appear in close contact with the cell walls (Honegger *et al.* 1996). During rehydration, the cytoplasm and organelles of the protoplast swell and cells recover their normal structure (Honegger 2006). In spite of the shrinkage of the protoplasts, cell membranes of the algal cells appear very well preserved (Honegger *et al.* 1996).

All *Trebouxia* photobionts have pyrenoids that contain electron-dense globules called pyrenoglobuli (Jacobs & Ahmadjian 1968). Morphology of pyrenoids may change between wet and dry state (Brown & Wilson 1968). Pyrenoglobuli have also shown *in situ* seasonal changes, i.e. *Bryoria capillaris* and *Hypogymnia physodes*, globules were smaller in autumn than in summer and were more peripheral (Holopainen 1982). Globules in *Xanthoparmelia conspersa* were numerous in the wet season and almost absent in the dry season (Ascaso & Galvan 1977). Movement of the pyrenoglobuli to the periphery of the pyrenoid matrix in lichens that have been dried or kept in the dark have been observed by some investigators, but the reason for this movement is not clear (Jacobs & Ahmadjian 1968; Brown & Wilson 1968; Ahmadjian 1993). Pyrenoglobuli store lipids and may be one reason why lichens can withstand extreme drying (Ahmadjian 1993). Lipids in the globules may be consumed under dry conditions to supply the bionts with energy and water. Pyrenoglobuli may be secondary storage products that are a result of shunting photosynthetic products to a lipid pool. This pool is used after the starch storage granules are used (Jacobs & Ahmadjian 1971). A decrease in the number of pyrenoglobuli in photobionts cells of *Parmelia sulcata* thalli that are kept in the dark supports the idea that

pyrenoglobuli are energy-storing reserves (Brown *et al.* 1988).

Changes in the ultrastructure of plasmalemma have also been reported during dehydration/rehydration cycles. Peveling and Robenek (1980) observed in the *Trebouxia* photobiont of *Hypogymia physodes* that the number and size of intramembranous particles were reduced in the dry state in comparison with the moist state. These particles are protein or lipoprotein molecules, which are inserted into the lipid part of the membrane (Bretscher 1973; Yu & Branton 1976), and, although their real function remains unclear, some authors associate them with stress responses (Peveling & Robenek 1980).

1.7. Production of reactive oxygen species (ROS) during desiccation/rehydration

Oxidative processes and increase of free radicals are usually supposed to be involved in molecular and cellular damage induced by a wide range of stresses including dehydration (Smirnoff 1993). 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 (Finkel & Holbrook 2000; Abele 2002). Several reactions in chloroplast and mithochondria 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 ($OH^{\bullet}$) (Elstner 1982). These reactive oxygen species (ROS) are accumulated during drying, especially in the presence of light (Fig. 1.4). On one hand, ROS production seems to be closely linked to respiration in a process involving desiccation-induced impairment of the mitochondrial electron chain (Leprince *et al.* 1994). 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 $^3O_2$, forming singlet oxygen ($^1O_2$), while superoxide and hydrogen superoxide can be produced at photosystem II and photosystem I by the Mehler reaction (Kranner & Lutzoni 1999; Halliwell 2006). Likewise, rehydration of lichens produces a burst of ROS during the first minutes and then decreases (Minibayeva & Beckett 2001; Weissman *et al.* 2005).

ROS are the most likely source of damage to nucleic acids, proteins and lipids. In particular, the $OH^{\bullet}$ radical can attack and damage almost every molecule found in living cells (Kranner & Birtic 2005). It 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). ROS such as $^1O_2$ and $OH^{\bullet}$ can also abstract hydrogen radicals from lipids thus initiating peroxidation. 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).

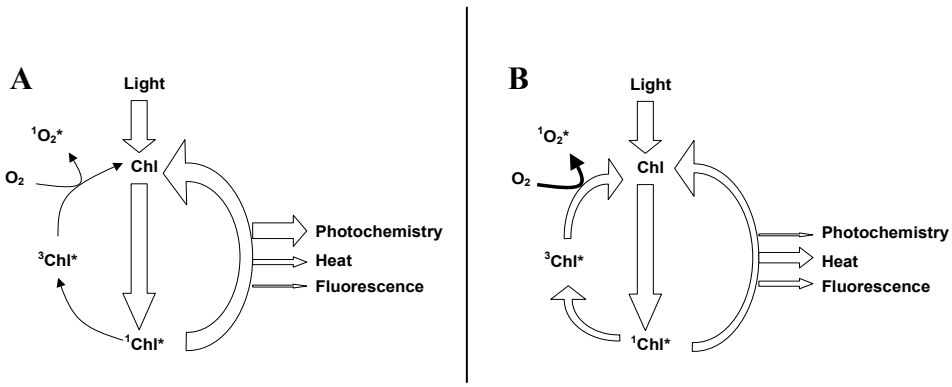


Figure 1.4.- The different pathways for de-excitation of excited chlorophyll molecules of PSII. A) Under optimal physiological conditions most of the absorbed energy is used in photosynthesis (80 – 90 %) or is thermally dissipated (5 – 15 %), only a residual fraction is re-emitted as fluorescence (0.5 – 2 %). B) Under stress conditions, such as desiccation, photochemistry may be impaired and the rate of energy dissipated by heat or fluorescence increase. In this condition excited chlorophylls may be de-excited producing reactive oxygen species.

1.8. Mechanisms of desiccation tolerance in lichens

Most of the recent studies on mechanisms on desiccation tolerance in lichens have been focused on the scavenging and prevention of the formation of reactive oxygen species. One of the principal formation centres of ROS are the photosystems, where the solar energy is absorbed and transformed into chemical energy. Under non-stressed conditions this energy is transformed into NADPH and ATP. However, when the organism is under some stress, such as high irradiation or dehydration, there is an excess of energy that can not be used in CO_2 fixation but can be transferred from photo-excited pigments onto $^3O_2$, raising it

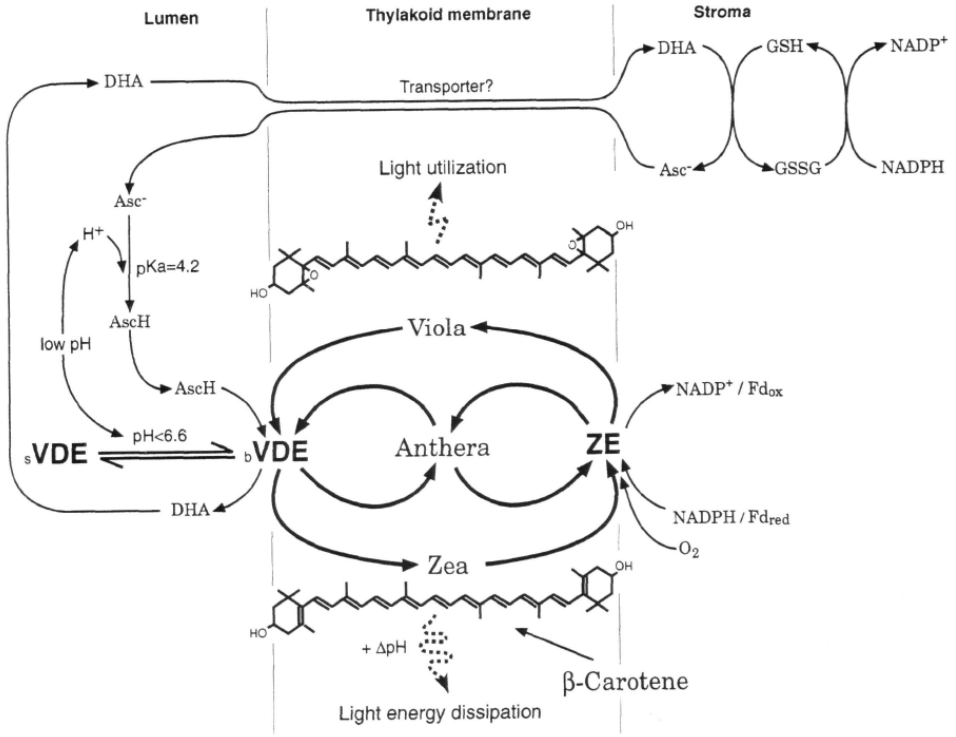


Figure 1.5. Model of the regulation of the xanthophyll cycle. sVDE, soluble VDE; bVDE, bound VDE; DHA, dehydroascorbate; Asc", ascorbate; AscH, ascorbic acid; GSH, glutathione; Viola, violaxanthin; Anthera, antheraxanthin; Zea, zeaxanthin; Fd, ferredoxin. Modified from Eskling *et al.* (1997).

to the more reactive excited-state $^1O_2$ (Halliwell 2006). Plants have developed mechanisms to prevent the formation of ROS by dissipating the excess of energy as heat, like the xanthophyll cycle (Fig. 1.5). Under excessive light, an elevated proton concentration in the thylakoid luminal space activates violaxanthin de-epoxidase, generating antheraxanthin and then zeaxanthin. This increase of violaxantin deepoxidation is correlated with an increase of thermal energy dissipation, measured as nonphotochemical quenching of chlorophyll fluorescence (NPQ) (Demmig-Adams *et al.* 1996). De-epoxidated xanthophylls binds to the light harvesting complex antenna in the photosysthem II subunit S (PsbS) (Spinall-O'Dea *et al.* 2002; Ruban *et al.* 2002) where can accept the excitation energy transferred from chlorophyll and thereby can be a direct quencher in NPQ (Holt *et al.* 2004). An alternative explanation is that PsbS alone can cause the quenching and zeaxanthin acts as an allosteric activator and is not the primary cause of the process (Horton *et al.* 2005; Crouchman *et al.* 2006). Relationship between NPQ and desiccation tolerance in lichens has seldomly been studied. In the thallus of *Parmelia quercina* NPQ increases during dehydration (Calatayud *et al.* 1997), which seems to be related with the conversion from violaxantin to zeaxanthin observed during the desiccation. These effects were also observed in *Ramalina maciformis* (Zorn *et al.* 2001) and *Cladonia vulcanii* (Kranner *et al.* 2005). Suprisingly, however, Kranner *et al.* (2005) did not find any xanthophyll cycle activity in the isolated photobiont of *Cladonia vulcanii* after dehydration.

In recent publications, evidence has been presented for new mechanisms of energy dissipation which protect desiccated mosses and lichens against photoinactivation (Kopecky *et al.* 2005; Veerman *et al.* 2007; Heber *et al.* 2007; Heber 2008). During drying of lichens chlorophyll fluorescence decrease and stable light-dependent charge separation in reaction centres of the photosynthetic apparatus is lost. Removal of structural water is suggested to produce a conformational change of a pigment-protein close to or in the core of photosystem II changing the position of bound pigments to one another within the protein. Under this configuration absorbed light energy is converted into heat within a picosecond or femtosecond depriving functional reaction centres of energy and thereby protecting them against photoinactivation. Desiccation-induced fluorescence quenching is produced by two different mechanisms, one that is activated by light, and another that is not light-dependent. The latter is induced during dehydration and requires a minimum drying

time; fast drying (<1h) produces less fluorescence quenching than slow drying. Likewise, rapidly dried lichens exposed to strong light show higher damage to the photosynthetic apparatus than lichens dried slowly. Energy dissipation is inactived and structural changes are reversed when water becomes available again facilitating the use of light for photosynthesis almost as soon as water becomes available (Heber *et al.* 2007; Heber 2008).

Scavenging of formed ROS can be carried out by enzymatic or non-enzymatic antioxidants (Fig. 1.6). Antioxidants enzymes include superoxide dismutase (SOD), catalases (CAT), peroxidases (POX) and auxiliary enzymes like mono- and dehydroascorbate reductases and glutathione reductase (GR) (Fig. 6). Within the few studies carried out with lichens, there is not a clear relationship between desiccation tolerance and levels of antioxidant enzymes. It could be expected that lichens showing higher levels of desiccation tolerance have higher antioxidant enzymatic activity than those less tolerant. However, Mayaba & Beckett (2001) observed that activities of SOD, CAT,

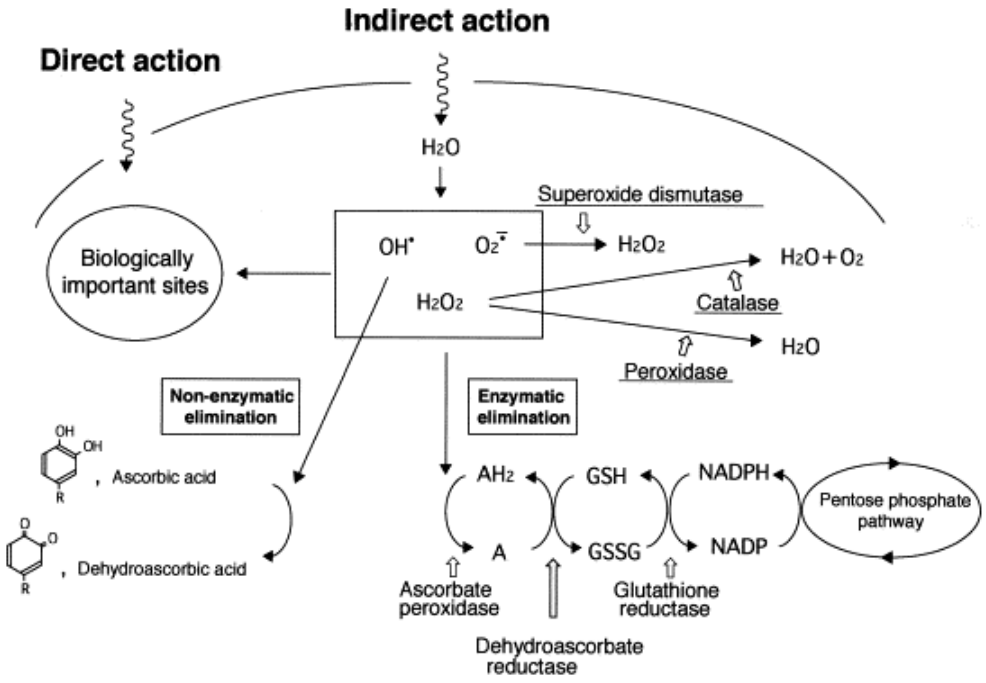


Figure 1.6.- A schematic representation of possible mechanisms operating in irradiated cells to eliminate radiation-produced oxidants. Modified from Wada *et al.* (1998).

and ascorbate peroxidase (AP) were similar during wetting and drying cycles in *Peltigera polydactila*, *Ramalina celastri* and *Teloschistes capensis*, which grow in moist, xeric and extremely xeric habitats, respectively. Kranner *et al.* (2002) neither observed a correlation between GR activity and the different degrees of desiccation-tolerance of three lichens, *Lobaria pulmonaria*, *Peltigera polydactila* and *Pseudevernia furfuracea*. Weissman *et al.* (2005) even reported that after rehydration *Ramalina lacera* loss almost all CAT activity and SOD decreases by 50-70%. By the other hand, in cultured isolated photobionts, antioxidants and antioxidants-related activities seems to be less effective, by orders of magnitude, than those of lichenized photobionts (Kranner *et al.* 2005). 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 (glutatione, 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). In lichens, it has been reported that the ascorbate play an important antioxidant role against oxidative stress, such as excessive light or atmospheric pollution (Calatayud *et al.* 1999; Caviglia & Modenesi 1999). However, Kranner *et al.* (2005) did not find any relationship between ascorbate and the desiccation tolerance of the lichen *Cladonia vulcanii* nor its photobiont. GSH can scavenge ROS reacting with $OH^{\bullet}$ to form $GS^{\bullet}$; it can also react with another $GS^{\bullet}$, forming glutathione disulphide (GSSG). GSSG can then be recycled by the NADPH dependent enzyme GR. In desiccation-tolerant organisms, GSSG accumulates during desiccation and is re-reduced to GSH during rehydration (Kranner *et al.* 2006). Desiccation caused oxidation of almost all GSH in the lichens *Lobaria pulmonaria*, *Peltigera polydactila* and *Pseudevernia furfuracea*, and rehydration caused the inverse effect (Kranner 2002). However, after a long desiccation period, in *P. furfuracea* the recovery of the initial concentration of GSH was very rapid, while *P. polydactylon* did not re-establish the GSH pool initial level. 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, which potentially supplies the NADPH required as a cosubstrate of GR (Kranner 2002). Thus, high concentrations of antioxidants, such as glutathione, or high antioxidant enzyme activities do

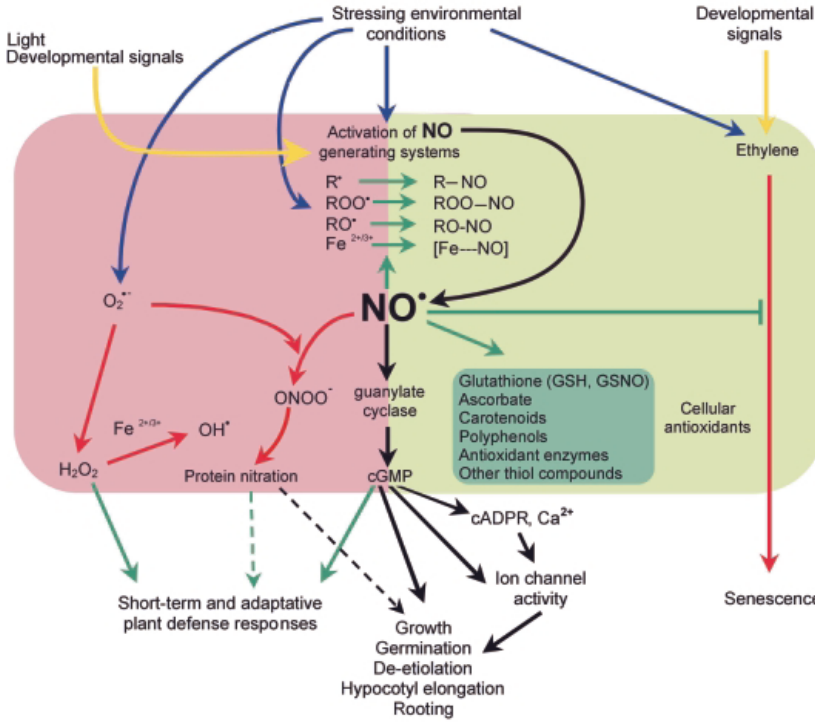


Figure 1.7.- Schematic representation of nitric oxide (NO) effects in plants. Stressing environmental clues trigger many responses within the plant cell (blue arrows). In those situations, NO actions may be either cytotoxic (pink area and red arrows) or cytoprotective (green area and arrows). NO reaction with reactive oxygen species would account for both toxicity and protection. Indirect methods of protection would also come from interaction with or activation of the cellular antioxidant system by NO. On the other side, light or developmental signals (yellow arrows) could also activate NO production and modulate processes such as greening, growth, germination or senescence. This could be accomplished through cGMP, cADPR and Ca2+/calmodulin pathways (black arrows) and/or by direct modification of target molecules (e.g. *S*-nitrosylation, ADP-ribosylation). Protein nitration by ONOO- is another putative mode for NO action. NO-mediated down regulation of ethylene-triggered responses could be a determining factor in delaying senescence (greentruncated arrow). R·, non oxygen free radicals; RO·, alcoxyl radicals; ROO·, peroxyl radicals; OH·, hydroxyl radical; ONOO-, peroxynitrite; GS-NO, nitrosoglutathione; cGMP, cyclic GMP; cADPR, cyclic ADP-ribose. Dashed lines correspond to NO-mediated effects whose chemical reaction has not been described in plants yet. Adapted from Beligni & Lamatitna (2001).

not necessarily indicate that a species is well adapted to tolerate desiccation-induced oxidative stress. Rather, the ability to rapidly re-establish the initial antioxidant concentrations, redox potentials and enzyme activities during rehydration is apparently characteristic of well-adapted species (Kranner 2002; Kranner *et al.* 2003).

In lichens, as we have seen above, there is a burst in the production of ROS during rehydration. Weissman *et al.* (2005) reported that this oxidative stress was coupled with the release of nitric oxide (NO) by the mycobiont in the lichen *Ramalina lacera* (Weissman *et al.* 2005). The bioactive gas NO has multiple biological functions in a very broad range of organisms. These functions include signal transduction, intracellular signalling, cell death, transport, basic metabolism, reactive oxygen species (ROS) production and degradation (Courtois *et al.* 2008, Palmieri *et al.* 2008), among others (Wilson *et al.* 2008) (Fig. 1.7). It is well-known that NO exerts both pro-oxidant and antioxidant effects, depending on the ambient redox status, the presence of other reactants, and the nature of the reaction (Darley-Usmar *et al.*, 2000). NO has been postulated as one of the first antioxidant mechanisms to have evolved in aerobic cells (Kröncke *et al.* 1997, Mallick *et al.* 2002). This idea builds on the work of Feelisch & Martin (1995), who suggested a role for NO in both the early evolution of aerobic cells and in symbiotic relationships involving NO efficacy in neutralizing ROS.

1.9. Mechanisms of desiccation tolerance in plants

Desiccation tolerance is widespread in the plant kingdom, including: ferns, mosses and their spores; pollen and seeds of angiospersms or even their whole organism in around 300 species; but is not found in gymnosperms (Oliver & Bewley 1997; Porembski & Barthlott 2000). There must be a differentiation between the plants that can tolerate a partial desiccation and those that can survive a complete desiccation. While “drought tolerance” can be considered as the tolerance to moderate dehydration, down to a moisture content below which there is no bulk cytoplamic water present, approximately 23% water on a fresh weight basis (Hoekstra *et al.* 2001), “desiccation tolerance” generally refers to the tolerance of further dehydration, 10% water content or less, when the hydration shell of molecules is gradually lost (Alpert 2006; França *et al.* 2006).

Desiccation tolerant plants can be divided broadly in two groups, those classified as "modified desiccation-tolerant plants" (resurrection traqueophytes), in comparison with the other group, the "fully desiccation-tolerant plants" (most bryophytes and some small pteridophytes), their ability to survive desiccation is rate-dependent (Oliver & Bewley

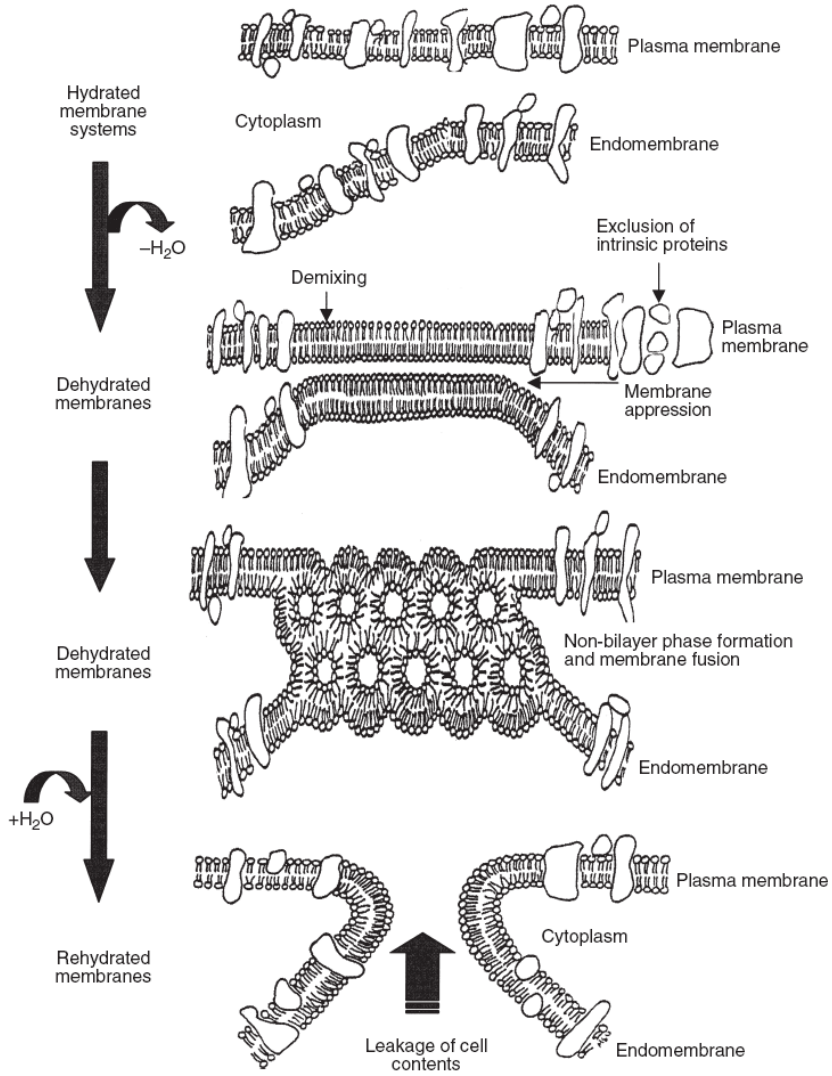


Figure 1.8.- Schematic drawing of the effect of dehydration on cellular membranes. Adapted from Adapted from Buitink *et al.* (2002).

1997). Tolerant traqueophytes only survive if the rate of water loss is very slow (in days to weeks) and take up to 24 hours or more to recover (Oliver & Bewley 1997; Proctor & Pence 2002; Alpert & Oliver 2002). This a consequence of an inducible desiccation tolerance, sufficient time is required for the development of the tolerance mechanisms based on protection during desiccation, rather than repair on rehydration (Oliver *et al.* 2000; Alpert & Oliver 2002). By the other hand, non-vascular resurrection plants can survive to very rapid drying events (less than 1 h) and recover the respiration and photosynthesis within a few minutes (Oliver & Bewley 1997; Proctor & Smirnoff 2000). Tolerance seems to be achieved predominantly by an ability to repair damage caused by desiccation, and is thus primarily based on constitutive mechanisms (Oliver & Bewley 1997). Drying rate appears to have little influence on ultimate survival, but recovery takes longer in rapidly dried tissue, perhaps suggesting the existence of inducible protection mechanisms (Schonbeck & Bewley 1981).

Upon water loss, the decrease in cellular volume causes crowding of cytoplasmic components and the cell contents become increasingly viscous, increasing the chance for molecular interactions that can cause protein denaturation and membrane fusion (Hoekstra *et al.* 2001) (Fig. 1.8). In order to prevent the hydrophobic interactions between membranes and proteins, the water molecules removed from the water shell of macromolecules can be replaced by a broad range of compounds such as polyols, mannitol, sorbitol, fructans, glutamate, glycine-betaine, carnitine trehalose, sucrose and oligosaccharides (Fig. 1.9) – this theory is known as the water replacement hypothesis (Faure *et al.* 1998; Hoekstra *et al.* 2001). Seeds, pollen and most tolerant plants accumulate high concentrations of the disaccharide sucrose (Buitink *et al.* 1998; Vicre *et al.* 2004), whereas in the fully desiccation-tolerant *Tortula ruralis* sucrose is approximately 10% of the dry mass and does not change in amount during dehydration/rehydration (Bewley *et al.* 1978). In tandem with specific proteins (Goyal *et al.* 2005), these sugars probably stabilize drying cells both by direct interaction with macromolecules and membranes and by reversibly immobilizing cytoplasm in an extremely slow-flowing liquid, a glass (Buitink & Leprince 2004).

Another critical component in the acquisition of cellular desiccation tolerance is the Late Embryogenesis Abundant (LEA) proteins. Although their actual function remains unclear, they are accumulated to high concentrations in seeds and in vegetative tissues of

desiccation-tolerant plants in response to desiccation, so they are thought to play a primary role in desiccation tolerance (Buitink *et al.* 2002; Kermode & Finch-Savage 2002; Phillips *et al.* 2002). Likewise, in the rapid-dehydration tolerant moss *Tortura ruralis*, dehydrins are constitutively expressed (Bewley *et al.* 1993). On the basis of the remarkably high number of polar residues within the structure, some LEAs are thought to coat intracellular macromolecules with a cohesive water layer. On further dehydration, LEAs would provide a layer of their own hydroxylated residues to interact with the surface groups of other proteins , acting as "replacement water" (Hoekstra *et al.* 2001).

Heat Shock Proteins (HSP) are another type of protein that has recently been associated with plant desiccation tolerance. These proteins are induced by the same stresses as LEAs and their synthesis coincides with the acquisition of desiccation tolerance (Bose *et al.* 1996; Wegele *et al.* 2004; Timperio et al. 2008). Generally, HSPs interact with other cell proteins to act as molecular chaperones and maintain partner proteins in a folding-competent, folded or unfolded state, or to prevent aggregation of non-native proteins, or to

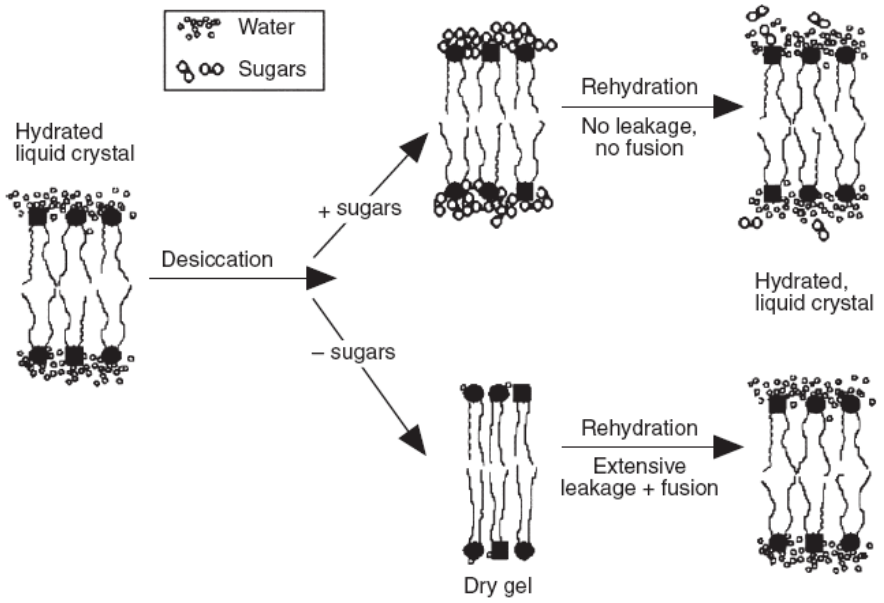


Figure 1.9.- Schematic representation of the phase behaviour of phospholipids in a bilayer as influenced by desiccation in the presence or absence of a disaccharide. Adapted from Buitink *et al.* (2002).

refold denatured proteins to regain their functional conformation, or to target non-native or aggregated proteins for degradation and removal from the cell (Perrot-Applanat *et al.* 1995; Rachmilevitch *et al.* 2006; Timperio *et al.* 2008).

Among plants, resistance to drought of desiccation tolerant species, seems to be associated with an activation of the ROS scavenging mechanisms (McKersie *et al.* 1999; Hsieh *et al.* 2002). In vegetative tissues of *Craterostigma wilmsii* and *Xerophyta viscose*, genes encoding enzymatic antioxidants, such as AP, GR and SOD, are up-regulated during drying or rehydration (Ingram & Bartels 1996; Sherwin & Farrant 1998). Furthermore, during dehydration are accumulated a lipoxygenase inhibitor (Bianchi 1992) and anthocyanins with antioxidant capability (Sherwin & Farrant 1998). The leaves of another resurrection plant, *Myrothamnus flabellifolius*, contain high levels (>90%) of a polyphenol, which protects cell membranes against desiccation and ROS (Moore *et al.* 2005). The desiccation of this tolerant species triggered substantial increases in zeaxanthin and redox shifts of the antioxidants glutahione and ascorbate towards their oxidized forms (Kranner *et al.* 2002). It should be realized that the enzymatic antioxidant systems can be active only under conditions of sufficient water and that, in the dried state; only the molecular antioxidants (e.g. glutathione, ascorbate, polyols, carbohydrates, tocopherol, quinones, flavonoids and phenolics) can alleviate oxidative stress (Vertucci & Farrant 1995). In order to prevent light-associated damage during desiccation poikilochlorophyllous desiccation-tolerant plants dismantle the photosynthetic apparatus and lost chlorophyll, whereas homoiochlorphyllous (chlorophyll retaining) fold leafs and shade chlorophyll (Tuba *et al.* 1996; Sherwin & Farrant 1998; Farrant *et al.* 1999).

In addition, other physiological, morphological and structural responses are produced in desiccation tolerant plants during desiccation. Mechanical damage associated with loss of turgor is proposed to be one of the major causes of irreversible desiccation-induced damage in plants. Desiccation-tolerant plants use two different approaches to minimize this mechanical stress: water replacement or a reduction in cellular volume (Gaff 1971). The desiccated cells become filled with vacuoles containing non-aqueous contents, replacing the volume occupied by water in the hydrated state, in this way the mechanical stress on the cellular substructure is reduced. (Farrant 2000). The formation of numerous small vacuoles during dehydration have been reported in several species (Farrant 2000;

Vander Willigen *et al.* 2001; Wang *et al.* 2009). An alternative strategy to prevent lethal mechanical stress on cellular desiccation is to reduce cellular volume. Extensive cell wall folding in some desiccation-tolerant species has been recorded to cause as much as a 78% reduction in cellular volume in dehydration (Farrant 2000; Vander Willigen *et al.* 2001). Recent works have shown that microtubular cytoskeleton plays a key role in the re-establishment of the elaborate cytoplasmic architecture during rehydration in the desiccation-tolerant moss *Polytrichum formosum* (Pressel *et al.* 2006; Wang *et al.* 2009).

1.10. Thesis objectives

The study of desiccation tolerance of lichens, and of their phycobionts in particular, has been mainly focused on the antioxidant system that protects the cell against the photo-oxidative stress produced during dehydration and rehydration. However, desiccation tolerance is a complex process that cannot be achieved in lichen phycobionts with antioxidant activity alone. The main objective of the thesis was to expand our knowledge of the poorly understood desiccation tolerant mechanisms that allow phycobionts to survive under continuous cycles of dehydration/rehydration and long desiccation periods. To achieve this general objective the thesis was divided into the following specific objectives:

- Few studies have dealt with the effect of desiccation in isolated phycobionts which, in addition, have reported controversial results. Hence, the first specific objective was to determine both the grade of desiccation tolerance of the isolated lichen phycobiont *Trebouxia erici* and the most suitable conditions to carry out dehydration/rehydration experiments.

- Literature show that the protective behaviour of the phycobiont antioxidant mechanisms after desiccation and/or rehydration depend on the lichen species, and vary between isolated and lichenized algae. Thus, we studied the responses of protective mechanisms such as, antioxidant enzymatic activities, xanthophyll cycle, other mechanisms of energy dissipation in the photosystem II and late embryogenesis abundant, and whether they are constitutively expressed or induced

during the dehydration/rehydration processes. This research was conducted to increase our understanding of the role of these mechanisms in desiccation tolerance of *T. erici*.

- Resurrection plants and mosses survive to desiccation activating a complex system of protection and/or repair mechanisms during drying and/or rehydration. We carried out proteomic and genetic expression analyses of the changes associated with desiccation and rehydration in the isolated phycobiont *Trebouxia erici.* The aim of these studies was to find out proteins that may be involved on desiccation tolerance mechanisms of phycobionts.

- During rehydration there is a burst in the production of reactive oxygen species together with the release of nitric oxide (NO) by the mycobiont. NO is a ubiquitous and multifaceted molecule involved in cell signalling and protection against abiotic stress, among other. In the present work, we investigated production and the antioxidant role of oxide nitrogen during rehydration of isolated phycobionts and lichens. This was conducted to gain insights into the role of the NO in the biochemical interaction between lichen symbionts and whether the NO may increase the endurance of lichenized phycobionts under desiccation.

- Some results of experiments carried out with isolated phycobiont cultures suggest that desiccation tolerance is higher in lichenized phycobionts than in free ones. The effect of cell ageing in desiccation resistance of *T. erici* was studied in order to know whether culture aging can contribute to the apparently lower desiccation tolerance observed in isolated phycobionts as compared with lichenized ones.

1.11. Resumen

Alrededor de 44 géneros de algas y cianobacterias han sido identificados como fotobiontes de líquenes. Los fotobiontes de algas verdes incluyen *Trebouxia*, *Trentepohlia*, *Cocoomyxa* y *Dyctiochloropsis*, entre otros (Gärtner 1992). Se ha estimado que más del 50 % de todas las especies de líquenes están asociadas con especies del género *Trebouxia* (Tschermak-Woess 1988).

Los miembros del género Trebouxia están descritos como algas verdes cocoides que pueden formar paquetes de autoesporas en forma de tétradas o mayor número de células. Las células tienen formas desde esféricas a elípticas. Las paredes celulares son finas, ocasionalmente engrosadas unilateralmente, y sin una cubierta gelatinosa. Los cloroplastos en las células adultas se sitúan en el centro de las células y desarrollan lóbulos y márgenes crenulados. Los cloroplastos tienen uno o múltiples pirenoides que, o bien están desnudos, o bien, como en algunas especies, están cubiertos de una capa de almidón. Este almidón se encuentra generalmente depositado en gránulos situados en el estroma del cloroplasto. El núcleo está siempre posicionado excéntricamente como consecuencia de la posición central del cloroplasto. Solamente se conoce la reproducción asexual por formación de autoesporas, o zoosporas, las cuales son lisas, con dos flagelos y un estigma (Ettl & Gartner 1995; Helms 2003).

Los líquenes se encuentran entre los organismos poiquilohídricos, es decir, entre aquellos que no pueden regular activamente su contenido hídrico. Así pues, el contenido hídrico del talo está principalmente determinado por la disponibilidad hídrica del ambiente. Cuando se secan, su contenido hídrico está frecuentemente en el rango del 10-20 %, respecto a su peso fresco. Este estado sería letal para la mayoría de las plantas vasculares y de los organismos, sin embargo, la gran mayoría de los líquenes son tolerantes a la desecación y pueden sobrevivir en un estado de animación suspendida hasta que el agua vuelve a estar disponible de nuevo, entonces reviven y reactivan de nuevo su metabolismo normal. Tras la rehidratación, los líquenes son capaces de recuperar la actividad fotosintética en un periodo de tiempo muy corto, entre 15 y 60 min. En la mayoría de los líquenes terrestres la inactivación y reactivación de la fotosíntesis va correlacionada con los

ciclos de deshidratación y rehidratación que se producen a causa de la niebla o del rocío nocturno. La desecación se produce relativamente lenta, más horas que minutos.

Los talos secos se encogen pero no se marchitan, aunque sí que se producen cambios en su consistencia y en el color. Los fotobiontes, los cuales tienen paredes finas e hialinas, colapsan (citorrisis), mientras que el micobionte, que tiene una pared gruesa y rígida, no pueden deformarse lo suficiente durante la pérdida de agua y cavitan. Durante la rehidratación, el citoplasma y los orgánulos se hinchan y recuperan su estructura normal. A pesar de la contracción de los protoplastos durante la deshidratación, las membranas celulares de las algas se conservan aparentemente en muy buen estado.

Los procesos oxidativos y el incremento de los radicales libres están generalmente implicados en el daño molecular y celular producido por un amplio rango de estreses ambientales, incluyendo la deshidratación. Por un lado, la producción de radicales libres parece estar estrechamente relacionada con la respiración como consecuencia del daño que se produce durante la desecación en la cadena de electrones mitocondrial (Leprince et al. 1994). Por otra parte, aunque la fijación de carbono se inhibe durante la desecación, el flujo de electrones en la fotosíntesis continua, y la energía de excitación puede ser transferida desde las clorofilas fotoexcitadas al $^3O_2$, y formar oxígeno singlete ($^1O_2$), así mismo, se puede formar superóxido de hidrógeno en los fotosistemas II y I por la reacción de Mehler (Kranner & Lutzoni 1999; Halliwell 2006). La formación de éstas especies reactivas de oxígeno (EROs) es una de las fuentes más importantes de daños en ácidos nucleicos, proteínas y lípidos. En los líquenes se ha comprobado que durante los primeros minutos tras la rehidratación se produce una explosión en la producción de EROs, aunque después disminuye.

La mayoría de los estudios recientes realizados sobre la tolerancia a la desecación de los líquenes han sido enfocados hacia los mecanismos de prevención y eliminación de las especies reactivas de oxígeno. Uno de éstos mecanismos estudiados ha sido el del ciclo de las xantofilas. Bajo un estrés lumínico el ciclo de las xantofilas se activa y la violaxantina se convierte en antheraxantina y posteriormente en zeaxantina. Esta última parece estar implicada en la captación de energía en el fotosistema y su disipación en forma de calor, lo que puede ser medido también como disipación no fotoquímica de la fluorescencia de la clorofila (NPQ). La activación del ciclo de las xantofilas durante la

deshidratación ha sido observada en líquenes como *Parmelia quercina*, *Ramalina maciformis* y *Cladonia vulcanii* (Calatayud *et al.* 1997; Zorn *et al.* 2001; Kranner *et al.* 2005). Sin embargo, no existen evidencias de que se active en fotobiontes aislados (Kranner et al. 2005).

En vegetales poiquilohídricos tolerantes a la desecación, como líquenes y musgos, existe otro mecanismo de disipación de energía que los protege de la fotoinactivación mientras se encuentran secos. Durante la deshidratación de los líquenes, la fluorescencia de la clorofila *a* disminuye y la separación de cargas en los centros de reacción desaparece. Esto parece ser debido a que la desaparición del agua estructural produce un cambio conformacional de una proteína-pigmento cercana o en el corazón del fotosistema II que provoca que la energía lumínica absorbida sea convertida en calor, de esta forma se priva a los centros de reacción de energía y los protege frente a la fotoinhibición. La disipación de energía en calor y los cambios conformacionales desaparecen en cuanto el talo vuelve a rehidratarse (Heber *et al.* 2007; Heber 2008).

La eliminación de las EROs formadas puede ser llevado a cabo por antioxidantes enzimáticos o no-enzimáticos. Entre los enzimas antioxidantes se encuentran las superóxidos dismutasas (SOD), las catalasas (CAT), peroxidasas (POX) y otros enzimas auxiliares como la mono- y de-hidroascorbato reductasa y la gluratation reductasa (GR). En los escasos estudios realizados en líquenes no se observa una clara relación entre los diferentes niveles de tolerancia a la desecación y la actividad enzimática antioxiadante (Kranner *et al.* 2008). En muchos organismos, los antioxidantes no-enzimáticos principales son el tripéptido GSH (glutation, γ-glutamil-cysteinil-glicina) y el ascorbato (Noctor & Foyer 1998). Tampoco una mayor concentración de antioxidantes se encuentra correlacionada con la mayor tolerancia a la desecación observada en algunos líquenes. Más bien, la característica que tienen en común las especies bien adaptadas a la desecación es la capacidad para reestablecer rápidamente las concentraciones iniciales de antioxidantes, los potenciales redox y las actividades enzimáticas durante la rehidratación (Kranner 2002; Kranner *et al.* 2003).

La tolerancia a la desecación se encuentra ampliamente representada en el reino de los vegetales: aparece en helechos, musgos y sus esporas; en angiospermas en sus semillas y polen, e incluso en todo el organismo en alrededor de 300 especies; pero no se encuentra

en gimnospermas (Oliver & Bewley 1996; Porembski & Barthlott 2000). Los vegetales tolerantes a la desecacion pueden ser clasificados a grandes rasgos en dos grupos. El primero lo forman las "plantas modificadas tolerantes a la desecación" (traquófitas de resurrección), las cuales no sobreviven si el tiempo de desecación no es suficientemente largo (de días a semanas) y la recuperación de su metabolismo y fotosíntesis puede llevarles más de un día (Oliver & Bewley 1997; Proctor & Pence 2002; Alpert & Oliver 2002). Esto es consecuencia de una tolerancia a la desecación inducible, ya que necesitan de un tiempo mínimo de deshidratación para la activación de los mecanismos de tolerancia, los cuales están más dirigidos a la protección que a la reparación tras la rehidratación (Oliver *et al.* 2000; Alpert & Oliver 2002). El segundo grupo lo forman las especies "totalmente tolerantes" (la mayoría de los briófitos y algunos pequeños pteridófitos), que sobreviven incluso a deshidrataciones rápidas (menores a 1 hora) y tienen una capacidad de recuperación es muy rápida (minutos) (Oliver & Bewley 1997; Proctor & Smirnoff 2000). Esta clase de tolerancia parece ser conseguida principalmente por mecanismos constitutivos y por la habilidad de reparar los daños causados durante la desecación (Oliver & Bewley 1997). La velocidad de la deshidratación parece tener una influencia muy pequeña en la supervivencia final, aunque la recuperación tarda más tiempo si la deshidratación es rápida, lo que sugiere también existen mecanismos de protección inducibles (Schonbeck & Bewley 1981).

Tras la pérdida del agua, la disminución del tamaño celular causa el apelotonamiento de los componentes celulares y el contenido celular se vuelve cada vez más viscoso, lo cual incrementa la probabilidad de interacciones moleculares que pueden causar desnaturalizaciones protéicas y fusiones de membranas (Hoekstra *et al.* 2001). En los vegetales tolerantes a la desecación, con el fin de prevenir las interacciones hidrofóbicas entre las proteínas y las membranas, las moléculas de agua perdidas durante la deshidratación pueden ser reemplazadas por un amplio rango de componentes tales como polioles, manitol, sorbitol, fructanos, glutamato, glicina-betaina, carnitina, trehalosa, sucrosa y oligosacáridos (Faure *et al.* 1998; Hoekstra *et al.* 2001). Otro componete crítico en la adquisición de la tolerancia celular a la desecación son las proteínas abundantes de la embriogénesis tardía (LEA, de su sigla en inglés). Aunque su función actual permanece desconocida, estas proteínas se acumulan en altas concentraciones en semillas y en tejidos

de vegetales tolerantes a la desecación, por lo que se considera que deben de jugar un papel esencial en la tolerancia a la desecación (Buitink *et al.* 2002; Kermode & Finch-Savage 2002; Phillips *et al.* 2002). Las proteínas de choque térmico (Hsp, de su sigla en inglés) son otro tipo de proteínas que han sido recientemente asociadas con la tolerancia a la desecación de los vegetales. Generalmente, las Hsps interactúan con otras proteínas celulares y entre otras funciones actúan como chaperonas moleculares, previenen la agregación de proteínas no-nativas, repliegan a las proteínas desnaturalizadas para que recuperen su configuración funcional y marcan proteínas no-nativas o agregadas para su degradación y eliminación de la célula (Perrot-Applanat *et al.* 1995; Timperio *et al.* 2008).

2. CULTURE AND EXPERIMENTAL CONTROL CONDITIONS

Abstract

The study of desiccation in lichens has generally been performed with intact thalli, however, working with the whole thallus makes it difficult to distinguish the independent physiological responses of each symbiont. Isolation and culture of the photobiont enables one to interpret the physiological changes and gain insight into the photobiont desiccation-tolerance mechanisms. Only a few studies using isolated photobiont cultures have focused on physiological responses, being *Trebouxia erici* the photobiont species for which there is the greatest amount of physiological data reported in the literature. Different authors have selected very varied culture conditions for *T. erici*, thus, the aim of this chapter is to establish the culture conditions to obtain optimal growth for further experimental work. An axenic strain of the lichen photobiont *T. erici* Ahmadjian (SAG 32.85 = UTEX 911) was used in our study. Algae were cultured in liquid or agar Trebouxia medium: 3 N Bold's Basal Medium (3NBBM) plus 10 g casein and 20 g glucose per litre. Several conditions, which affect to inoculation cell density, temperature, growing light and photoperiod, were checked. In liquid culture, *T. erici* showed linear growth phase extended from the third to the 19th day. Light saturation point varied between 300 and 500 μmol m^{-2} s^{-1} and the highest electron transport rate value was reached on the 19th day, after this point ETR decreased. Algae cultures should be grown at 20 ºC, under this temperature the growth rate is two-fold higher than at 17 ºC, whereas at 23 ºC, growth rate was similar, but under 50 μmol m^{-2} s^{-1} PAR it showed photo-inhibition and a decline in growth. We selected an irradiance of 30 μmol m^{-2} s^{-1} PAR for further experiments, since under this light intensity the growth rate of *T. erici* is good and photoinhibition is avoided. Experiments will be carried out with 21 day-old algal discs, because photosynthetic capacity is the highest at this moment and the culture is still in the log growth phase. Our results do not reveal whether the isolated photobiont *T. erici* has daily photosynthesis rhythms, but if it occur, no effects were found on the fluorescence measurements, thus, photosynthesis measurements can be carried out at any hour of the day.

2.1. Introduction

The study of desiccation in lichens has generally been performed with intact thalli, thereby preserving the conditions and interactions between the symbionts. The relationship between thallus hydration and photosynthesis and respiration has been the object of numerous studies (e.g., Farrar 1976; Lange & Tenhunen 1981; Sancho & Kappen 1989; Lange *et al.* 2006; del Prado & Sancho 2007). The morphological and ultrastructural changes that symbionts undergo during dehydration and rehydration have also been studied in the whole thallus (e.g., Jacobs & Ahmadjian 1971; Green *et al.* 1985; Scheidegger *et al.* 1995; Honegger *et al.* 1996; Honegger 2006); as well as, the mechanisms that may be involved in lichen desiccation-tolerance, including the antioxidant system (Kranner 2002; Kranner *et al.* 2005; Weissman *et al.* 2005) or mechanisms that dissipate excess energy received by the photobiont during desiccation (Calatayud *et al.* 1997; Veerman *et al.* 2007; Heber 2008). However, there are only a few reports concerning the effects of desiccation on the isolated photobiont (Vaczi & Bartak 2006; Kosugi *et al.* 2009, Brock 1975; Kranner *et al.* 2005; Weissman *et al.* 2005). The results of the above studies suggest that desiccation tolerance in photobionts is enhanced by symbiosis, although this issue is far from clear. Working with the whole thallus makes it difficult to distinguish the independent physiological responses, such as respiration or antioxidant enzymatic activities, of each symbiont. Isolation and culture of the photobiont enables to interpret the physiological changes and gain insight into the photobiont desiccation-tolerance mechanisms. Examination of the photobiont responses to desiccation and rehydration processes may also broaden our understanding of the physiological responses of lichens as symbiotic organisms.

Symbionts have previously been cultured directly in liquid and on agar solid media, mainly for identification and phylogeny purposes and resynthesis experiments. Only a few studies using isolated photobiont cultures have focused on physiological responses, most of which have used the isolated photobiont *Trebouxia erici* to investigate heavy metal stress (e.g., Backor & Vaczi 2002; Backor *et al.* 2004; Bud'ova *et al.* 2006; Guschina & Harwood 2006; Backor *et al.* 2007) and the effects of irradiance and osmotic stress on photosynthesis (Vaczi & Bartak 2006). Goldsmith *et al.* (1997) used this species to develop

a new technique for photobiont culture and manipulation. Generally speaking, the technique involves growing the photobiont on cellulose-acetate discs placed on nutrient agar medium, which allows the photobiont cells to access nutrients, while making them easy to handle.

As far as we know, *T. erici* is the photobiont species for which there is the greatest amount of physiological data reported in the literature and for this reason we have selected this species for our studies. However, different authors have selected very varied culture conditions for *T. erici*; hence, it has been cultured at 18 ºC (Goldsmith *et al.* 1997; Backor & Fahselt 2005), 20 ºC (Vaczi & Bartak 2006) or 24 ºC (Backor & Vaczi 2002; Guschina & Harwood 2006); and under a radiation of 300 μmol m^{-2} s^{-1} (Goldsmith *et al.* 1997), 100 μmol m^{-2} s^{-1} (Backor *et al.* 2003) or 30 μmol m^{-2} s^{-1} (Backor *et al.* 2007). Thus, the aim of this chapter is to describe how we established the culture conditions to obtain optimal growth in the experimental work, object of this Ph.D. Dissertation, and to analyse the possible effect of growing conditions on photosynthetic measurements.

2.2. Materials and methods

2.2.1.Growth in liquid culture

An axenic strain of the lichen photobiont *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) was used in this study. Algae stock cultures were maintained in 10 ml tubes containing *Trebouxia* medium: 3 N Bold's Basal Medium (3NBBM) plus 10 g casein and 20 g glucose per litre (Ahmadjian 1973). The 3NBBM contained per litre: 0.75 g $NaNO_3$; 0.175 g KH_2PO_4; 0.075 g K_2HPO_4; 0.075 g $MgSO_4.7H_2O$; 0.025 g CaCl and 0.025 g NaCl. In addition, 1 l of medium contained the following micronutrients: 11.42 mg H_3BO_3; 4.98 mg $FeSO_4.7H_2O$; 8.82 mg $ZnSO_4.7H_2O$; 1.44 mg $MnCl_2.4H_2O$; 0.71 mg MoO_3; 1.57 mg $CuSO_4.5H_2O$; 0.49 mg $Co(NO_3)_2.6H_2O$; 50 mg EDTA and 31 mg KOH. Initial stock culture conditions were selected on the basis of previous works carried out with *T. erici* (Goldsmith *et al.* 1997; Backor & Vaczi 2002). Liquid cultures were maintained at 20ºC under a 12 h photoperiod of 30 μmol m^{-2} s^{-1} white light.

As the main subject of this chapter, several conditions, such as inoculation cell density, temperature (17 – 23 ºC), growing light (15 – 50 μmol m^{-2} s^{-1} PAR) and

photoperiod, were checked out in order to establish the optimal growing conditions and to determine how these working procedures could modify the results of further experiments.

Cell number was determined using a Neubauer haemocytometer. The growth rate of algal cultures, also called *intrinsic rate of increae*, *the Malthusian parameter*, or the *instantaneous rate of increase* (Gotelli 1995) was calculated following the equation for arithmetic growth:

$$r = 1/\Delta t \, . \, (N_t\text{-}N_0/N_0)$$

where N_0 is the population size at the beginning of a time interval, N_t is the population at the end of the interval and Δt is the length of the interval ($t_t - t_0$).

Doubling time, T_2, for the culture, expressed in the same units of time as *r*, can be calculated from an estimate of *r* using of the equation:

$$T_2 = 1/r$$

To asses the fresh weight of the algal biomass, a fraction of the 10 ml culture was centrifuged (10000 g, 10 min) into a pre-weighted tube, then the supernatant was discarded and the tube weighed again.

For chlorophyll *a* and *b* determination, an aliquot of the culture was centrifuged (10000 g, 10 min), the supernatant discarded and the pellet resuspended in 1 ml of dimethyl sulfoxide (DMSO). The suspension was kept in the dark for 30 min at 65 ºC, vortex twice during the incubation and then centrifugated (10000 g, 5 min). The supernatant was transferred to another tube and the extraction was repeated until the pellet lost the green colour Absorbance of the extracts was then read at 665 and 648 nm. Turbidity of the extract was checked at 750 nm to confirm an absorbance lesser than 0.01. Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) concentrations were calculated using the following equations (Barnes *et al.* 1992):

- μg Chl *a/ml* $= 12.19A_{665} - 3.45A_{648}$
- μg Chl *b/ml* $= 21.99A_{648} - 5.32A_{665}$

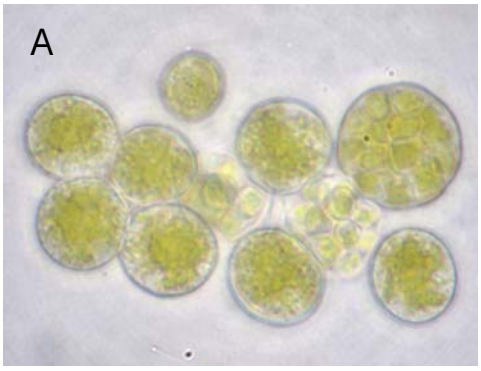
2.2.2. Inoculation on cellulose-acetate discs placed on nutrient agar medium

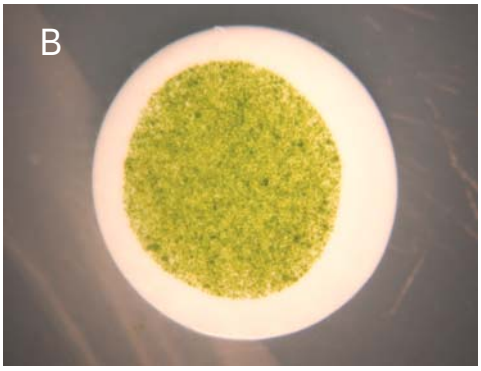
Two-week-old liquid cultures were stirred with a pipette under sterile conditions and then were let stay for 10 min to allow remaining algal clumps to be settled from the

suspension. Then, 1 ml from the top of the tube was taken and the cell number was quantified using a haemocytometer. The final cell density was adjusted with sterilized medium to 10^6 cells per millilitre. Fifty µl of this suspension were inoculated on sterile cellulose-acetate discs (13 mm diame ter, 0.45 µm pore size) and placed on agar *Trebouxia* medium in Petri plates (Goldsmith *et al.* 1997; Backor & Vaczi 2002). Discs were kept without any movement until the excess of water from the inoculation drained out onto the medium (Fig. 2.1).

2.2.3. Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence was measured *in vivo* with a modulated light fluorometer (PAM-2000, Walz, Effeltrich, Germany). The discs were placed on a microscope slide wrapped with moist filter paper to maintain a hydrated state. All the samples were kept in the dark for 30 min just before fluorescence measurements were





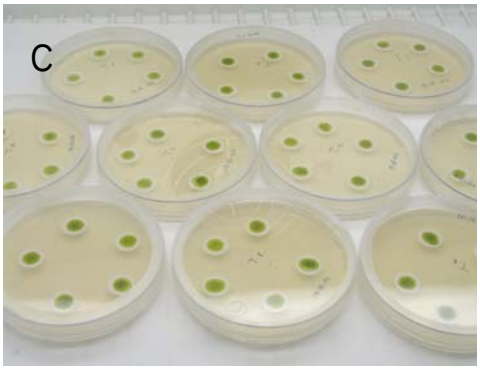


Figure 2.1 - An axenic strain of the lichen photobiont *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) (A) was cultured on sterile cellulose-acetate discs and placed on agar *Trebouxia* medium (B & C).

taken. The minimum (dark) fluorescence yield (F_o) was obtained after excitation of the algae with a weak measuring beam from a light-emitting diode. The maximum fluorescence yield (Fm) was determined with an 800 ms saturating pulse of white light (SP, 8000 µmol m^{-2} s^{-1}). Maximal variable fluorescence (F_v) was calculated as F_m-F_o. The parameter F_v/F_m represents the maximum quantum efficiency of PSII photochemistry when all the reactions centres are open after a period of dark adaptation.

Light curves kinetics were carried out to determine the best actinic light intensity for the slow induction fluorescence kinetics that provide a good balance between photochemical and non-photochemical quenching. A series of 30 sec actinic lights (44, 65, 87, 139, 210, 315, 460, 700, 1100, 1700 µmol m^{-2} s^{-1}) followed by a SP and a brief interruption (3 s) of actinic illumination in the presence of 6 µmol m^{-2} s^{-1} far red (FR, 730 nm) light was applied to determine the Chl *a* fluorescence yield during actinic illumination (F_s), the rmaximum fluorescence yield during actinic illumination (F_m'), and the minimum fluorescence yield (F_o'), respectively. Relative electron transport rate (ETR) was calculated as (F_m'-F_s)/F_m'×PAR×0.84×0.5 (Schreiber *et al.* 1986; Jensen 2002).

For slow fluorescence kinetics, after F_v/F_m measurement, samples were two-minute dark readjustment, actinic light (AL, 210 µmol m^{-2} s^{-1}) was switched on, and SP were applied at 1 min intervals for 11 min to determine the maximum fluorescence yield during actinic illumination (F'_m), the Chl *a* fluorescence yield during actinic illumination (F_s) and the level of modulated fluorescence during a brief interruption (3 s) of actinic illumination in the presence of 6 µmol m^{-2} s^{-1} far red (FR, 730 nm) light (F'_o). The effective quantum efficiency of PSII photochemistry, ϕPSII, which is closely associated with the quantum yield of non-cyclic electron transport, was estimated from (F'_m-F_s)/F'_m (Genty *et al.* 1989; Kramer *et al.* 2004). The coefficient for photochemical quenching, q_P is non-linearly related to the fraction of open PSII reaction centres and was calculated as (F'_m-F_s)/(F'_m-F'_o) (Schreiber *et al.* 1986). Quenching due to the non-photochemical dissipation of absorbed light energy (NPQ) was calculated at each saturating pulse, according to the equation NPQ= (Fm-F'm)/F'm (Bilger & Bjorkman 1990).

2.2.4. Statistical analysis

For each experiment several plates with 5 algal discs that were randomly chosen. Every experiment was repeated several times starting from new stock material and the preparation of fresh new cultures, dishes and plates. We used one-way ANOVA, or two-way ANOVA when more than one factor was analysed, in combination with the LSD test (Statgraphics Plus® (4.11) for windows), calculated at 95% confidence level, to determine significant differences among samples groups.

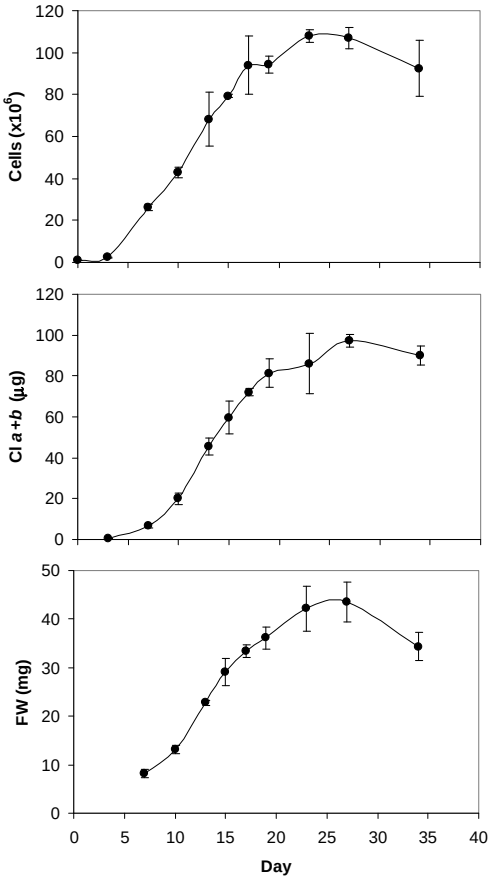


Figure 2.2 - Evolution of the total cell number (A), total chlorophyll *a* + *b* (B) and fresh weight (C) of *T. erici* liquid cultures maintained at 30 µmol m^{-2} s^{-1} (12h photoperiod) and 20 ºC in 10 ml of *Trebouxia* Medium. Data represent average ± SD, n = 5.

2.3. Results and discussion

2.3.1. *Growth of T .erici in liquid cultures*

As show in Fig 2, growth of *T. erici* in liquid cultures maintained at 30 µmol m^{-2} s^{-1} PAR (12h photoperiod) and 20 ºC showed a typical sigmoid curve (Salisbury and Ross, 1991): the first three days after inoculation corresponded to the initial logarithmic growth phase; the linear growth phase extended from the third to the 19th day and later, the number of cells started to decline gradually during the senescence phase.

During the linear growth phase the population rose from the initial inoculum of 10^5 cells to around 1.1 10^8 cells. During this phase the maximum growth rate (*r*), 0.253, and the doubling time (T_2), 3.960 days, rose between the 7th and the 17th day; the same growth pattern can be depicted as a function of the total amount of chlorophylls or the algal biomass (Fig. 2.2). In fact, measurements of chlorophyll concentration and algal biomass were strongly correlated with cell density, R^2 = 0.985 and R^2= 0.947, respectively. Thus, any of these three parameters can be used to assess culture growth.

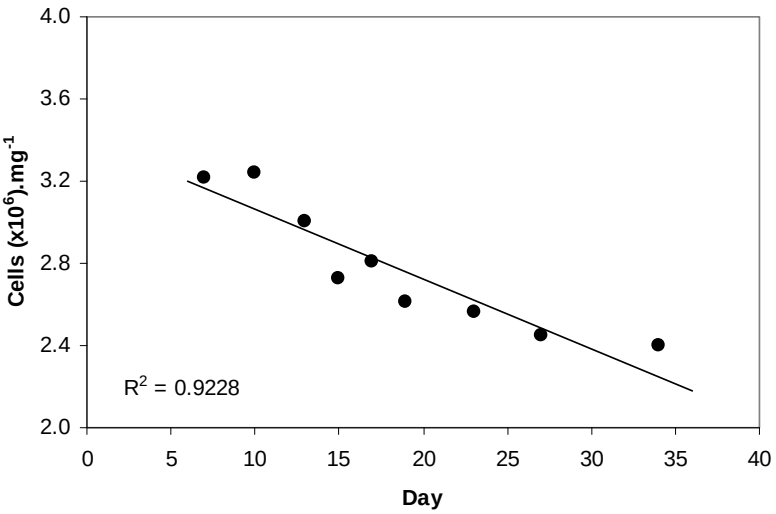


Figure 2.3 - Relationship between the number of cells per miligram (cells.mg^{-1} in fresh weight) and the age of a 10 ml *T. erici* culture maintained at 30 µmol m^{-2} s^{-1} PAR (12h photoperiod) and 20 ºC.

The number of cells per milligram of fresh weight decreased during the linear growth phase and later (Fig. 2.3). The sixth day after inoculation, a milligram of algae fresh weight contained around 3.2 10^6 cells, while the 25th day of growth a milligram contained 2.4 10^6 cells, (25 % less), indicating that cell growth predominates on cell division after the logarithmic growth phase. Likewise, zoospores were only observed until the 19th day. Zoospores are asexual reproductive cells formed by protoplast division and their formation is dependent on nutrient availability (Slocum *et al.* 1980). Thus, between the 19th to the 25th growth days, the culture started to become senescent. After the 25th day of growth, cell death can be assessed by the decay of total cell number, total chlorophyll and fresh weight (Fig 2.2).

2.3.2. Light saturation point

Light response curves of the relative electron transport rate (ETR) displayed the characteristic photosynthetic response to irradiance, divided into three phases (Fig. 2.4): the first phase, where photosynthesis is limited by irradiance; the second phase, where

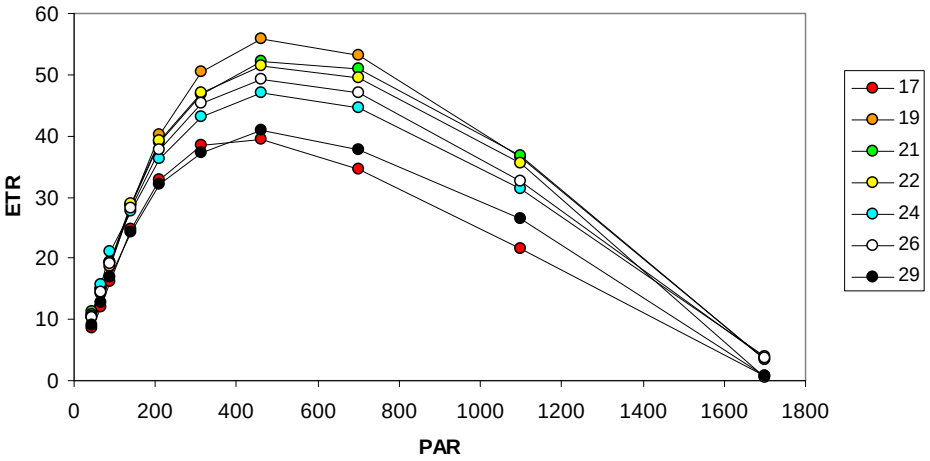


Figure 2.4 - PAR response curves of the relative electron transport rate (ETR) in *T. erici* cultures growing in cellulose-acetate disc on agar medium at 30 µmol m^{-2} s^{-1} (12h photoperiod) and 20 ºC, after 17, 19, 21, 22, 24, 26 and 29 days of growing. Values are mean ± SD, n = 5.

photosynthesis reaches light saturation point; and the third phase, where photosynthesis is limited by carboxilation efficiency. Light saturation point varied between 300 and 500 μmol m^{-2} s^{-1} and the highest ETR value was reached on the 19th day, after this point ETR decreased. The photosynthetic photon flux density (PAR) required to saturate net photosynthesis is highly variable in lichens. In general, lichens occurring in sheltered habitats exhibit saturation at lower PAR than lichens from exposed habitats. *Pseudocyphellaria* species that grow in evergreen-broadleaf forests, where light rarely exceed 10 μmol m^{-2} s^{-1}, saturate at about 120 to 150 μmol m^{-2} s^{-1} (Green & Lange 1991). Whereas, soil exposed species, like *Collema* ssp, have light saturation points over 1000 μmol m^{-2} s^{-1} PAR (Lange *et al.* 2001). For this reason a light curve response should be carried out prior to photosynthetic measurements. Generally, in lichens, an actinic light intensity of 100 μmol m^{-2} s^{-1} PAR provides a good balance between photochemical and non-photochemical quenching (Jensen 2002), but our results in *T. erici* indicate that an actinic light between 100-300 μmol m^{-2} s^{-1} PAR should be used for slow induction fluorescence kinetics.

2.3.3. Effects of temperature and light on agar solid cultures

All the experiments, carried out within the framework of this Ph.D., use cultures of *T. erici* growing in cellulose-acetate discs on agar nutrient medium, for this reason it is critical to establish the best growing conditions and those factors that could influence the results of further experiments.

The effects of temperature and light were analysed in 21-day-old cultures, when the algal culture began to grow beyond the edges of the acetate-cellulose disc at 20 and 23 ºC.

Temperature had a significant effect ($P_v<0.01$) on all the fluorescence parameters and growth of *T. erici* agar cultures (Table 2.1). Maximum quantum yield of PSII (F_v/F_m) was higher at 17 ºC. However, the photochemical efficiency in light (ϕ_{PSII}) and photochemical quenching (q_P) were higher in the cultures maintained at 20 ºC than in those cultured at 17 ºC or 23 ºC. Maximum non-photochemical quenching (NPQ) was observed at 17 ºC. This high NPQ at 17 ºC is due to slow growth rate or to far from optimum

Table 2.1 - Chlorophyll *a* fluorescence parameters and fresh weight (mg) of *T. erici* agar solid agar cultures maintained at different temperature (17, 20 and 23 ºC) and light (15, 30 and 50 µmol m^{-2} s^{-1}) during 21 days. F_v/F_m, maximal quantum efficiency, Φ_{PSII}, effective quantum yield of photochemical conversion in PSII; q_P, photochemical quenching; NPQ, quantum yield of regulated energy dissipation in PSII. Values are mean ± SD, n = 5. Values followed by the same letter indicate significant differences (LSD test, 95 % confidence levels).

T	Light	F_v/F_m	ϕ_{PSII}	qP	NPQ	Weight
17	15	0.687 de	0.472 bcd	0.740 a	0.201 cd	38.6 a
	30	0.682 d	0.457 bc	0.715 a	0.219 d	39.0 a
	50	0.703 e	0.462 bcd	0.713 a	0.233 d	39.6 a
20	15	0.680 cd	0.514 e	0.825 c	0.142 ab	72.4 b
	30	0.672 bcd	0.498 de	0.797 bc	0.120 a	79.8 cd
	50	0.672 cd	0.490 cde	0.800 c	0.173 bc	80.4 d
23	15	0.663 bc	0.451 ab	0.769 b	0.133 a	78.3 cd
	30	0.655 ab	0.412 a	0.722 a	0.141 ab	83.3 d
	50	0.642 a	0.441 ab	0.731 a	0.161 ab	74.7 bc
T		**	**	**	**	**
Light		-	-	**	*	*
TxLight		-	-	-	-	*

* Pv < 0.05, ** Pv < 0.01 significant effect of the culture condition (Two way- ANOVA test)

conditions. At the latter temperature the algal biomass was 50 % lower than that obtained at 20 or 23 ºC. Younger cultures have higher NPQ than older, and this mechanism can compete with photochemistry for light energy, that may explain the lowers values of ϕ_{PSII} observed at 17 ºC. According to Ahmadjian (1993) the optimal culture temperature for isolated photobionts ranges between 15-20ºC, but there are exceptions like *Trebouxia excentrica* with an optimal temperature at 12ºC (Kranner *et al.* 2005) or 24ºC for *Trebouxia* isolated from Brazilian lichens (Cordeiro *et al.* 2005).

Light intensity only had a significant effect on qP and NPQ (Pv<0.01 and Pv<0.05, respectively (Table 2.1). While qP values were higher as light intensity decreased, NPQ demonstrated the opposite behaviour. qP and NPQ are competitive energy dissipation mechanisms, whereas qP is related with the light energy that is transformed into chemical energy through photosynthesis and NPQ is related with the light energy that is dissipated as

non-radiative light, thus, an increase in one parameter causes a decrease in the other. Increased NPQ, as a protection mechanism against photo-oxidative damage, represents the algal acclimation to higher light intensities. Small increases at low light intensities stimulated growth (Pv<0.01) at 17 and 20 ºC, but at 23 ºC an irradiance as low as 50 µmol m^{-2} s^{-1} produced a decrease in F_v/F_m and growth. Thus, at 23 ºC an irradiance of 50 µmol m^{-2} s^{-1} may be close to the light tolerance limit of *T. erici*. At the same time, the small differences in growth responses at 20ºC under the studied light intensities, assures us that the slight irradiance variation in a culture chamber tray (Appendix 1) can not cause significant effects on photosynthesis and growth. The optimum light intensity range of most lichen algae is between 16-27 µmol m^{-2} s^{-1}; some *Trebouxia* strains even lose their colour when cultured at light intensities above 11 µmol m^{-2} s^{-1} (Ahmadjian 1993). This range of light intensities is surprisingly narrow and low, but may be explained by lichen ecophysiology. First, lichens are rarely active, and only briefly under full sunlight. Most of them are mainly photosynthetically active when the thallus is lightly moistened by overnight dew. They have a brief but high peak of net photosynthesis after sunrise and then the lichen dry. If they become rehydrated by rain, the irradiance under this weather condition is also usually very low (Palmqvist *et al.* 2008). Secondly, because the photobiont is usually found in a layer beneath cortical hyphal tissue, the light reaching the photobiont is reduced compared with surficial light measurements by 54–79% when dry and 24–54% when fully hydrated (Ertl 1951; Dietz *et al.* 2000), and even less in some cases (Büdel & Lange 1994). The presence of the fungal cortex and extracellular compounds provides protection against excess PAR and UV radiation.

2.3.4. Photosynthesis and growth of agar cultures at 20 ºC and 30 µmol m^{-2} s^{-1} PAR

Figure 2.5 shows the time-course increase in algal biomass of *T. erici* cultures growing in cellulose-acetate disc on agar medium at 30 µmol m^{-2} s^{-1} (12h photoperiod) and 20 ºC. Cultures grew linearly until the 29th day. From the 19th day to the 26th day, the *r* and T_2 were 0.604 and 1.655, respectively, a little lower than for liquid cultures. It should be pointed out that after the 21st day of growth, algae passed beyond the cellulose-acetate disk

edges and when the discs were removed from the agar media, part of the algal culture was left, so the weight for the last days was underestimated. Actually, cultures keep growing slowly on the margins until the 35th day.

Healthy vascular plants generally have F_v/F_m values of around 0.830 (Björkman & Demmig 1987), while lichens have values of between 0.630 and 0.760 (Jensen & Kricke 2002). Fluorescence measurements of agar cultures of *T. erici* maintained at 30 µmol m^{-2} s^{-1} and 20 ºC gave mean F_v/F_m values of 0.650 for the 17th day of growth, then increased up to the 21st day to 0.698, and decreased to 0.572 for 29th day (Fig. 2.6). The initial low value may be due to technical problems with fluorescence measurements. We have noticed, working with *T. erici* and with other algal species, that if the algal biomass is under 30 mg FW the fluorescence signal is low and the fluorometer PAM-2000 displays incorrect data; under these conditions, F_v/F_m is always underestimated. The decline in F_v/F_m after the 21st day of culture may be explained by a decrease in photosynthesis activity as a consequence of culture ageing (Thompson 1988; Bond 2000; Munne-Bosch & Alegre 2002). Similar reasons may be argued for the changes in the effective quantum efficiency of PSII photochemistry (ϕ_{PSII}) during the culture period. These data indicate that NPQ decrease is

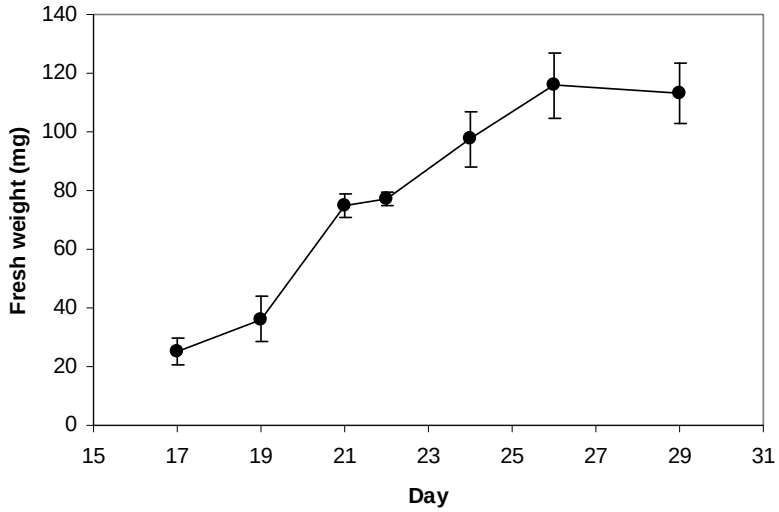


Figure 2.5 - Fresh weight of *T. erici* cultures growing in cellulose-acetate disc on agar medium in at 30 µmol m^{-2} s^{-1} (12h photoperiod) and 20 ºC. Values are mean ± SD, n = 5.

associated with ageing. A decline in the protective mechanisms against photo-oxidative stress has previously been related with vegetative cell ageing (Thompson 1988; Munne-Bosch & Alegre 2002; Dertinger *et al.* 2003).

2.3.5. Daily rhythm of photosynthesis

Circadian rhythms have been reported to occur in a wide variety of eukaryotic taxa, ranging from unicellular organisms to mammals and were discovered in cyanobacteria among prokaryotes (Lüning 2005). Among algae the ubiquity of the circadian rhythm has been demonstrated and reviewed for unicellular algal species, which present rhythmic

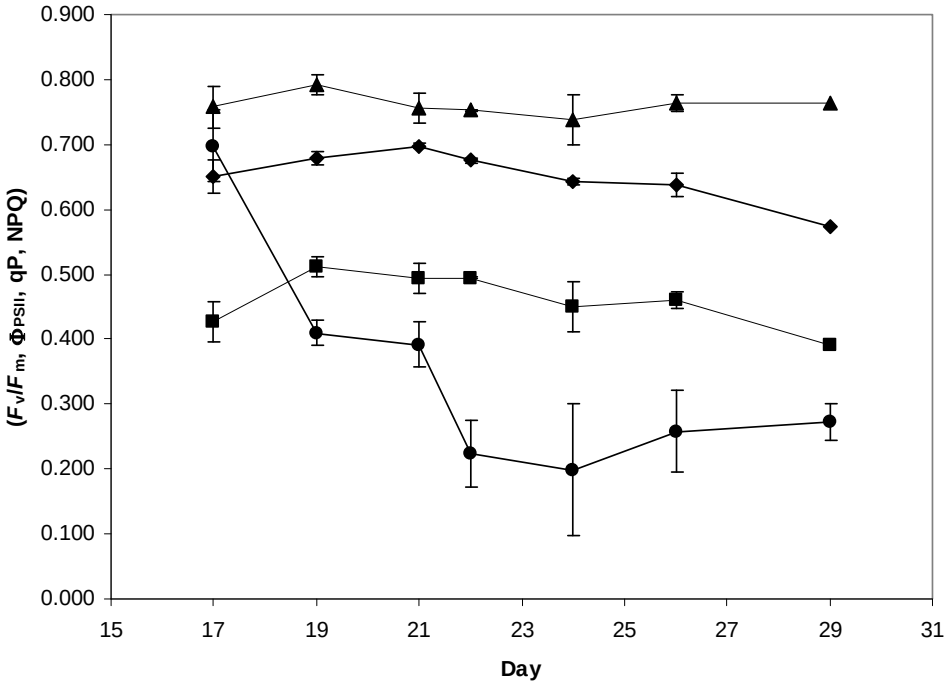


Figure 2.6 - Chlorophyll *a* fluorescence parameters from the 17th to the 29th day after inoculation of *T. erici* cultures growing in cellulose-acetate discs on agar medium at 30 mmol m^{-2} s^{-1} (12h phototoperiod) and 20 ºC. F_v/F_m, maximal quantum efficiency (rhombus); Φ_{PSII}, effective quantum yield of photochemical conversion in PSII (squares); q_P, photochemical quenching (triangles); NPQ, quantum yield or regulated energy dissipation in PSII (circles). Values are mean ± SD, n = 5.

Table 2.2 - Variation in chlorophyll fluorescence parameters throughout the illumination time in *T. erici* agar cultures maintained at 20 ºC and 30 µmol m^{-2} s^{-1} PAR (12 h photoperiod) for 21 days. Measurements were carried out 1, 3, 5, 9 and 11 hours after the culture chamber light was switched on. Values are mean ± SD, n = 5. Values followed by the same letter indicate no significant differences (LSD test, 95 % confidence level).

Ilumination time	F_v/F_m	Φ_{PSII}	q_P	NPQ
1	0.667 a	0.482 a	0.813 a	0.288 a
3	0.693 b	0.497 a	0.812 a	0.246 a
5	0.682 ab	0.493 a	0.812 a	0.260 a
7	0.682 ab	0.494 a	0.810 a	0.271 a
9	0.680 ab	0.496 a	0.814 a	0.257 a
11	0.667 a	0.485 a	0.812 a	0.245 a

phenomena such as phototaxis, timing of cell division, photosynthetic capacity, bioluminescence, gene expression, or sensitivity to ultraviolet radiation (Sweeney 1987; Johnson *et al.* 1998; Mittag 2001; Suzuki & Johnson 2001; Johnson 2001). Endogenous cell clocks prepare, for example, the algal cell before dawn for photosynthesis and before dusk for various reactions that occur preferably during the night, such as cell division or growth (Lüning 2005). Rhythms of photosynthetic capacity were found, for example, in white light for unicellular algae such as *Lingulodinium polyedrum* or *Euglena* sp., and in red light for the filamentous brown alga *Ectocarpus* sp. (Hastings *et al.* 1961; Walther & Edmunds 1973; Schmid *et al.* 1992). Our results do not reveal whether the isolated photobiont *T. erici* has circadian rhythms, but should it occur, no effects were found on the fluorescence measurements, since all the fluorescence parameters remained constant all during the entire day (Table 2.2).

2.3.6. Confidencial limits for our experimental conditions

Cultures used in our experiments are always in linear growth, since they are in liquid cultures until they are inoculated and grow in cellulose-acetate disc in agar medium, thus a minimal methodological error might produce a great variation in the results of the

Table 2.3 - Effect of the initial cell density inoculation on chlorophyll *a* fluorescence paramenters and weight (mg) of *T. erici* cultures growing in cellulose-acetate discs on agar medium at 30 µmol m^{-2} s^{-1} PAR and 20 ºC after 21 days. Cell density of the stock cultures was assessed and adjusted to 10^6 cell ml^{-1} using the mean value (M) or the mean ± the standard error (SE) of the cell count. Values are mean ± SD, n = 5. Values followed by the same letter indicate no significant differences (LSD test, 95 % confidence level).

Density	F_v/F_m	Φ_{PSII}	q_P	NPQ	Weight
M	0.666 a	0.496 a	0.815 a	0.277 a	89.6 a
M+SE	0.668 a	0.496 a	0.801 a	0.237 a	84.5 a
M-SE	0.671 a	0.504 a	0.809 a	0.252 a	83.0 a

experiments. We observed that an error in the estimation of the stock cell density, thus in the number of cells inoculated, did not have any effects on photosynthetic parameters or on algal growth (Table 2.3). Nor did a possible mistake in the amount of nutrients in the Petri dishes produce any variation (Table 2.4), thus Trebouxia Medium nutrients are not a limiting growth factor, at least during the first three weeks of culture.

2.4. Conclusions

- Cellulose-acetate discs for agar cultures should be inoculated with 14-day-old liquid cultures, when they reach maximum growth rate.

- For fluorescence measurements, we should use an actinic light of 210 µmol m^{-2} s^{-1} PAR. Since, *T. erici* has a light saturation point of around 300 µmol m^{-2} s^{-1} PAR and the closest lower light intensity that the fluorometer PAM-2000 enables us to use is 210 µmol m^{-2} s^{-1} PAR.

- Algae cultures on acetate-cellulose disc should be grown at 20 ºC. Under this temperature the growth rate is two-fold higher than at 17 ºC, which allows us to obtain enough biomass for experiments (pigment analysis, protein extraction, determination of enzymatic activities, DNA/RNA isolation, etc) in less time.

Table 2.4 - Effect of the nutrient concentration on chlorophyll *a* fluorescence parameters and weight (mg) of *T. erici* cultures growing in cellulose-acetate discs on agar medium at 30 µmol m^{-2} s^{-1} PAR and 20 ºC after 21 days. Agar medium was prepared with a half of the normal nutrient concentration (0.5 X), normal concentration (1 X), or a one and a half normal nutrient concentration (1.5 X). Values are mean ± SD, n = 5. Values followed by the same letter indicate no significant differences (LSD test, 95 % confidence level).

Nutrient	F_v/F_m	Φ_{PSII}	q_P	NPQ	Weight
0.5 X	0.679 a	0.478 a	0.783 c	0.157 a	74.5 a
1 X	0.680 a	0.471 a	0.764 b	0.176 a	83.2 a
1.5 X	0.671 a	0.464 a	0.743 a	0.185 a	84.5 a

Furthermore, we observed the higher photosynthetic rates at 20 ºC. At 23 ºC, growth rate was similar but under 50 µmol m^{-2} s^{-1} PAR *T. erici* showed photo-inhibition and growth decline. We selected an irradiance of 30 µmol m^{-2} s^{-1} PAR. Under this light intensity the growth rate of *T. erici* is good and photoinhibition is avoided.

- Experiments should be carried out with 21-day-old algal discs, because photosynthetic capacity is the highest at this moment and the culture is still in the log growth phase.

- Fluorescence measurements can be carried out during the entire day since we did no observed daily rhythms of photosynthesis.

2.5. Resumen

Introducción

El estudio de la desecación en líquenes ha sido generalmente llevado a cabo con talos intactos, por lo tanto, preservando las condiciones e interacciones entre los simbiontes. Solamente unos pocos estudios se han centrado en el estudio de los efectos de la desecación en fotobiontes aislados (Brock 1975; Lange et al. 1990; Kranner et al. 2005; Weissman et al. 2005; Vaczi & Bartak 2006; Kosugi et al. 2009). La experimentación con talos íntegros hace difícil distinguir por separado las respuestas fisiológicas, como la respiración o las actividades enzimáticas antioxidantes, de cada uno de los simbiontes. El aislamiento y cultivo del fotobiontes facilita la interpretación de sus cambios fisiológicos e incrementa nuestros conocimientos sobre sus mecanismos de tolerancia a la desecación. Así mismo, el estudio de las respuestas de los fotobiontes a los procesos de desecación y rehidratación también pueden ayudarnos a entender mejor el funcionamiento fisiológico de los líquenes como organismos simbióticos.

Los diversos estudios realizados con fotobiontes aislados han sido centrados en la respuesta al estrés por metales pesados (p.e., Backor & Vaczi 2002; Backor *et al.* 2004; Bud'ova *et al.* 2006; Guschina & Harwood 2006; Backor *et al.* 2007), y en el efecto de la irradiancia y del estrés osmótico en la fotosíntesis (Vaczi & Bartak 2006). Hasta donde llega nuestro conocimiento, en la literatura encontramos que *Trebouxia erici* es la especie de fotobionte de la que existe mayor número de datos fisiológicos. Por esta razón hemos seleccionado esta especie para nuestros estudios. Pero, por el contrario, las condiciones de cultivo utilizadas para esta especie varían según los autores de estos trabajos. Así pues, el objetivo de este capítulo es la descripción de cómo establecimos las condiciones de cultivo óptimas para los experimentos a realizar en esta Tesis, así como la discusión y el análisis de los posibles efectos de las condiciones de crecimiento en las mediciones de la fotosíntesis.

Materiales y métodos

Una cepa axénica del fotobionte liquénico *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) fue utilizada en nuestro estudio. Los cultivos stock de algas fueron mantenidos en tubos de 10 ml conteniendo medio de *Trebouxia*: 3N Bold Basal Médium (3NBBM) más 10 g de caseina y 20 g de glucosa por litro (Ahmadjian 1973). Los cultivos líquidos fueron mantenidos a 20º C bajo un fotoperiodo de 12 h de luz blanca con una intensidad de 30 μmol m^{-2} s^{-1} (PPFD).

Cultivos líquidos crecidos durante dos semanas fueron agitados con una pipeta en condiciones estériles, y después dejados reposar durante 10 min para dejar sedimentar los agregados de células. A continuación, se tomó un mililitro de la parte superior del tubo y el número de células fue cuantificado con un hematocitómetro. La densidad celular final fue ajustada con medio estéril a 10^6 células por mililitro. Cincuenta microlitros de esta suspensión fueron inoculados en discos de acetato de celulosa estériles (13 mm de diámetro, 0.45 μm de tamaño de poro) y puestos en medio de *Trebouxia* con agar en placas Petri (Goldsmith *et al.* 1997; Backor & Vaczi 2002).

La fluorescencia de la clorofila *a* fue medida in vivo en los discos de algas con un fluorómetro de luz modulada PAM-2000 siguiendo los protocolos descritos por Calatayud *et al.* (2006).

Resultados

Los cultivos líquidos de *T. erici* mantenidos a 30 μmol m^{-2} s^{-1} (PPFD) (12 h de fotoperiodo) y 20 ºC mostraron una fase de crecimiento lineal desde el tercer día al 19º. Durante esta fase la tasa de crecimiento máxima (*r*, 0.253), y la del tiempo de duplicación (T_2, 3.960 días), se alcanzaron entre los días 7 y 17 de cultivo.

En los cultivos en agar, el punto de saturación lumínica varió entre los 300 y los 500 μmol m^{-2} s^{-1} (PPFD), y máxima tasa de transporte de electrones (ETR) se alcanzó el día 19, después de este momento la ETR disminuyó.

Los efectos de la temperatura y la luz fueron analizados en cultivos sólidos de 21 días, cuando las algas comenzaron a sobrepasar los límites de los discos de acetato de celulosa. La temperatura tuvo un efecto significativo ($Pv < 0.01$) en todos los parámetros fotosintéticos medidos y en el crecimiento. El rendimiento fotosintético máximo (F_v/F_m) fue mayor a 17 ºC. Sin embargo, el rendimiento fotosintético en luz (Φ_{PSII}) y la disipación fotoquímica (q_P) fueron mayores en los cultivos mantenidos a 20 ºC. A 17 ºC la biomasa de las algas fue aproximadamente la mitad de la obtenida a 20 ºC ó a 23 ºC. Las algas crecidas a 50 μmol m^{-2} s^{-1} (PPFD) y a 23 ºC comenzaron a mostrar signos de fotoinhibición indicando que esa intensidad lumínica puede estar cercana a los límites de tolerancia lumínica de *T. erici*.

La biomasa de las algas cultivadas en discos de acetato de celulosa a 30 μmol m^{-2} s^{-1} (PPFD) creció linealmente hasta el día 29. Desde el día 19 al 24, la *r* y la T_2 fueron 0.604 y 1.655, respectivamente. Las mediciones de fluorescencia dieron unos valores medios de Fv/Fm de 0.650 para el día 17, después se incrementó hasta el día 21 llegando a 0.698, para después comenzar a disminuir, reduciéndose hasta 0.572 el día 29.

Nuestros resultados no revelaron si el fotobionte aislado *T. erici* tiene ritmos circadianos, pero si los tiene no se encontró efecto alguno en los parámetros de la fluorescencia, ya que éstos permanecieron constantes durante todo el día.

Conclusiones

- Las inoculaciones en los discos de acetato de celulosa serán llevados a cabo con cultivos líquidos de 14 días, cuando se encuentran en una tasa de crecimiento máxima.
- Para las mediciones de fluorescencia se utilizará una luz actínica de 210 μmol m^{-2} s^{-1} (PPFD), ya que, como hemos visto anteriormente, *T. erici* tiene un punto de saturación de alrededor de 300 μmol m^{-2} s^{-1} (PPFD) y esta intensidad es la menor más cercana que puede utilizarse en el PAM-200.
- Las algas cultivadas sobre discos de acetato de celulosa deberán ser mantenidas a 20 ºC. Bajo esta temperatura la tasa de crecimiento es el doble que a 17 ºC, lo que

nos permite obtener una biomasa suficiente para los futuros experimentos (análisis de pigmentos, extracción de proteínas, determinación de actividades enzimáticas, aislamiento de ADN/ARN, etc) en menos tiempo. Así mismo, a 20 ºC se registraron las máximas tasas fotosintéticas. A 23 ºC, la tasa de crecimiento fue similar que a 20 ºC, pero a 50 μmol m^{-2} s^{-1} (PPFD) aparecieron síntomas de fotoinhibición y un menor crecimiento.

- Los experimentos serán llevados a cabo con discos de algas de 21 días, debido a que en este momento la capacidad fotosintética es máxima y el cultivo esta todavía en la fase de crecimiento lineal.
- Las mediciones de fluorescencia pueden realizadas en cualquier hora del día ya que el fotoperiodo no afecta a la fotosíntesis.

3. DEHYDRATION RATE AND TIME OF DESICCATION AFFECT RECOVERY OF THE LICHENIC ALGAE *TREBOUXIA ERICI*: ALTERNATIVE AND CLASSICAL PROTECTIVE MECHANISMS

Abstract

Desiccation tolerant plants can be divided broadly in two groups, those classified as "modified desiccation-tolerant plants" (resurrection vascular plants), because in comparison with the other group, the "fully desiccation-tolerant plants" (most bryophytes and some small pteridophytes), their ability to survive desiccation is rate-dependent. The mechanisms involved in desiccation tolerance of lichens and their photobionts are still poorly understood. The aim of the present work is to analyse the effect of the drying rate on the viability and further recovery, of the isolated lichen photobiont *Trebouxia erici*, studying the response of the protective mechanisms and whether these ones are constitutively expressed or induced during the dehydration process. Our findings indicate that the drying rate has a profound effect on the recovery of photosynthetic activity of algae after rehydration, greater than the effects of desiccation duration. The basal fluorescence (F'_0) values in desiccated algae were significantly higher after rapid dehydration (< 60 min), than after slow dehydration (4 – 5 h), suggesting higher levels of light energy dissipation in slow-dried algae. Higher values of PSII electron transport were recovered after rehydration of slow-dried *Trebouxia erici* compared to rapid-dried algae. The main component of non-photochemical quenching after slow dehydration was energy dependent (q_E), whereas after fast dehydration it was photoinhibition (q_I). Although q_E seems to play a role during desiccation recovery, no significant variations were detected in the xanthophyll cycle components. Desiccation did not affect PSI functionality. Classical antioxidant enzymes activities like superoxide dismutase or peroxidase decreased during desiccation and early recovery. Dehydrins were detected in the lichen-forming algae *T. erici* and were constitutively expressed. There is probably a minimal period required to develop strategies which will facilitate transition to the desiccated state in this algae. In this process, the xanthophyll cycle and antioxidant mechanisms play a very limited role, if any. However, our results indicate that there is an alternative mechanism of light energy dissipation during desiccation, where activation is dependent on a sufficiently slow dehydration rate.

3.1 Introduction

Lichens are symbiotic associations (holobionts) between fungi (mycobionts) and certain groups of cyanobacteria or unicellular green algae (photobionts). This association has played an essential role in enabling the colonisation of terrestrial and consequently dry habitats. Lichens are found among poikilohydric organisms, capable of surviving long periods in a desiccated state. This desiccated state is defined by a water content below 10% of the fresh weight, which would be fatal for most vascular plants and organisms (Bewley 1979; Rundel 1988; Kranner 2002). The ability of cells to survive from the air-dry state is referred to as desiccation tolerance (Bewley 1979) or anhydrobiosis (Crowe *et al.* 1992). 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, in some algae, fungi and bacteria, in ca. 350 species of flowering plants and ferns, and in most bryophytes and seeds of flowering plants (Proctor & Tuba 2002; Alpert 2006).

Desiccation-tolerant plants can be divided into two groups (Oliver & Bewley 1997): those classified as ‘‘modified desiccation-tolerant plants’’ (resurrection vascular plants) and the ‘‘fully desiccation-tolerant plants’’ (most bryophytes and some small pteridophytes). Tolerant angiosperms (modified desiccation-tolerant plants) only survive if the rate of water loss is very slow (in days to weeks) and they take up to 24 h or more to recover (Oliver & Bewley 1997; Alpert & Oliver 2002; Proctor & Pence 2002). This is a consequence of an inducible desiccation tolerance, with sufficient time being required for tolerance mechanisms to develop, which are based on protection during desiccation, rather than repair on rehydration (Alpert & Oliver 2002). In contrast, non-vascular resurrection plants (fully desiccation-tolerant plants) can survive very rapid drying events (less than 1 h) and recover respiration and photosynthesis within a few minutes (Oliver & Bewley 1997; Proctor & Smirnoff 2000). Tolerance seems to be achieved predominantly by an ability to repair damage caused by desiccation, and is thus primarily based on constitutive mechanisms (Oliver & Bewley 1997). Drying rate appears to have little influence on ultimate survival, but recovery takes longer in rapidly dried tissue, perhaps suggesting the existence of some inducible protection mechanisms (Schonbeck & Bewley 1981). At the cellular level, various protective biochemical mechanisms against desiccation damage have

been suggested. Non-reducing di- and oligosaccharides contribute to ‘‘vitrification’’, the formation of a glassy state in cytoplasm (Buitink *et al.* 2002). In addition, they appear to substitute for water, thus maintaining the membrane structure during desiccation. The late embryogenesis abundant (LEA) proteins are also considered critical in the acquisition of cellular desiccation tolerance because, although their function remains unclear, in desiccationtolerant angiosperms they are accumulated during dehydration and in bryophytes, like the moss *Tortula ruralis*, they are constitutively synthesised (Bewley *et al.* 1993; Ingram & Bartels 1996). Another important process under stress conditions is the generation of non-photochemical quenching that dissipates excess light energy, which cannot be used for photosynthesis and is potentially switched to generate photooxidative damage, in the form of heat (Müller *et al.* 2001). In vascular plants the dissipation of light energy excess is associated with acidification of the thylakoidal luminal space, the xanthophyll cycle and the action of the PSII-associated pSBS protein (Demmig-Adams & Adams 1992; Adams & Demmig-Adams 2004; Krause & Jahns 2004; Niyogi *et al.* 2004). Alternative mechanisms of energy dissipation have been described in mosses and lichens, where new quenching centres are functional during desiccation (Kopecky *et al.* 2005; Heber *et al.* 2006a,b; Heber 2007; Heber 2008). Upon desiccation, a light-dependent mechanism is activated which requires neither protonation nor zeaxanthin, where the recombination between P680 and a neighbouring Chl- is suggested to form the molecular basis of a desiccation- induced energy dissipation in photosystem II (PSII) reaction centres (Heber *et al.* 2006a,b). Another alternative mechanism found in mosses and lichens may be dependent on the desiccation rate, independent of light and probably associated to conformational changes in a chlorophyll–protein complex (Heber *et al.* 2007; Heber 2008). Finally, the antioxidant system protects the cell against the photooxidative stress produced by the increase in reactive oxygen species (ROS) during dehydration and rehydration (Minibayeva & Beckett 2001; Kranner & Birtic 2005; Weissman *et al.* 2005).

Studies of drought and desiccation tolerance of lichens and their photobionts are usually focused on the antioxidant system. Thus, the antioxidant mechanisms common in vascular plants have been analysed in lichens: antioxidant enzymes, such as, superoxide dismutase (SOD) (Weissman *et al.* 2005), peroxidases (POX) (Silberstein *et al.* 1996; Kranner *et al.* 2005), glutathione reductase (GRX), ascorbate peroxidase (Kranner 2002;

Kranner *et al.* 2005); and low molecular weight substances like glutathione, a-tocoferol, ascorbic acid (Kranner 2002), carotenoids and specially the components of the xanthophyll cycle (Deltoro *et al.* 1998; Kranner *et al.* 2005). However, it has not been possible to establish a clear relationship between the different levels of desiccation tolerance in most of the lichens and antioxidant mechanisms. Several studies show controversial results and reveal that the behaviour of the antioxidant mechanism after desiccation and/or rehydration depends on the lichen species, pre-treatment of the thalli, time course of the experiment, as well as the experimental methodology used for the dehydration and rehydration (Deltoro *et al.* 1998; Kranner *et al.* 2005; Weissman *et al.* 2005). The aim of the present work is to analyse the effect of the drying rate on the viability and further recovery of the isolated lichen photobiont *Trebouxia erici*, studying the response of the protective mechanisms and whether they are constitutively expressed or induced during the dehydration process.

3.2 Materials and methods

3.2.1. *Biological material*

An axenic strain of the lichen photobiont *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) was cultured on sterile cellulose-acetate discs as is described in the Chapter 2 (Section 2.2.2.).

3.2.2. *Desiccation and rehydration treatments*

For slow dehydration (SD) experiments, the discs supporting the algae culture were picked from the agar plates and placed on a microscope cover-glass inside a phytotron at 20ºC, 30 μmol m^{-2} s^{-1} and constant relative humidity (r.h.) of 67%. For rapid dehydration (RD) the conditions were similar but the discs were maintained at a constant r.h. of 20%. The discs on the microscope cover-glasses were placed on precision scales to monitor the loss of fresh weight until it remained constant.

To study the effect of continuous desiccation over a period of time, the algae were kept dried inside sealed plates, stored in a culture chamber under photoperiod conditions.

Algae were rehydrated at different times by adding a volume of distilled water equivalent to the water lost.

3.2.3. *Chlorophyll* a *fluorescence measurements*

Chlorophyll *a* fluorescence was measured *in vivo* with a modulated light fluorometer (PAM-2000, Walz, Effeltrich, Germany). The discs were placed on a microscope slide wrapped with moist filter paper to maintain a hydrated state. All the samples were kept in the dark for 30 min just before fluorescence measurements were taken. The minimum (dark) fluorescence yield (F_o) was obtained after excitation of the algae with a weak measuring beam from a light-emitting diode. The maximum fluorescence yield (Fm) was determined with an 800 ms saturating pulse of white light (SP, 8000 μmol m^{-2} s^{-1}). Maximal variable fluorescence (F_v) was calculated as F_m-F_o. The parameter F_v/F_m represents the maximum quantum efficiency of PSII photochemistry when all the reactions centres are open after a period of dark adaptation (Baker & Oxborough, 2004). Following a two-minute dark readjustment, actinic light (AL, 200 μmol m^{-2} s^{-1}, unless otherwise stated) was switched on, and SP were applied at 1 min intervals for 11 min to determine the maximum fluorescence yield during actinic illumination (F'_m), the Chl *a* fluorescence yield during actinic illumination (F_s) and the level of modulated fluorescence during a brief interruption (3 s) of actinic illumination in the presence of 6 μmol m^{-2} s^{-1} far red (FR, 730 nm) light (F'_o). The parameter F'_v/F'_m (where, $F'_v = F'_m - F'_o$) provides an estimate of the maximum quantum efficiency of PSII photochemistry at the point of measurement under light conditions (Baker & Oxborough, 2004). Quenching due to the non-photochemical dissipation of absorbed light energy was calculated as described by Schreiber *et al.* (1986) like $q_N = 1-(F'_m-F'_o)/(F_m-F_o)$. Relaxation kinetics of q_N in the dark can be decomposed in a fast component, related to high trans-thylakoidal pH gradient (q_E); a middle component, sometimes related to state I-state II transitions (q_M); and a third slow component (q_I) related to photosystem II photoinhibition (Quick & Stitt, 1989; Walters & Horton, 1991; Krause & Weis 1991). These components of non-photochemical quenching were determined following the dark relaxation kinetics of q_N as described by Walters & Horton (1991). The effective quantum efficiency of PSII photochemistry, ϕPSII, which is closely associated

with the quantum yield of non-cyclic electron transport, was estimated from $(F'_m-F_s)/F'_m$ (Genty *et al.* 1989; Kramer *et al.* 2004). The coefficient for photochemical quenching, q_P is non-linearly related to the fraction of open PSII reaction centres and was calculated as $(F'_m-F_s)/(F'_m-F'_o)$ (Schreiber *et al*., 1986).

3.2.4. *Measurement of the Absorbance Change of P700*

The redox change of P700 assessed by monitoring absorbance at 820 nm was measured with a PAM-101 chlorophyll fluorometer (Walz, Effeltrich, Germany) equipped with an ED 800T emitter-detector unit as described by Munekage *et al.* (2002), but using a white actinic light of 200 μmol $m^{-2}s^{-1}$. All measurements were performed after 10 min dark adaptation.

3.2.5. *Estimation of the integrity of cell membranes*

Membrane integrity was assessed by immersing the algal disc in water and measuring the electrical conductivity, which expresses electrolyte leakage (Pearson & Rodgers 1982; Garty *et al.* 1998). Dehydrated algal discs were immersed in 3 ml of Milli-Q water and stirred gently every 5 min. The electrical conductivity of the water was measured with an electrical conductimeter (Crison GLP 32, Spain) for about 30 min, until the measurement was constant. The electrical conductivity of five discs without algae, which remained in the culture plates during the growing period, were measured to remove the effect of the ions absorbed by the cellulose-acetate discs, and the average value was subtracted from each measurement.

3.2.6. *Inmunodetection of dehydrins*

Electroblotting and immunodetection were performed as described in Guéra *et al.* (2000) using a commercial anti-dehydrin antibody (Agrisera, Vänäs, Sweden) against a conserved C-terminal peptide sequence (K segment) from dehydrin (Close *et al.* 1993). Quantification was obtained by ImageJ software based analysis (http://rsb.info.nih.gov/ij/).

3.2.7. Enzyme analytical methods

Algal disks were ground with liquid nitrogen by using mortar and pestle and resuspended in 1 ml of 100 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 0.1 % (w/v) PVP and 0.1 % (v/v) Triton X-100. The slurry was centrifuged at 13500 rpm for 5 min, the supernatant collected and frozen in liquid nitrogen and stored at –80ºC until the enzymatic determinations. All operations (until analysis) were carried out at 3-5 ºC. The protein concentration was determined using the Bradford method (Bradford, 1976) with bovine serum albumin standard.

Peroxidase (POX) activity was analyzed following the protocol described by Zapata *et al.* (1998) with slight modifications. The assay was performed in a cuvette containing 50 mM Tris-acetate buffer (pH 5.0), 40 μM 3,3',5,5'-tetramethylbenzidine (TMB) and an appropriate volume of the algal extract. Reaction assays were started by the addition of 10 mM H_2O_2. Activity was determined by the increase in the absorbance at 652 nm as a result of the TMB oxidation.

Superoxide dismutase (SOD) Superoxide dismutase (SOD) activity was measured spectrophotometrically as described by Beyer & Fridovich (1987). The reaction volume contained 50 mM Tris-HCl buffer (pH 7.8) Triton X-100 0.025 % (v/v), 9.9 mM methionine, 57 μM nitroblue tetrazolium (NBT) and rivoflavin 1 μM and was performed in the dark. Reaction assays were started by the addition of an appropriate volume of the algal extract and placing them in light. In this assay, 1 U of SOD is defined as the amount required to inhibit the photoreduction of NBT by 10% (Backor *et al.* 2003).

3.2.8. *Assessment of TTC reduction*

Conversion of triphenyltetrazolium chloride (TTC) to red triphenylformazan (TPF) was considered an indication of dehydrogenase activity and thus of respiration (Towill & Mazur 1975). To evaluate the reduction of TTC a modified method of Backor *et al.* 2005 was used which involved homogenizing biological test material in dimethyl sulfoxide (DMSO) rather than water. Photobiont cell masses were weighed in sterilized 2 ml

centrifuge tubes and 2 ml of 0.6% TTC in 0.05 M phosphate buffer (pH 6.8) containing 0.005% Triton X 100 was added. Closed tubes were incubated in the dark for 20 h at 25 °C to permit TPF production. Photobiont cells were collected by centrifugation at 400 × *g* for 15 min, the supernatant was removed and the pellets were re-suspended in 2 ml distilled water. After 5 min, cells were again collected by centrifugation. Water-insoluble formazan was extracted from all tested samples with 2 ml of DMSO at 65 °C in the dark for 30 min, vortex twice during the incubation and then centrifugated (10000 g, 5 min). The supernatant was transferred to another tube and the extraction was repeated until the pellet lost the red colour. After centrifugation, the absorbance of the supernatants was spectrophotometrically measured at 492 nm, for TPF quantification. Turbidity of the extract was checked at 750 nm to confirm an absorbance lesser than 0.01. Pure DMSO was used as a blank.

3.2.9. *Ascorbate determination*

Ascorbate was determined spectrophotometrically by monitoring the decrease in the absorbance at 265 nm induced by the complete oxidation of ascorbate acid (AsA) to dehydroascorbate (DHA) by AsA oxydase (A0157 Sigma), as described by Luwe *et al.* (1993). *T. erici* discs were were ground to powder with liquid nitrogen by using mortar and pestle and resuspended with ice-cold metaphosphoric acid (2 ml, 2 % w/v). The homogenate was centrifuged at 10000 g x 10 min (4 ºC). Reaction assays contained 0.5 ml of algal extract and 1 ml of 0.1 M sodium phosphate buffer (pH 6.8). AsA oxidation was started by the addition of 10 µl of AsA oxidase (0.25 units μl^{-1}).

3.2.10. *Algal pigments analysis*

Algal disc were frozen with liquid nitrogen inmediatedly after the treatments, ground to powder by using mortar and pestle and resuspended in cold 100% acetone. After a centrifugation (13500 rpm, 5 min, 4 ºC) the supernatant was filtered with a nylon filter (0.15 µm pore size). Extracts were frozen with liquid nitrogen and stored with an N_2 atmosphere in darkness at –80ºC. Algal pigments were analysed by HPLC following the method described by (Mantoura & Llewellyn 1983) (Appendix 2).

3.2.11. *Statistics*

For each experiment several plates were randomly chosen, every plate containing 5 cellulose-acetate discs that were also randomly chosen to apply the different treatments. Every experiment was repeated four to nine times starting from new stock material and the preparation of fresh new cultures, dishes and plates. We used one-way ANOVA in

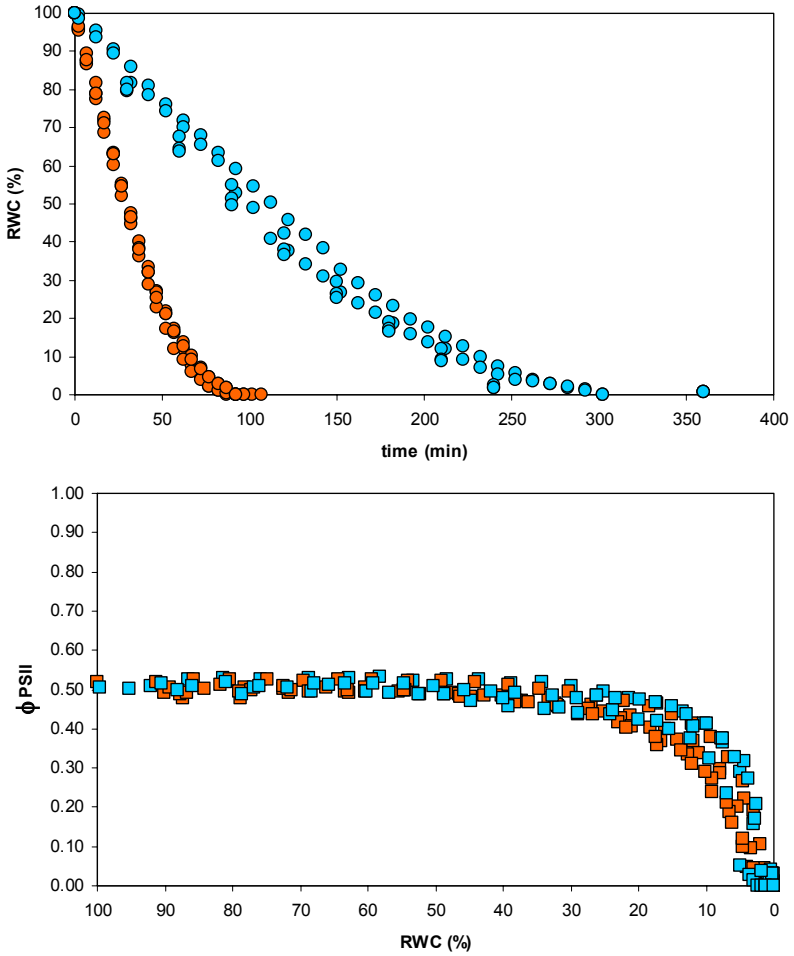


Figure 3.1 - Time-course of water content (a) and Φ_{PSII} (b) during slow (blue symbols) and rapid (black symbols) dehydration in *T. erici*. Water content is expressed as a percentage of the water content (RWC) of fully hydrated algae disc. Results are from 3 – 6 different experiments.

Table 3.1 - Levels of F_m, F'_m, F_o and F'_o fluorescence[a] in hydrated and desiccated colonies of *T. erici.*

	$F_{m\ hydrated}$	$F_{o\ hydrated}$	$F'_{m\ desiccated}$**	$F'_{o\ desiccated}$**	$F_{m\ hydrated}$ / $F'_{o\ desiccated}$*	$F_{o\ hydrated}$ / $F'_{o\ desiccated}$*
Slow	0.575 ± 0.100	0.223 ± 0.020	0.079 ± 0.017	0.073 ± 0.013	7.972 ± 1.424	3.143 ± 0.743
Rapid	0.642 ± 0.111	0.230 ± 0.033	0.129 ± 0.013	0.125 ± 0.014	5.121 ± 0.701	1.829 ± 0.129

[a] Fluorescence values in arbitrary units. Mean values ± SD. Slow, n = 9. Rapid, n = 12.
* P < 0.005, ** P < 0.001, significant differences between slow and rapid drying.

combination with Tukey HSD test to compare the means of drying treatments during desiccation and rehydration time curses.

3.3. Results

3.3.1. *Influence of the drying rate and desiccation on fluorescence parameters*

Slowly dehydrated (SD) algae became completely desiccated in 300 min, whereas rapid dehydration (RD) produced a complete loss of water content in approximately 85 min (Fig. 3.1 a). Attempts to accelerate the dehydration rate under vacuum in the presence of silica gel gave a drying time longer than 90 min, thus slower to RD under our atmospheric pressure conditions. It has to be taken into account that the nitrocellulose filters retain a high amount of water and drying of these filters embedded in water without cell cultures on them consumed a similar time than drying of disc algal cultures under vacuum conditions. Actual quantum yield of PSII (ϕ_{PSII}) is maintained during most of the dehydration process (Fig. 3.1b) both under SD and RD conditions. Then, when reached a value of less than 20% RWC, falls abruptly. This behaviour was dependent only on the dehydration state and was independent of the drying rate. At the beginning of experiments in full hydrated algae (Table 3.1) the differences among *T. erici* colonies were not significant for F'_m or F'_o. Under our experimental conditions, at the end of the dehydration (RWC≤3%) only very low residual charge separation levels were observed both in SD algae (F'_v/F'_m=0.060±0.040) and RD algae (F'_v/F'_m =0.029±0.017). The F'_o values were significantly higher (P<0.001) in desiccated algae at the end of RD than at the end of SD (Table 3.1), suggesting higher

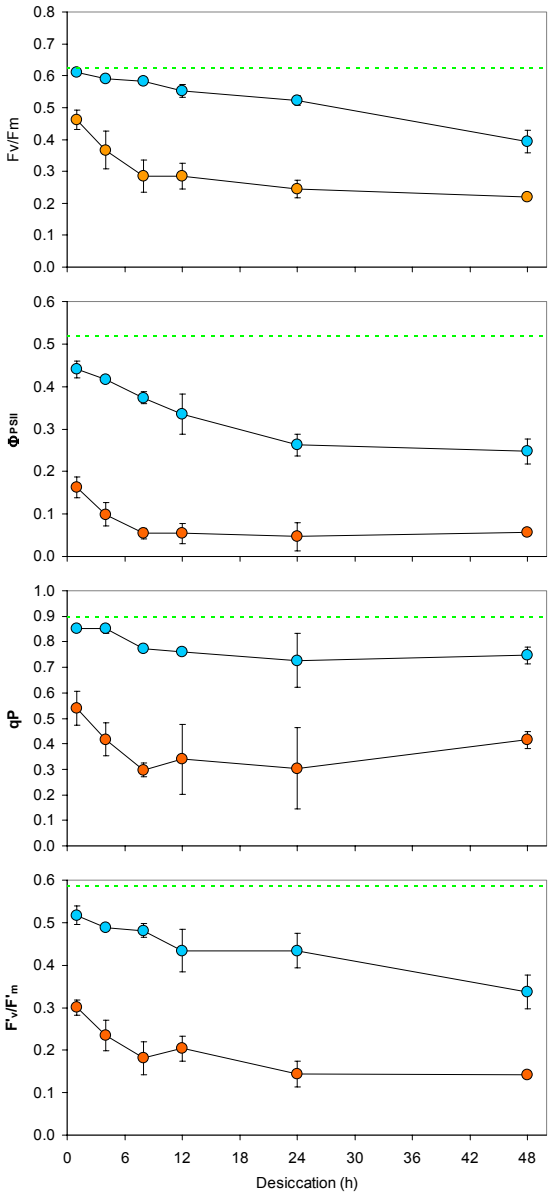


Figure 3.2 - Effect of the desiccation period on chlorophyll fluorescence parameters. For fluorescence measurements SD or RD, algal disc cultures were rehydrated after the indicated time, kept under culture conditions for 15 min, and then dark-adapted for 30 min. Recovery of F_v/F_m; Φ_{PSII}; q_P; F'_v/F'_m, after SD (blue squares) or RD (oranges squares). Green dotted lines denote control values of well-hydrated samples. Data represent the mean ± standard deviation (n = 5)

levels of light energy dissipation in SD algae that are confirmed by the statistically significant differences ($P<0.05$) between the $F'_{ohydrated}/F'_{odesiccated}$ ratios of RD and SD. Ratios of maximum pulse-induced fluorescence $F_{mhydrated}$ to $F_{odesiccated}$ (Table 3.1) were significantly higher ($P<0.05$) in SD than in RD algae. According to Heber *et al.* (2007) this ratios are indicative of differences in the extent of photoprotection, which would be higher in SD than in RD algae.

Dehydration rate and desiccation phase duration decreased the primary processes of photosynthesis after rehydration (Fig. 3.2). When algae were rehydrated immediately after SD, maximal PSII efficiency (F_v/F_m) was similar to the control non-dehydrated algae (0.625, $P=0.425$), but after RD F_v/F_m was only about 74% of the control value ($P<0.001$). During desiccation F_v/F_m values slowly decreased for SD algae to 83.6% and 63.1% of the control after 24 and 48 h, respectively. These data agree with metabolic activity tests based on the oxidation of triphenyl-tetrazolium chloride (Bačkor & Fahselt, 2003), which indicated a cell viability of 95% after 1h of SD and about 50% after 48h. Following RD the decay of F_v/F_m was faster during the first hours of desiccation, falling to 39.3% of the control value after 24 h and to 35% after 48 h (Fig. 3.2a). Based on the oxidation of tetrazolium, the viability of cells after 1h RD was about 50% and after 48h about 40%. When algae were rehydrated immediately after SD 85% of the control value of Φ_{PSII} was recovered (0.518) and decreased to 48% (0.293), 48 h after desiccation. Meanwhile, in RD algae only 31% of the Φ_{PSII} control value was recovered immediately after desiccation and fell to 10.9% after 8 h (Fig. 3.2b). Photochemical quenching (q_P) values were higher for SD than for RD algae ($P<0.01$, Fig. 3.2c). The parameter F'_v/F'_m was higher for SD than for RD algae and decreased for both during desiccation time ($P<0.001$, Fig. 3.2d), indicating when compared with ϕ_{PSII} and q_P values a higher level of photoinhibition and lower efficiency of energy dissipation processes for RD algae.

3.3.2. *q_E and the xanthophyll cycle*

Relaxation of q_N in the dark enables the components of non-photochemical quench to be distinguished (Krause & Weis 1991). As shown in Fig. 3.3a, when the light was switched off once q_N had reached a steady-state level, a very fast decay was observed for

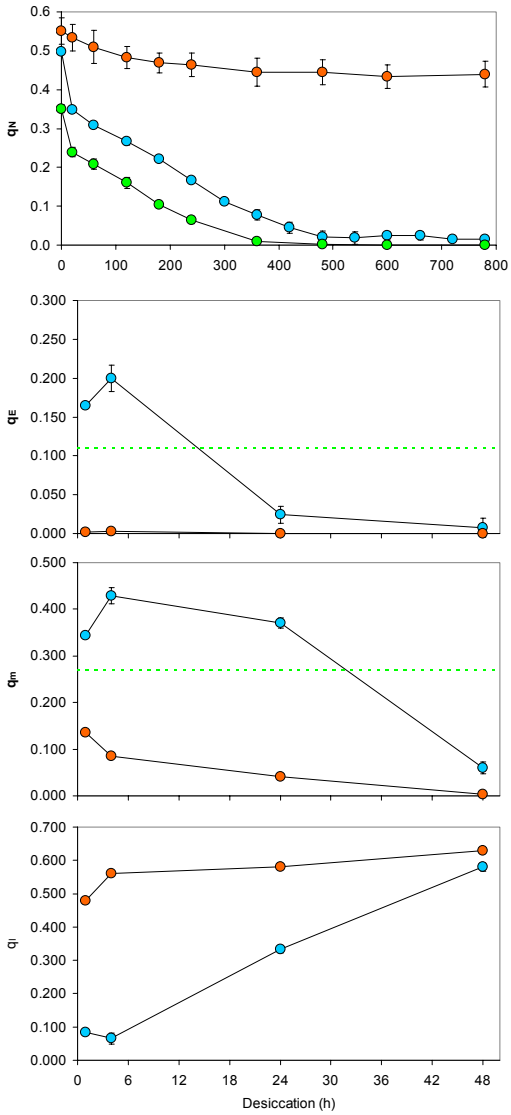


Figure 3.3 -Relaxation kinetics of q_N. a After reaching q_N steady-state values, the actinic light was turned off and saturation pulses were applied at the indicated time. Green circles correspond to well hydrated control algae; blue circles to SD algae maintained for 1 h in the desiccated state, and, orange circles to RD algae maintained for 1 h in the desiccated state. At the end of the desiccation period, algae were rehydrated. Variation of q_E during the desiccation period, blue symbols SD, orange symbols RD, and the green dotted line represents the control value. Variation of q_M during the desiccation period. Variation of q_I during the desiccation period, where the control value was zero. Data represent mean ± standard deviation (n = 5)

the first seconds in the dark in both control and SD algae. This first phase of fast decay during q_N relaxation in the dark is related to the rapid relaxation kinetics of the energy dependent (q_E) component of non-photochemical quenching (Horton & Hague 1988; Quick & Stitt 1989; Walters & Horton 1990). In higher plants this initial decay can be easily recorded within the first 3-4 min of dark relaxation (Munekage *et al.* 2002; Li *et al.* 2002; Guera *et al.* 2005). However in *Trebouxia* the half-life of q_E was shorter (about 8 s for control cells). This fast decay was not present in RD algae. Because q_N dropped to zero in less than 4 min in the dark for control algae and in less than 8 min for SD algae after 1h of desiccation, could be deduced nil or very low values for the photoinhibition component (q_I) of non-photochemical quenching. A middle-decay-velocity factor, previously denoted as q_M (Quick & Stitt 1989; Krause & Weis 1991) was apparently the main component of non-photochemical quenching for control and SD algae. In contrast, after RD the algae did not show a fast relaxation phase of q_N, nor a clear decline to zero values after more than 30 min in the dark, indicating that q_I is the main component of non-photochemical quenching. When the components of non-photochemical quenching were calculated according to (Walters & Horton 1991), results showed that q_E disappeared immediately after RD and was maintained or moderately increased immediately after SD. Furthermore, a minor initial rise of q_E was recorded during the first hours after SD followed by a clear decline during longer periods in the dehydration-state (Fig. 3.3b). The q_M component, seems to be the main component of non-photochemical quenching in control or SD algae after short desiccation times (Fig. 33c). In coherence, q_I values were higher in RD than in SD algae (Fig. 3.3d). Xanthophyll levels were only barely affected by the dehydration process (Table 3.2). Significant variations in the total amount of chlorophyll or the carotenoid/chlorophyll ratio were not found in these experiments. The total amount of the xanthophyll cycle components (V+A+Z) was less after dehydration, but without significant differences between SD or RD algae. The deepoxidation state (DPS) of xanthophylls as indicated by the ratio (A+Z)/(V+A+Z) did not change significantly with respect to control with either desiccation treatments. When well-hydrated, control algae were maintained for 24 h in the dark (Table 3.2, CD), a condition in which q_N falls to a near-zero value, only a small decrease was observed in the xantophyll DPS, in contrast with the clear increase observed after 2 h of high light (1000μmol $m^{-2}s^{-1}$) exposure (Table 3.2, CHL). Otherwise the levels

Table 3.2 - Changes in the proportion of carotenoid pigments in *T. erici*

		V	A	Z	V+A+Z	DPS	Lutein	β-carotene
C		46.048 a	7.531 ab	19.994 b	73.574 b	0.373 b	95.041a	5.358 a
CD		44.876 a	3.994 b	14.213 bc	63.085 b	0.288 b	94.501 a	5.625 a
CHL		22.546 bc	12.144 a	65.376 a	100.068 a	0.774 a	92.811 a	5.522 a
SD	1h	31.1493 b	10.4183 a	9.6015 c	51.169 c	0.387 b	85.263 a	7.025 a
	24h	27.224 bc	10.207 a	12.885 bc	50.316 c	0.453 b	80.672 a	5.718 a
	R 24h	26.733 bc	5.772 b	19.257 b	51.763 c	0.4853 b	80.316a	5.440 a
RD	1h	31.8616 b	10.217a	10.198 c	52.277 c	0.391 b	83.157a	6.745 a
	24h	28.142 bc	9.881 ab	12.882 bc	50.905 c	0.447 b	86.409 a	3.323 a
	R 24h	19.072 c	7.263 ab	13.850 bc	40.187 c	0.525 b	72.082 a	2.242 a

Carotenoid content of whole cell extracts is expressed as mmol mol^{-1} Chl *a* + *b*. C accounts for non-dehydrated control samples under growing light conditions (30 μmol m^{-2} s^{-1}). CD refers to 24 h dark-adapted control samples. CHL accounts for control samples after 120 min exposure to 1000 μmol m^{-2} s^{-1} white light. Pigment measurements in slowly (SD) and rapidly (RD) dried samples were carried out 1 h and 24 h from desiccation, and after 24 h of desiccation and 24 h recovery of the sample (R 24 h). V / A / Z = violaxanthin + antheraxanthin + zeaxanthin; DPS = (A + Z)/(V + A + Z). The results are expressed as mean ± standard deviation (n = 4). The values denoted by different letters differ significantly when subjected to a one-way ANOVA and Tukey HSD test (P<0.05)

of lutein (another xanthophyll, but not-related to the xanthophyll cycle), paralleled those of V+A+Z and the levels of β-carotene did not undergo any significant variation. Ascorbate levels were under the limit of detection following the method of Luwe *et al.* (1993).

3.3.3. *Dehydration did not produce photoinhibition in PSI, but affected membrane integrity*

Twenty-four hours after dehydration, the extent of P700 oxidation (Δ_{P700}^{+}) in actinic light was significantly (P<0.05) higher in the RD algae than in the control, but there was no significant difference for SD algae (Fig. 3.4). When the samples were treated with DCMU, an inhibitor of the electron transport from PSII to PSI, Δ_{P700}^{+} increased, reaching similar values for all treatments. In the case of dehydration-dependent photoinhibition of PSI, a lower Δ_{P700+} in dehydrated algae than in the control would be expected after DCMU

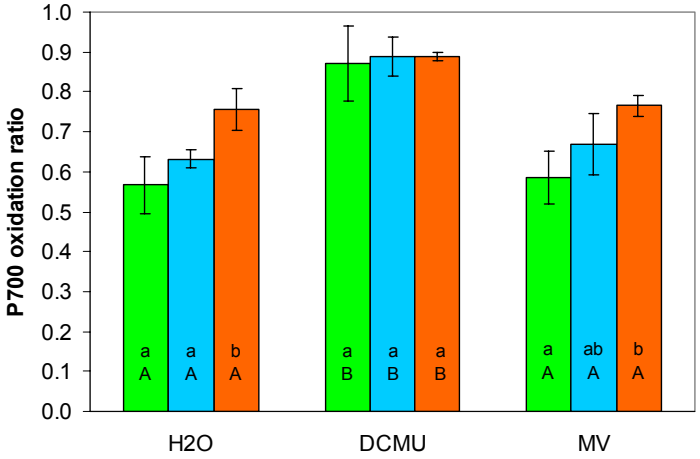


Figure 3.4 - Redox change of P700 assessed as A/P, where A is the maximum absorbance at 820 nm with actinic light of 200 μmol m^{-2} s^{-1} and P with a saturating pulse. Control (green bars), slow-dried (blue bars) and rapid-dried (orange bars) samples, previously desiccated for 24 h, were imbibed with water, 25 μM 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU) or 5 mM methyl viologen (MV) and kept in culture conditions for 15 min prior to the P700+ measurement. Results are expressed as mean ± standard deviation (n = 5). Different lowercase and capital letters are indicative of significant differences between desiccation and chemical treatments, respectively (P<0.05; Tukey HSD test)

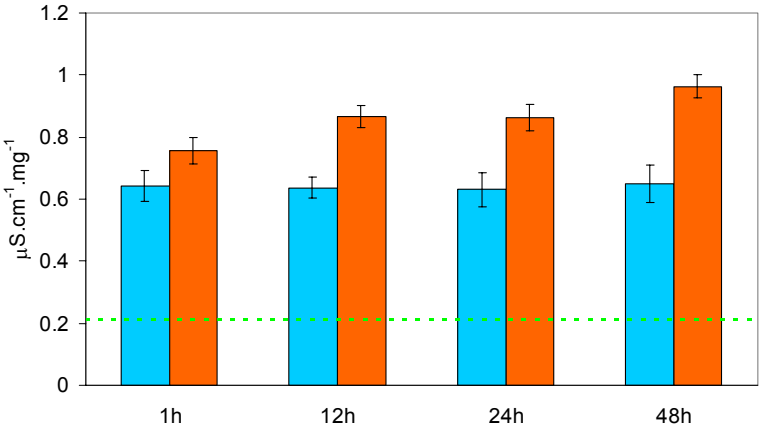


Figure 3.5 -Electrolyte leakage from *T. erici* dried slowly (blue bars) and rapidly (orange bars) measured as the electrical conductivity (μS cm^{-1} mg^{-1}) of MilliQ®-water, in which disc samples were immersed. Results are expressed as mean ± standard deviation (n = 5). The control value is represented as a green dotted line.

treatment (Kim *et al.* 2001). To confirm that electron transport from P700 to the final acceptors was not inhibited, dehydrated and non-dehydrated algae were treated with methyl-viologen, an electron acceptor from the PSI donor site competing with ferredoxin. Results were similar to those obtained for non-inhibitor treated samples, indicating that photoinhibition was produced on the PSII and was higher in the rapidly dehydrated algae than in those dehydrated slowly.

When desiccated algae were immersed in water, electrical conductivity was greater than in controls (Fig. 3.5) indicating a higher leakage of plasmatic ions. Thus, membrane damage was significantly greater after RD than after SD ($P<0.05$). Electrical conductivity was three times higher than the control after SD and remained constant throughout desiccation. By contrast, leakage of ions after RD rose gradually from 3.5 times the control value immediately after desiccation to 4.5 times after 48 h.

3.3.4. *Recovery phase*

The photosynthetic activity in algae desiccated for 24 h was not recovered immediately after rehydration, but maximal PSII efficiency (F_v/F_m) continued decreasing (Fig. 3.6a), reaching a minimum value around 8 h after rehydration (71.3 % and 25.7 % of the control value after SD and RD, respectively). Later, F_v/F_m started to rise, although recovery was slower in the SD algae than in RD. Nevertheless, at the end of the experiment (48h rehydrated), F_v/F_m was still significantly higher ($P<0.05$) in SD than in RD. The ϕ_{PSII} (Fig. 3.6b) of SD algae did not recover after rehydration and remained constant (around 60 % of the control). In contrast, RD algae started a considerable recovery of ϕ_{PSII} after 12h. At the end of the experiment no significant differences were observed between SD and RD ϕ_{PSII} values and both were far from reaching the control value ($P<0.001$). F'_v/F'_m was higher for SD than for RD algae ($P<0.01$, Fig. 3.6c). During the first 24 h of recovery F'_v/F'_m clearly decreased in RD algae, but only barely for the case of SD algae. Later the values for RD algae increased and after 48h became similar to SD algae. After 24 h of recovery, the xanthophyll cycle pigments (Table 3.2) presented similar values to those obtained at the end of the desiccation time. No significant differences were found either for the total amount of V+A+Z or DPS, irrespective of the preceding dehydration treatment

employed. Moreover, mean lutein values were lower than after the end of desiccation, although this decay was only statistically significant for the case of RD algae. Otherwise, β-carotene seemed to have a tendency to decline during desiccation and recovery, but again the Analysis of Variance did not show significant differences at the $P \leq 0.05$ level for these values.

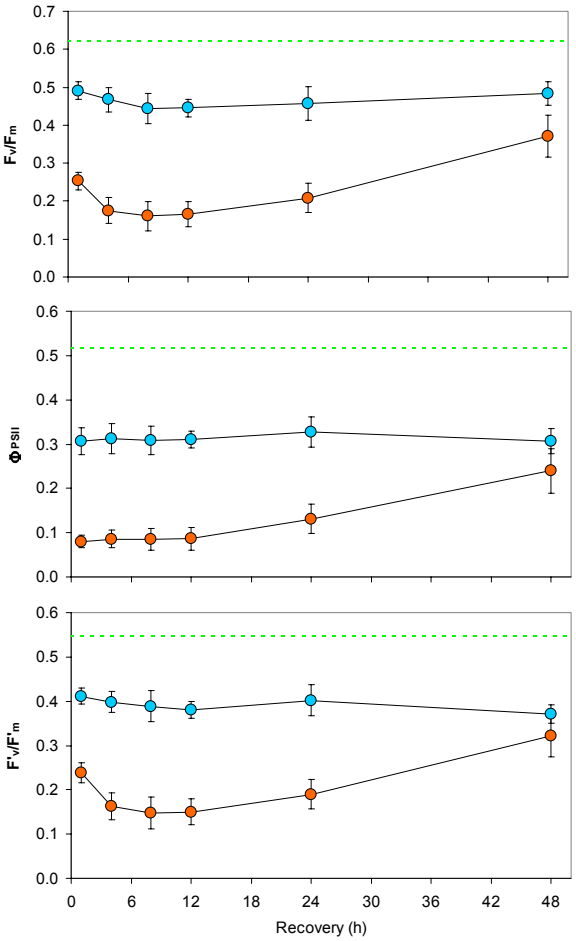


Figure 3.6 - Recovery of fluorescence parameters after SD and RD. Culture discs of T. erici desiccated for 24 h were rehydrated and kept under culture conditions during the indicated times. Recovery of F_v/F_m, Φ_{PSII} and F'_v/F'_m in SD algae (blue circles) and RD (orange circles). Green dotted lines denoted control values of well hydrated algae. Data represent mean ± standard deviation (n = 5)

3.3.5. *Antioxidant activities and dehydrins*

We found that both POX and SOD declined during the desiccation period of *T. erici* (Fig. 3.7a and 3.7b, respectively). Although the mean SOD and POX activities were slightly higher after SD than after RD, the differences were not significant. The same happened during recovery (Fig. 3.7c,d), POX and SOD declined during the first 24 h after rehydration and only a recovery in the antioxidant activity was observed for SD algae after 48 h of rehydratation.

The synthesis of specific proteins (dehydrins) during dehydration is thought to be involved in resistance to desiccation and recovery after rehydration (Oliver *et al.* 2005). Western blotting and immunodetection using a commercial antibody against dehydrins (Agrisera, Vänäs, Sweden) allowed the detection of five main bands of 15, 18, 34, 40 and 120 kDa in both control and treated samples (Fig. 3.8). Similar amounts of dehydrins were quantified in control, RD and SD, and 24 h rehydrated algae.

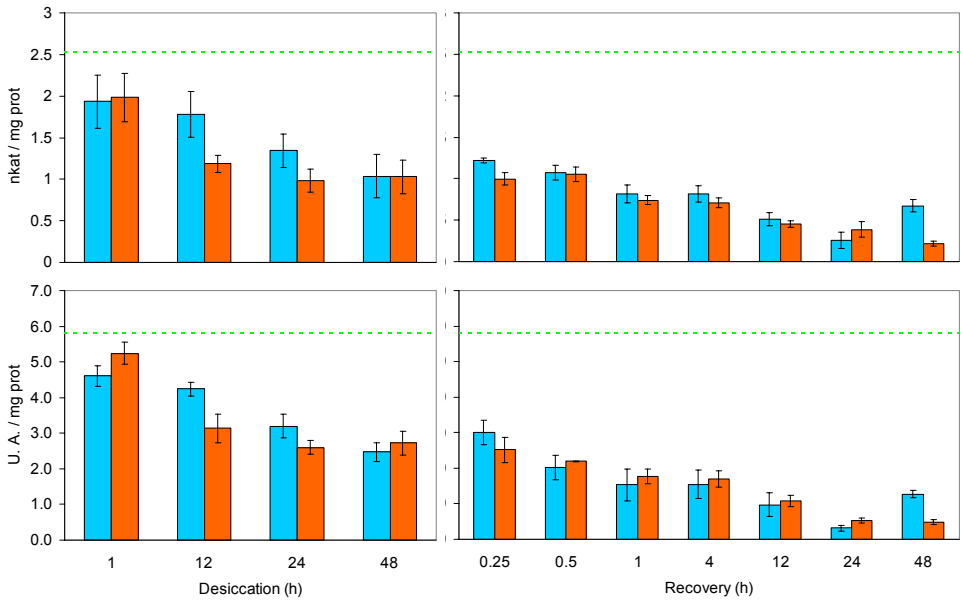


Figure 3.7 - Peroxidase (a, b) and SOD (c, d) activities in *T. erici* slowly (blue bars) and rapidly dried (orange bars) during desiccation (a, c) and recovery (b, d). Samples used for recovery were kept desiccated for 24 h. Green dotted lines denoted control values of well-hydrated algae. Data represent mean ± standard deviation (n = 5)

3.4. Discussion

The mechanisms that allow survival of poikilohydric organisms under desiccation conditions are still poorly understood. Our study on the physiological responses to dehydration of lichenized algae firstly characterizes the responses of *Trebouxia erici* under different conditions which can affect desiccation. During dehydration, the decrease of photosynthesis in *T. erici* was dependent on the RWC and independent of the dehydration rate (Fig. 3.1), but differential effects of faster dehydration on photosynthesis, cell viability and membrane damage are detectable during desiccation and rehydration. After the end of dehydration SD algae presented lower values of F_o than RD algae (Table 3.1). This effect

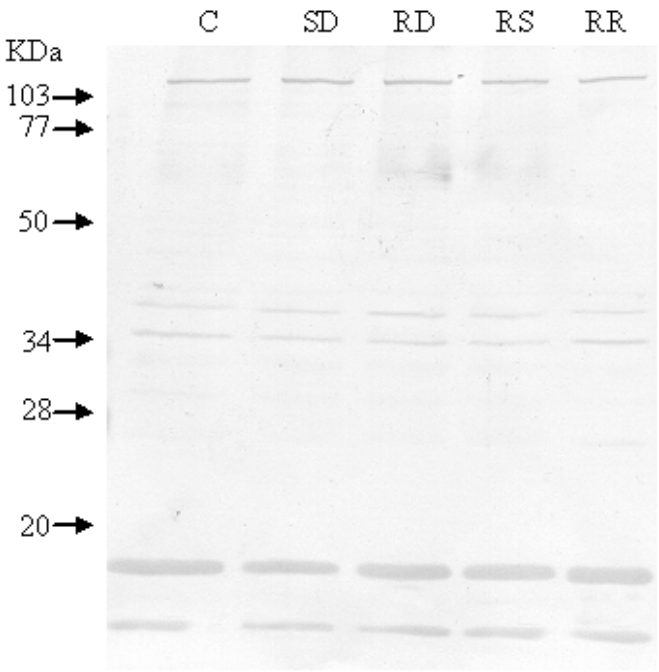


Figure 3.8 - After SDS-PAGE and Western blotting of polypeptides from non-dehydrated T. erici samples (C), dehydrated slowly (SD), rapidly (RD), and recovered for 24 h after SD (RS) or RD (RR), the dehydrin proteins were immunodetected (n = 4). As much as 15 μg of total protein was loaded per lane. The mobility of MW markers is indicated on the left. Data from one representative experiment are shown

has been extensively studied and discussed by Heber *et al.* (2007) and Heber (2008) as indicative of the existence of a photoprotection mechanism characteristic of mosses and lichen that is dependent of the desiccation rate and independent of light. According to these authors, such a mechanism requires conformational changes in a protein and could be more protective than other mechanisms of light dissipation as zeaxanthin –dependent or quencher formation within the reaction centre of PSII. Very recently, Kosugi *et al.* (2009) have proposed that a Trebouxoid algae isolated from the chlorolichen *Ramalina yasudae*, conserved dehydration induced quenching of PSII fluorescence, but no a lesser extent then in the lichen holobiont, suggesting the (partial) loss of a substance or mechanism to dissipate light energy to heat. According to our results, this mechanism seems to be present in the isolated *T. erici* photobionts (SAG 32.85 = UTEX 911) that have been replicated and conserved under culture conditions for very long periods. As shown in Fig. 3.2, the recovery of F_v/F_m and Φ_{PSII} was lower after RD than after SD. In both cases the recovery of photosynthetic electron flow diminished for longer periods of desiccation, but it was always higher in the cells previously subjected to SD treatments. The higher inhibition of electron transport in PSII after RD was accompanied by a higher decrease in cell viability (assessed by the Tetrazolium test) and a higher membrane leaking (Fig. 3.5). Similar effects caused by different drying rates have been reported in poikilohydric plants (Bewley & Krochko 1982; Oliver & Bewley 1984; Farrant *et al.* 1999; Cooper & Farrant 2002), which are usually interpreted as indicative that a minimal time is required to implement the cellular mechanisms implied both in the resistance to desiccation as in the recovery capacity after rewetting. Some of these mechanisms should participate in the defence and prevention of photooxidative stress including those regulating the dissipation of excess light energy and those avoiding the formation and accumulation of ROS.

The degree of non-photochemical quenching calculated from a Stern-Volmer approach as NPQ is a useful measure of the allocation of absorbed light to thermal energy dissipation, a protective mechanism (Logan *et al.*, 2007). However, comparison of NPQ values after stress treatments must be taken with caution, because a decrease in F_m (taking the value of post-desiccation dark-acclimated saturation pulse) may be related to photoinhibition itself and affects the calculated value of NPQ. Thus it underestimates the total photoinhibition component of NPQ. As an alternative, we have taken the F'_v/F'_m

values, a parameter which provides an estimate of the maximum quantum efficiency of PSII photochemistry (Baker & Oxborough, 2004). A decrease of F'_v/F'_m measures the extent to which the photochemistry of PSII is limited by a competition with thermal decay processes (Oxborough & Baker 1997). Results of F'_v/F'_m (Fig. 3.2d) when compared with ϕ_{PSII} (Fig. 3.2b) and q_P (Fig. 3.2c) indicated higher levels of electron transporters reduction after RD than after SD and also lower levels of energy dissipative protection after RD than after SD. Previous cautions might not affect the comparison of the relaxation kinetics of non-photochemical quenching in the dark, expressed as q_N, between different treatments (Walters & Horton 1991). In vascular plants it is assumed that q_E (usually the main component of non-photochemical quenching) affords a protection system for the photosynthetic mechanism, through the light-dependent formation of $\Delta\mu_H^+$ activating the xanthophyll cycle (Demmig-Adams & Adams 1992); Adams and Demmig-Adams (2004) and/or the action of the PSII associated protein psbS (Niyogi *et al.* 2004). In our experiments (Fig. 3.3) the RD algae lost q_E after 1h of desiccation and q_I, related to photoinhibition, becomes the main non-photochemical quenching component in RD algae. In SD algae q_E increased over the control value during the first hours in the desiccated state, but later decreased reaching almost zero value after 48 h of desiccation. These results imply that if the dehydration process of *T. erici* is delayed for approximately 250 min (Fig. 3.1), the components involved in the formation of q_E are temporarily preserved, but not after more rapid drying rates. However, no significant differences were found when comparing the *T. erici* xanthophyll content and DPS after the different dehydration/desiccation/recovery treatments (Table 3.2). In contrast, it was observed that after exposure to 1000 μmol m^{-2} s^{-1} hydrated algae had a 150% increase in the xanthophyll DPS (Table 3.2). Nonetheless, increases of up to 1300% have been reported in vascular plants regarding similar changes in light intensity (Demmig-Adams & Adams 1993). Therefore, our results infer a limited role of the xanthophyll cycle in q_E formation in *T. erici* and the higher q_E values found for SD algae after a short desiccation time cannot be explained by a higher DPS of xanthophylls. According to Bukhov *et al.* (2001) the disappearance of q_E after RD in *Rhytidiadelphus squarrosus*, where the presence of a few zeaxanthin molecules per reaction centre is enough to permit efficient light dissipation, could be a consequence of protonation capacity loss, probably associated with higher

membrane damage. Thus, our data could be explained, in part, by the greater membrane damage produced by rapid drying treatments (Fig. 3.5), suggesting that factors expressed and accumulated during a sufficiently slow dehydration period contribute to membrane stability. However, this reduced membrane damage would not ensure functionality of q_E after a few hours of desiccation, as its value decreased about 50% after 24 h for SD algae and decayed to near-zero values after 48 h (Fig. 3.3); notwithstanding, membrane permeability was apparently not affected further after this desiccation time (Fig. 3.5). Then, the light-independent formation mechanism of energy-dissipation proposed by Heber *et al.* (2007) and supported by the data of Table 3.1, can explain the effects found in isolated colonies of *T. erici*. In addition to q_E and q_I, a third q_M component has usually been attributed to state-transitions and denoted as q_T (Quick & Stitt 1989; Krause & Weis 1991), but with certain criticisms (Walters & Horton 1991). As shown in Fig. 3.3, q_M is the main component of q_N in control well-hydrated algae. It is also the main component after SD and is more resistant to the desiccation than q_E. A possible relevant role of state-transitions during desiccation has been described for the lichen *Lobaria pulmonaria* during dehydration (Chakir & Jensen 1999). If state transitions were to play an important role during recovery, an increase in the oxidation state of P700 would be expected as a consequence of the increased number of excitons impelling on P700. We found (Fig. 3.4) that upon rehydration there was an increase in the oxidation state of P700 that was greater after RD than after SD. However, results obtained using DCMU or methyl-viologen indicated that this increased oxidation of P700 is due to PSII photoinhibition while PSI remains functional during the dehydration/rehydration processes. This agrees with the fact that the main q_N component after RD or after SD following 24 h desiccation is q_I. Hence, the reduction of the energy used in photochemistry, accompanied by an increment in the energy dissipated thermally, observed in the samples RD, is a consequence of the photoinhibition of the PSII, not of the PSI, which remains functional during the desiccation/rehydration processes. Therefore, it is likely that the increase of thermal dissipation developed during the first hours after SD, when the levels of q_I are still very low, was generated in an independent way of the classical mechanisms described for vascular plants: xanthophyll cycle, protonation or state-transitions. Although the activation of the xanthophyll cycle during dehydration has been reported in some lichens (Calatayud

et al. 1997; Kranner *et al.* 2003), it cannot be assumed as a general response for lichenic algae because other lichen species, like *Pseudoevernia furfuracea* or the isolated lichen photobiont *Trebouxia excenctrica* do not increase the xanthophyll DPS upon dehydration or rehydration (Kranner *et al.* 2003; Kranner *et al.* 2005).

The consequences of a faster dehydration rate were also observable during the recovery after rehydration. Following rehydration, *T. erici* showed lower F_v/F_m, ϕ_{PSII}, q_P and F'_v/F'_m values after RD than after SD (Fig. 3.6). There was a depression of these parameters during the first 12 h of recovery that was more intense in RD than in SD samples. It is well documented (Minibayeva and Beckett 2001; Kranner *et al.* 2005; Weissman *et al.* 2005) that after rehydration the respiration rate abruptly increased from near null to normal values and as a consequence of this metabolic burst are generated higher levels of ROS that potentially lead to injuries that can explain the observed depression of the photosynthetic activity during the first hours of recovery. Thus, the higher levels of photosynthetic activity found in SD algae could be hypothetically attributed to a higher accumulation of antioxidant activities allowed by a longer dehydration process. We found that during both the desiccation period and recovery of *T. erici* the levels of superoxide dismutase and peroxidase were slightly but not significantly greater after SD than after RD and both decreased along desiccation and during the first hours of recovery (Fig 3.7). Furthermore, ascorbate levels were undetectable after all treatments and the lutein or β-carotene levels did not undergo significant changes (Table 3.2). In lichens such as *Ramalina lacera* (Weissman *et al.* 2005) and *Peltigera polydactyla* (Kranner *et al.* 2003) desiccation and/or rehydration is also accompanied by a decrease in antioxidant activities. Thus, in *T. erici*, a longer dehydration time apparently does not allow a higher accumulation of classical antioxidants during dehydration or, alternatively, preservation of activity during desiccation. Consequently the higher values of PSII electron transport registered for SD algae during the first hours of recovery cannot be related to these antioxidant activities or substances.

Other factors, like the accumulation of dehydrin/rehydrin (Group II, LEA proteins) might contribute to explaining the differences found in recovery of photosynthetic activity, membrane damage and cell viability after SD or RD treatments Although their actual function remains unclear, they are accumulated to high concentrations in seeds and in

vegetative tissues of desiccation-tolerant plants in response to desiccation, so they are thought to play a primary role in desiccation tolerance (Buitink *et al.* 2002; Kermode & Finch-Savage 2002; Phillips *et al.* 2002). On the basis of the remarkably high number of polar residues within the structure, some LEAs are thought to coat intracellular macromolecules with a cohesive water layer. On further dehydration, LEAs would provide a layer of their own hydroxylated residues to interact with the surface groups of other proteins , acting as "replacement water" (Hoekstra *et al.* 2001). Our results (Fig. 3.8), obtained using a commercial anti-dehydrin antibody (Agrisera, Vänäs, Sweden), show the presence of five putative dehydrin polypeptides (Mr 15, 18, 34, 40 and 120 kDa). The size of dehydrins ranges from about 15 to 150 kDa, and their number can vary widely among plant species (Close *et al.* 1993). Our results also show that in *T. erici*, like in the desiccation tolerant moss *Tortura ruralis* (Bewley *et al.* 1993), dehydrins are constitutively expressed, because similar amounts of immunodetected polypeptides were quantified in control, RD and SD and 24 h rehydrated algae.

All data presented suggest that dehydration tolerance in *T. erici* depends both of constitutively expressed mechanisms (like dehydrins, SOD or peroxidase) and others that are induced, which should need a sufficient time of dehydration to be expressed improving cell endurance (as those allowing higher levels of energy dissipation under desiccation or the maintenance of q_E and the recovery of higher levels of photosynthetic electron transport immediately after rehydration). In the bryophyte *Tortula ruralis*, dehydration induces changes in gene expression that becomes evident upon rehydration (Oliver 1991). During a sufficient slow drying, rehydrins transcripts are accumulated, and the proteins are quickly synthesized upon rehydration. In contrast, after a rapid dehydration the protein synthesis is delayed because new gene expression is needed (Wood & Oliver 1999; Velten & Oliver 2001).

In conclusion, the importance of the dehydration rate clearly indicates that a minimal time is required to implement the cellular mechanisms implied both in the resistance to desiccation as in the recovery capacity after rewetting. This suggests that "*de novo*" synthesis of desiccation resistance factors occurs during the dehydration process and that not only constitutive mechanisms are involved in the desiccation tolerance of

Trebouxia. Further genomic, proteomic and/or metabolomic analysis will be needed to answer this question.

3.5 Resumen

Introducción

Los líquenes se encuentran entre los organismos poiquilohídricos capaces de sobrevivir largos periodos de tiempo en estado seco. La habilidad de las células de sobrevivir en estado seco es denominada tolerancia a la desecación (Bewley 1979) o anhidrobiosis (Crowe *et al.* 1992).

Los vegetales tolerantes a la desecación pueden ser divididos en dos grupos. Uno formado por las angiospermas tolerantes, las cuales solamente sobreviven si la pérdida de agua es muy lenta (de días a semanas) ya que necesitan activar los mecanismos de tolerancia a la desecación durante la deshidratación (Oliver & Bewley 1997; Proctor & Pence 2002; Alpert & Oliver 2002). En el otro grupo se encuentran los vegetales poiquilohídricos y pequeños helechos, los cuales resisten deshidrataciones muy rápidas (menos de 1 h) ya que la tolerancia a la desecación está basada en mecanismos constitutivos (Oliver & Bewley 1997; Proctor & Smirnoff 2000).

A nivel celular, han sido sugeridos varios mecanismos bioquímicos protectores para hacer frente a los daños producidos por la desecación. Las proteínas abundantes de la embriogénesis abundante son consideradas como críticas en la adquisición de la tolerancia a la desecación celular. Otro proceso importante bajo condiciones de estrés es la disipación en forma de calor del exceso de energía lumínica que podría generar daños fotooxidativos, (Müller et al 2001; Heber *et al.* 2007). También el sistema antioxidante protege a la célula contra el estrés fotoxidativo producido por el incremento de la generación de especies reactivas de oxígeno durante la deshidratación y la rehidratación (Minibayeva & Beckett 2001; Weissman *et al.* 2005; Kranner & Birtic 2005).

El estudio de la tolerancia a la desecación de los líquenes y de sus fotobiontes ha ido generalmente enfocada hacia el sistema antioxidante. Sin embargo, no ha sido posible establecer una relación clara entre los diferentes niveles de tolerancia a la desecación de los líquenes y los mecanismos antioxidantes. El objetivo del presente capítulo fue analizar el efecto de la velocidad de la deshidratación en la viabilidad y posterior recuperación del fotobiontes liquénicos *Trebouxia erici*.

Material y métodos

Un cultivo axénico del fotobionte liquénicos *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) fue utilizado en nuestro estudio y cultivado en discos de acetato de celulosa como se describe en el capítulo 2.

Para las deshidrataciones lentas (DL), los discos de algas fueron separados de las placas con agar y puestos dentro de un fitotrón a 20 ºC y 30 μmol m^{-2} s^{-1}, con una humdad relativa del 67 %, con lo que las algas quedan secas en 4 – 5 h. La deshidratación rápida (DR) se realizó con una humedad relativa del 20 % y tardó alrededor de 60 min.

La fluorescencia de la clorofila *a* fue medida in vivo con una fluorímetro de luz modulada PAM-2000. Los cambios en el estado redox del fotosistema I (PSI) fueron calculados midiendo la absorbancia a 820 nm con un fluorímetro PAM-101. La integridad de la membrana fue estimada mediante la inmersión de los discos de algas en agua y la medición de la conductividad eléctrica. La detección de las dehidrinas se llevó a cabo a través de la técnica del Western-Blot utilizando anticuerpos comerciales antidehidrina (Agrisera). La actividad peroxidada (POX) fue analizada siguiendo el protocolo descrito por Zapata el al. (1998). La actividad supoeróxido dismutasa fue medida como se describe en Beyer & Fridovich (1987). Para evaluar la reducción del TTC se utilizó el método descrito por Backor *et al.* (2005). El ascorbato fue determinado espectrofotométricamente siguiendo la metodología utilizada por Luwe *et al.* (1993). Los pigmentos de las algas fueron analizados por HPLC siguiendo el protocolo descrito por Mantura & Llewellyn (1983).

Resultados y discusión

Durante la deshidratación, la disminución de la actividad fotosintética en *T. erici* fue dependiente del contenido hídrico, pero independiente de la tasa de deshidratación. Al final de la DL las algas presentaron unos valores de la fluorescencia basal menores que tras la DR. Este efecto indica la presencia del mecanismo de fotoprotección característico de musgos y líquenes, y que requiere una deshidratación lenta para una mejor funcionalidad (Heber *et al.* 2007; Heber *et al.* 2008). La recuperación del flujo de electrones de la

fotosíntesis de las algas fue menor tras la DR que tras la DL, y en ambos casos el tiempo de permanencia en estado seco influyó negativamente. La elevada fotoinhibición del transporte de electrones en el fotosistema II (PSII) observada después de una DR fue acompañada de una mayor disminución de la viabilidad celular (menor reducción del TTC) y un mayor daño en las membranas celulares. Estos resultados parecen indicar que las algas necesitan un tiempo mínimo para incrementar los mecanismos celulares implicados tanto en la resistencia a la desecación como en la capacidad de recuperación tras la rehidratación. En las algas DR el componente de disipación no fotoquímica principal fue q_I, el cual está relacionado con la fotoinhibición. En las algas DL el componente principal fue q_E, el cual es dependiente de la energización de la membrana y ha sido clásicamente relacionado con el ciclo de las xantofilas (Adams and Demmig-Adams 2004), aunque este componente decreció a lo largo tiempo en el que las muestras permanecían secas desapareciendo a las 48 h. Sorprendentemente, no se encontraron diferencias significativas en el contenido de las xantofilas ni en la proporción de deepoxidación después de los diferentes tratamientos de deshidratación, desecación y recuperación. La reducción de la energía utilizada en fotoquímica, junto con el incremento de la energía disipada térmicamente, observada en las muestras RD, es una consecuencia de la fotoinhibición del PSII principalmente, ya que el PSI permaneció funcional durante los procesos de desecación y rehidratación.

En nuestros experimentos también encontramos que, después de desecar las algas, los niveles de SOD y POX disminuyeron, tanto tras la DL como tras la RD, cuanto mayor fue el tiempo de desecación, así como durante las primeras horas tras la rehidratación. Además, los niveles de ascorbato fueron indetectables después de cualquier tratamiento y el las concentraciones de β-caroteno no mostraron cambios significativos. Así pues, en *T. erici* una deshidratación lenta no permite aparentemente una acumulación de los antioxidantes clásicos, e incluso disminuyen.

Nuestros resultados también muestran que en *T. erici*, como en el musgo tolerante a la desecación *Tortula ruralis* (Bewley *et al.* 1993), las dehidrinas (LEA, grupo II) son constitutivamente expresadas, ya que cantidades similares de polipéptidos inmunodetectados fueron obtenidas en las muestras control, en las desecadas y en las rehidratadas.

En conclusión, todos estos datos sugiere que la tolerancia a la deshidratación de *T. erici* depende tanto de mecanismos constitutivamente expresados (como las dehidrinas, las SOD y POX), como de inducidos, los cuales necesitarían de un tiempo mínimo de deshidratación para ser expresados e incrementar la resistencia de la célula.

4. PROTEOMIC AND EXPRESSION ANALYSIS OF *TREBOUXIA ERICI*. RESPONSE TO DEHYDRATION AND REHYDRATION

Abstract

The study of desiccation tolerance of lichens, and of their photobionts in particular, has frequently focused on the antioxidant system that protects the cell against photo-oxidative stress, produced by an increase in ROS during dehydration and rehydration cycles. However, desiccation tolerance cannot be achieved in lichens with antioxidant activity alone. In other desiccation tolerant poikilohydric organisms changes in the synthesis of proteins have been implicated in recovery/repair mechanisms following rehydration. As far as we know, few studies have dealt with the regulation of the responses during dehydration and/or rehydration in lichen photobionts at a molecular level. Thus, we decided to carry out proteomic and genetic expression analyses of the changes associated with desiccation and rehydration in the isolated photobiont *Trebouxia erici*. Algae were dried slowly (5 - 6 h) and rapidly (< 60 min), and after 24 h of desiccation were rehydrated. To identify those proteins that accumulate during the drying and the rehydration process, we have employed a strategy of 2-D Difference Gel Electrophoresis (DIGE) coupled with individual protein identification using trypsin digestion (from silver stained replicates) and LC-MS/MS. The DIGE strategy allowed us to identify those proteins that increased or decreased in relation to the fully hydrated photobiont. Proteomic analysis showed that desiccation caused up-regulation of around 19 proteins and down-regulation of 43 proteins in *T. erici*. Some of the up-regulated proteins in the desiccated and rehydrated algae were identified as proteins involved in transport, protection, cytoskeleton, cell cycle and targeting and degradation. Three and two of the most highly up-regulated proteins were Heat Shock Protein 90 (Hsp90) and β-tubulin proteins, respectively. The synthesis of all of these proteins was increased significantly during dehydration, and although it was around 20 % higher in slowly dried samples this difference was not significant, neither before nor after the rehydration. β-tubulins and Hsp90 are a gene family, thus, previous to quantify the expression of these genes with qRT-PCR, we used the GeneRacer and cloning techniques to know the number of functional genes of each family and the 3' end sequence of each gene, respectively. We observed that five Hsp90 and two β-tubulin genes were activated during dehydration and mRNA was accumulated until the cell was completely dried. The Hsps ensure maintenance of homeostasis, protect cells and help to return to equilibrium during recovery after stress. In poikilohydric desiccation tolerant plants, microtubule skeleton seems to play a key role in the recovery of the ultrastructure of cells, as in our case *T. erici*.

4.1. Introduction

Plants, as sessile organisms, depend on proteomic plasticity to remodel themselves during periods of developmental change, to endure varying environmental conditions and to respond to biotic and abiotic stresses. Desiccation tolerant plants need to resist three major subcellular stresses: a) mechanical stress, e.g. shrinkage of the plasma membrane away form the cell wall as a consequence of water loss and subsequent cytorrhysis; b) oxidative stress caused by the production of reactive oxygen species (ROS); c) damage to macromolecules such as DNA and proteins (Farrant 2000; Alpert 2006). When subjected to dehydration stress, plants change gene expression and metabolism.

In order to identify genes that may be responsible for desiccation tolerance mechanisms, several studies based on RNA expression have examined changes that occur during dehydration and/or rehydration in the transcriptome of several resurrection plants; *Xerophyta humillis* (Collett *et al.* 2003; Collett *et al.* 2004), *Craterostigma plantagineum* (Bartels *et al.* 1990; Bockel *et al.* 1998), *Sporobolus stapfianus* (Blomstedt *et al.* 1998; Neale *et al.* 2000), *Selaginella moelendorfii* (Weng *et al.* 2005; Iturriaga *et al.* 2006) and in the desiccation-tolerant moss *Tortula ruralis* (Oliver *et al.* 2004). However, mRNA abundance may only represent a putative function since there is still a questionable correlation between mRNA and protein levels. mRNA may not be translated, or changes in protein level or enzyme activity may occur without any detectable change in transcript abundance due to translational or other levels of control (Gygi *et al.* 1999). A further consideration in the case of certain resurrection plants and mosses is that mRNAs appear to be stored during drying and only translated during dehydration (Velten & Oliver 2001; Collett *et al.* 2003). By contrast, proteomics can reveal the actively translated portion of the genome that performs the enzymatic, regulatory and structural functions of the cell at a particular moment. Comparative proteomic investigation of plants before and after specific and interactive stresses provides information about which and how defensive mechanisms are activated. Separation of proteins, using techniques such as bidimensional electrophoresis (2-DE) or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), enables protein identification by comparison of the intact protein mass measurement with that expected from the DNA sequence. Furthermore, the increasing

amount of protein sequence data (over 300,000 sequences in the National Centre for Biotechnology Information, NCBI, Viridiplanta database) facilitates identification of homologous proteins in non-model plant species. Proteomic studies carried out in resurrection plants and mosses have shown that during dehydration there may be an increase in reactive oxygen species (ROS) scavenging enzymes (Ingle *et al.* 2007; Jiang *et al.* 2007; Wang *et al.* 2009), chaperone proteins (Wang *et al.* 2009), energy metabolism enzymes (Ingle *et al.* 2007; Jiang *et al.* 2007; Wang *et al.* 2009), targeting and degradation proteins (Ingle *et al.* 2007; Deeba *et al.* 2009) and proteins involved in signalling (Wang *et al.* 2009). On the contrary, since the photosynthetic system is blocked during desiccation, there is generally a decrease in proteins related with photosynthetic activity to avoid ROS formation (Ingle *et al.* 2007; Wang *et al.* 2009; Deeba *et al.* 2009).

The study of desiccation tolerance of lichens, and of their photobionts in particular, has frequently focused on the antioxidant system that protects the cell against photo-oxidative stress, produced by an increase in ROS during dehydration and rehydration cycles (Fridovich 1984; Minibayeva & Beckett 2001; Weissman *et al.* 2005). However, desiccation tolerance cannot be achieved in lichens with antioxidant activity alone. As reported in Chapter 3, there are other proteomic mechanisms involved in desiccation tolerance that are constitutively expressed, like late embryogenesis, abundant proteins or those induced during dehydration, such as the ones that cause conformational changes in the PSII, and certainly other mechanisms as yet undescribed in photobionts. Furthermore, as far as we know, few studies have dealt with the regulation of the responses during dehydration and/or rehydration in lichen photobionts at a molecular level. Accordingly, we decided to carry out proteomic and genetic expression analyses of the changes associated with desiccation and rehydration in the isolated photobiont *Trebouxia erici*.

4.2. Materials and methods

4.2.1. *Biological material*

An axenic strain of the lichen photobiont *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) was used for our study cultured on sterile cellulose-acetate discs as is described in Chapter 2 (Section 2.2.2.).

4.2.2. *Desiccation and rehydration treatments*

Slow drying (SD) was achieved by placing algal disks into a closed container with a saturated solution of ammonium nitrate (R.H. 66%), and maintained under culture conditions. Under this regime, dried samples were obtained within 6 h. Rapid dehydration (RD) was achieved under the same conditions but changing the ammonium nitrate for silica gel (R.H. 20%). The air-dried state was reached within 1 h under these conditions. The algae remained dried for 24 h and were then frozen with liquid nitrogen. Recovery after slow and rapid drying (RS and RR, respectively) was studied with samples desiccated for 24h, which were rehydrated by adding a volume of distilled water equivalent to the water lost, maintained under culture conditions for 3 h and then frozen with liquid nitrogen. All the samples were kept at -80ºC until protein or RNA extraction.

4.2.3. *Protein extraction and two-dimensional electrophoresis*

For protein extraction, 100 mg of algae was ground to a powder with liquid nitrogen in a mortar and pestle and resuspended in 2.5 ml of Tris pH 8.8 buffered phenol and 2.5 ml of extraction media (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4 % 2-β-mercaptoethanol, 0.9 M sucrose). The suspension was kept at 4 ºC for 30 min in continuous agitation and centrifuged at 4000 g x 20 min at 8 ºC. Phenol phase was removed ant back-extracted aqueous phase with 2.5 ml of extraction media and phenol by vortexing and centrifuged again. Phenol extracted proteins were precipitated by adding 5 volumes of 0.1 M ammonium acetate in 100% methanol (at -20ºC) to phenol phase, vortexed and incubate

at -20ºC overnight. Solution was centrifuged at 4000 g for 20 min at 4 ºC. The pellet was washed twice with 0.1 M ammonium acetate in 100 % methanol, twice with ice-cold 80 % acetone and finally once with cold 70 % ethanol. The final pellet was air-dried and resuspended in 100 µl of extraction media and stored at –80 ºC until isoelectric focusing (IEF).

The protein concentration was determined using the Bradford method (Bradford 1976) and then adjusted with lysis buffer (30 mM Tris-HCl pH 8.5, 7 M Urea, 2 M Thiourea, 4 % (w/v) CHAPS) to 5 mg/ml. An internal standard was used for the experimental design, which was prepared by pooling equal amounts of the control and treated samples. 50 µg of each sample was labelled with either Cy3 or Cy5 and the internal standard with Cy2 CyDye DIGE Fluor minimal dye following the manufacturer's recommended protocols (GE Healthcare). The labelling reaction was incubated in ice in the dark for 30 min and the reaction was terminated by addition of 10 nmol lysine. The labelling experiment was independently repeated four times for each treatment, and the dyes Cy3 and Cy5 alternatively used. Equal volumes of 2X sample buffer (8 M Urea, 130 mM DTT, 4 % (w/v) CHAPS) were added to each of the labelled protein samples and three samples with different Cydye were mixed. IEF sample extraction media (8M Urea, 2M Thiourea, 2 % (w/v) CHAPS, 2 % (v/v) Triton X-100, 50 mM DTT, a trace of Bromophenol blue) was added to make up the volume to 450 µl. Prior to isoelectric focusing 10 µl of DeStreak rehydration solution and 2.25 µl of IPG buffer 4-7 (GE Healthcare) were added.

Labelled samples were loaded onto the rehydration trays and covered with 24 cm immobilised pH gradient (IPG) strips (pH range, 4-7; GE Healthcare). Strips were submitted to active rehydration at 50 V for 14 h at 12ºC, followed by isoelectric focusing using an PROTEAN IEF Cell (BioRad) to a total of 80 kVh (step 500 V for 1 h, step 1000 V for 2 h, step 8000 V for 3 hl). After isoelectric focusing, disulfide bridges were reduced by submerging the strips in equilibration buffer (6M urea, 50mM tris, pH 8.8, 30% glycerol, 5% SDS) supplemented with 2 % DTT for 15 min. The strips were then incubated for 10 min in freshly prepared equilibration buffer supplemented with 2.5 % iodoacetamide. IPG strips were transferred onto 12% polyacrylamide gels and overlaid with 1% agarose SDS running buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS) containing 0.2

mg/ml bromophenol blue. SDS-PAGE gels were prepared using low fluorescence glass plates (24 cm, GE Healthcare). Each gel was run at 2 W for 14 h using a Hoeffer SE-600 Ruby system (GE Healthcare).

Individual images of proteins labelled Cy2, Cy3 and Cy5 in each gel were obtained by scanning on a Typhoon 9410 (GE Healthcare) with 480/530 nm excitation/emission wavelengths for Cy2, 520/590 nm for Cy3, and 620/ 680 nm for Cy5. After imaging, gels were stained with silver following the protocol described by Shevchenko *et al.* (1996).

Image analysis was performed in an automated mode using DeCyder Differential Analysis Software (GE Healthcare). Spot patterns from different gels were matched using the internal standard sample present on every gel to allow comparison and statistical analysis of spot-volume ratios.

The spots of those proteins more up-expressed respect the control were excised from the preparative gels, washed three times with Mili-Q water, silver reduced with 50 mM sodium thiosulfate and 15 mM potassium ferricyanide and washed again three times with water. Gel pieces were equilibrate with a serie of 100 mM ammonium bicarbonate, 50 mM ammonium bicarbonate/50% acetonitrile and 100 % acetonitrile. Gel pieces were dehydrated with acetonitrile and digested overnight at 37 ºC with 0.02 mg/ml trypsin (Promega modified porcine TPCK-treated) in 50 mM ammonium bicarbonate. The peptides were extracted twice with 1 % TFA in 60 % acetonitrile. Extracts were combined and frozen at –80 ºC. Once frozen, the peptides were dry by centrifugal evaporation. The tryptic peptides were resuspended in 0.1 % TFA in 50 % acetonitrile, mixed with 10 mg/ml α-cyano-4-hydrosycinnamic acid in 0.1 % TFA and 50 % acetonitrile, and spotted onto MALDI target plates

Peptides were analysed by using the LC-MS/MS technique. The National Center for Biotechnology Information (NCBI;http.ncbi.nih.gov/blast) non-redundant protein database was used for querying all data. The FASTA dababase utilities and indexer of the BioWorks Rev 3.3, software was used to create an algae database from the NCBI and JGI non-redundant database and to index it for trypsin cleavage with cysteine modified by carboxyamidomethylation (+57Da). The searching was performed taking *Trebouxia*, *Chlorella*, *Helicosporidium*, *Chlamydomonas*, *Ostreococcus*, *Physcomitrella* as taxonomy.

Table 4.1 - List of primers used for the Nested and RACE-PCR.

	Direction	
Primer	Forward	Reverse
GeneRacer™ 3'		GCAATGCATCGCATAGCAACTGTCG
GeneRacer™ 3' Nested		GTGACAGTACGGCAATGCATCGC
Tub 4	CARATYGGWKCBAARTTYTGG	
Tub 3	GTNYTNGAYGTBGTBCGBAAGGA	
Tub 2	HWSVAAGATYCGYGAGGARTACC	
Tub 1	ACRARGCNYTDTAYGAYATCT	
HSP90 4	GAGTGGDMNNWSRTGAACAAG	
HSP90 3	BMRSGAHGAGTAYGCYGCNTTCTA	
HSP90 2	TTYGAYMTSTTYGACRSHMRSAA	
HSP90 1	AGTTYTAYGARKCYTTCKSHAAGA	

Protein hits were filtered with the following criteria> peptide probability less than 0.001, Xcorr values greater than 1.5, 2.0 and 2.5 for +1, +2, and +3 charged ions, respectively.

4.2.4. *mRNA quantification of* β-tubulin *and* HSP90 *genes*

RNA extraction was carried out using QiaGen's Plant RNeasy Plant extraction kit according to the manufacturer's guidelines. The final pellet was resuspended in DEPC-treated water, quantified using a NanoDrop ND-1000TM spectrophotometer (Daemyung, Korea). DNA was degraded using the DNA Free RNA kit (Zymo Research) and the complementary DNA (cDNA) synthesized with the SuperSript™ II RNase-H RT kit (Invitrogen) following the manufacturer's guidelines.

β-tubulins and *Heat Shock Protein 90s* (HSP90) are a gene family widely characterized in the Plant Kingdom, from *Arabidopsis thaliana* to *Chlamydomonas reinhardtii*, but not yet studied nor sequenced in the *Trebouxia* genus. For this reason, previous to quantify the expression of these genes with qRT-PCR, we used the GeneRacer and cloning techniques to know the number of functional genes of each family and the 3' end sequence of each gene, respectively.

Degenerate forward gene specific primers (GSP) were designed on the basis of the consensus sequence generated with the sequences of these two gene families that are available at the GenBank of the NCBI (Table 4.1). A RACE-PCR was performed with 1 µl of template cDNA (RT product), 1.5 µl of 10 µM forward GSPs Tub4, Tub3, Tub2, HSP4,

Table 4.2 - List of primers used for RT Q-PCR and PCR product size.

Genes	PCR (bp)	Forward	Reverse
TubCon1	62	GGAGGAGGCTGCATGATAGC	CCCAATGGGTTGATGAGCAT
TubCon2	76	AACCCCCGAGTACAATTTCAATT	CTTGGCACAGCATGCAACAC
HSPCon6Clone37	65	CGAATGTTTGGGCCATCAG	GGCATAGGTGTAGGCATGATAAGA
HSPCon6Clone43	62	GGGCTCCCGCATGGA	CAGCAGCAGATGTCGTGTCA
HSPCon6	75	GCACTAGGTGTAGGCACTGATAAG	ACGAGACGCGAATGTTTGG
HSPCon7	59	CTGTGGTTCCCTTGATACGAACT	TTCACGCACGATGGTTTCAG
HSPCon8	65	CACGCCACAAAAGTACAAATGG	TTGCCTGAGCAGGGTGAGA
18S	89	TGATTCTATGGGTGGTGGTG	GCAGGTTGAGGTCTCGTT

HSP3 and HSP2 and 4.5 µl of 10 µM GeneRacer 3' primer (Table 4.1), 5 µl of the 10X PCR Buffer, 0.5 µl of Platinum® *Pfx* Polymerase (2.5 U/µl), 1.5 µl of 10 mM dNTPs, 1 µl of 50 mM $MgSO_4$, and 35 µl of sterile water. After a denaturing step at 94ºC for 2 min, 5 cycles of 94 ºC for 30 s and 72 ºC for 1 min, followed by 5 cycles of 94 ºC for 30 s, 55 ºC for 1 min and 68 ºC for 1 min, and 25 cycles of 94 ºC for 30 s, 50 ºC for 30 s and 68 ºC for 1 min. A final extension step at 72 ºC for 2 min was carried out. In order to increase the specificity and sensitivity Nested PCRs were performed with the RACE products and all the possible combinations with the internal GSPs. Nested PCRs were carried out with 1 µl of the RACE-product, 1 µl of 10 µM GeneRacer 3' Nested primer, 1 µl of internal GSP, 5 µl of 10X PCR Buffer, 1.5 µl of 10 mM dNTPs, 1 µl of 50 mM $MgSO_4$, and 40 µl of sterile water. After a denaturing step of 94 ºC for 2 min, 25 cycles at 94 ºC for 30 s, 55 ºC for 30 s and 68 ºC for 2 min were performed, followed by a extension step at 68 ºC for 10 min.

The twelve Nested-PCR products were ligated into pCR4-TOPO plasmid vector and transformed into chemically competent TOP 10 *Escherichia coli* cells (TOPO TA Cloning kit; Invitrogen). Four colonies per transformant were selected after growing in LB-kanamycin medium. Isolation of plasmid DNA was carried out following the Alkaline Lysis Minipreps procedure (Sambrook *et al.* 1989). Sequencing of plasmid using M13 sense and antisense primers (Invitrogen) was performed using Big Dye v.3.1. DNA sequences were assembled into contigs and checked for similarities to β-tubulins and HSP90 genes using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, NIH, http://www.ncbi.nlm.nih.gov/BLAST) score system.

In order to analyze the expression of each gene during the dehydration and the rehydration, the RNA extraction was done each hour during the slow drying treatment and

during the recovery. cDNA was synthesized with the Quantitect Reverse Transcription Kit (Quiagen) following the manufacturers guideline. New specific primers were designed for each gene (Table 4.2) in order to quantify the cDNA by a real-time PCR and specifity checked by electrophoresis 2% agarose gel (Appendix 3, Fig. 4.1). The gene of the 18S rRNA (AB080310) was used as housekeeping gene for the normalization of the quantification. PCR amplification was done in a 10 µl total volume containing 1 µl of 10x diluted cDNA, 0.5 µM each primer and 5 µl of 2x FluoCycle^{TM} SYBR-Green hot-start Taq ready-mix using an ABI PRISM® 7700 instrument. The thermal profile used for real-time PCR consisted of a DNA polymerase activation step at 95 ºC for 10 min and 40 cycles of two steps at 95 ºC for 15 s and at 58 ºC for 60 s. After the last cycle, temperature increased to 95 ºC and then decreased to 58 ºC. Then it was increased gradually to 95 ºC to obtain the dissociation curves. Absence of non-specific PCR products and primer dimmers was checked by the dissociation curves (Appendix 3, Fig. 4.2) and by electrophoresis on 2% agarose/GelRed gel. Two negative controls missing total RNA template or reverse transcriptase were included in each experiment.

4.2.5. *Statistical analysis*

Four protein extractions and CyDye labelling were carried on for each dehydration/rehydration treatment. One-way ANOVA in combination with Tukey HSD test was performed with the Standardized log abundance ratios (R_i) to identify spots with significantly different expression. A Principal Component Analysis (PCA) was performed with the R_i data of the those spots for which significant differences were expressed. PCA involves a mathematical procedure that transforms a number of possibly correlated variables (desiccation treatments in our experiment) into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. PCA is a useful tool to identify relationships among variables and among samples.

4.3. Results

4.3.1. *Two-dimensional electrophoretic analysis of total proteins*

In order to investigate the changes in protein profiles during dehydration and recovery, and to know the response-time, we applied two different drying rates to cultures of *T. erici*. Ten percent of the RWC was achieved in around 60 min when the rapid-drying method was applied; meanwhile using the slow-drying method it took 300 min. We carried out 2D-DIGE analysis of the total proteins from four replicates per treatment, and they showed a high level of reproducibility. A representative gel is shown in Figure 4.1. More

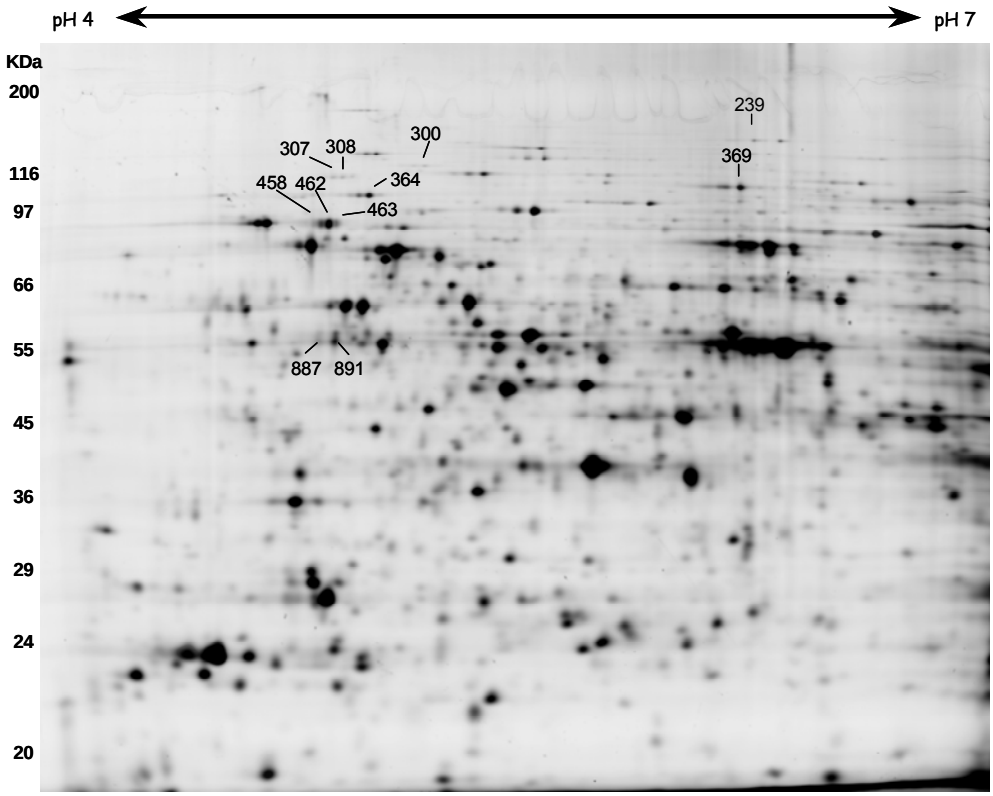


Figure 4.1 - Example output from DeCyder showing the Cy2-labelled standard gel image. Annotated spots numbered correspond to that were more significant up-regulated after dehydration/rehydration ($Pv < 0.05$).

than 2000 protein spots were detected by DeCyder 2D 6.3 Software, and more than 500 were confirmed manually. Quantitative image analysis revealed a total of 62 protein spots that changed their intensities significantly ($P < 0.05$) at least in one treatment. The Figure 4.2 (A) represents a principal component analysis performed with the Ri values of these spots. The first component of the PCA is not represented in the graphic because, although it accounted the 65.42 % of the total variance in data set, this variable can just be explained by the differences among the relative abundance of the proteins in the five treatments. By the contrary, the second and third principal component, which accounted the 22.50 and 6.89 % of the total variance respectively, represent better the changes among the different proteomes after each treatment. In this way, the PCA shows the Eigenvectors of the control and the dehydrated/rehydrated samples in opposite directions in the diagram, showing a clear difference between the proteome non-dehydrated samples and those treated. The spots with a negative value are indicating those proteins that are more abundant in control samples, whereas a positive value is indicating a higher expression after dehydration. Those spots up-regulated after dehydration formed two groups: one with the proteins that were more abundant after a slow dehydration, spots 458, 462, 887 and 891; and other group with proteins that were more abundant after a rapid drying, spots 300, 307, 308 and 364. Only the variable RS has also a negative value in the second principal component like the control, indicating that the samples slowly dried began of the recovery to the initial proteome, but far to be complete after three hours of rehydration. Figure 4.2 (B) shows the PCA performed with the Ri values of the treated samples respect those on non-dehydrated samples. In this diagram, the first component absorbs the 86.98 % of the total variance, and allows differentiating easily those proteins that were up or down-regulated in the treated samples respect the control ones. Most of the 62 spot proteins had a negative value in this axe, thus down-regulated. Only 19 proteins had a positive value, among these, the 10 more up-regulated proteins formed a defined group in a region of the PCA. This change respect the surprisingly homogeneity of the proteome suggest that these proteins may help us to elucidate the mechanism of desiccation tolerance in *T. erici*.

Those 10 up-regulated proteins with relatively greater changes in abundance were excised from the gel to be analysed by LS-MS/MS (Fig. 4.1). However, peptides from only

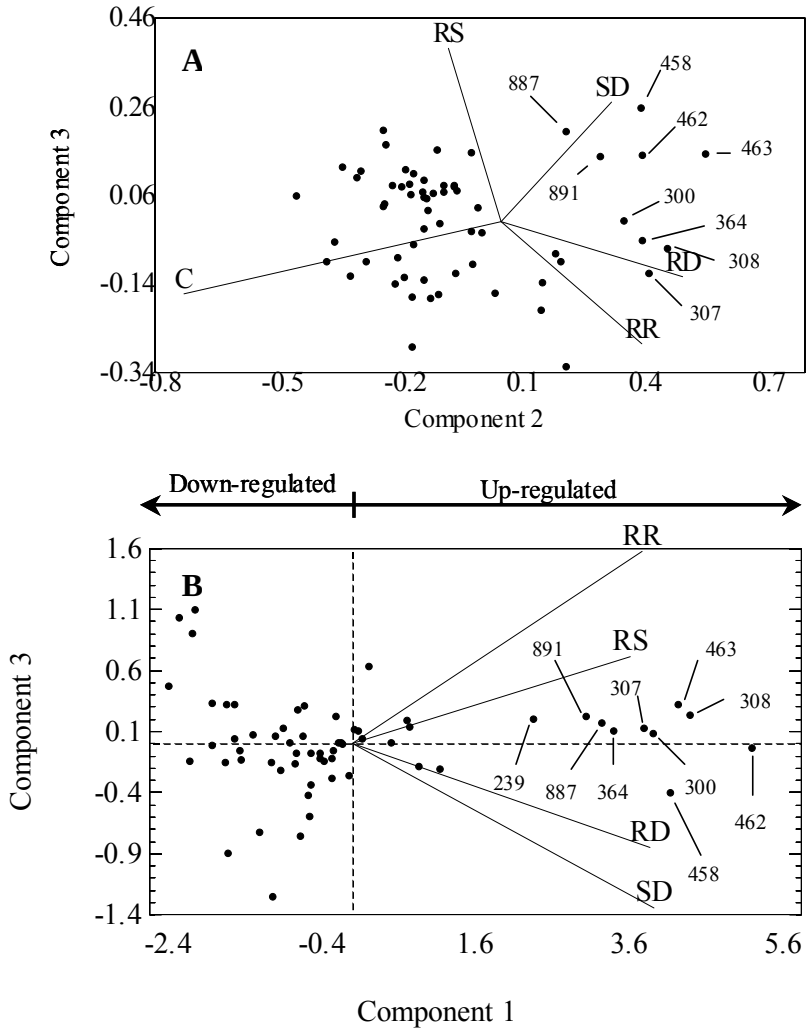


Figure 4.2 - (A) PCA analysis with the Standard Log Abundant Ratio (Ri) of the spots that had significant different expression in at least one treatment (Pv < 0.05). C, Control; SD, slow drying; RS, recovery after slow drying; RD, rapid drying; RR, recovery after rapid drying. (B) PCA analysis with treated Ri respect control Ri values. Positive and negative values are indicating up-regulation and down-regulation, respectively. Annotated spots numbered in B correspond to that were more up-regulated and were selected for protein identification.

Table 4.3 - Identification of some proteins of interest selected from a 450 μg system preparative gel using BioWorks Rev 3.3, software and the MASCOT system.

Spot no.	Database	Accesion no.	Protein name	Organism	Score	Ca
300	JGI	193017	Ion transport protein, Cation (not K+) channel, TM region	*Chlamydomonas reinhardtii*	10.15	0.97
307	JGI	131411	Putative protein with F-box domain	*P. patens*	10.13	3
364	NCBir	AAR20845	Cell division cycle protein 48	*Chlorella ellipsoidea*	35.2	9
458	JGI	147333	Heat shock protein 90	*P. patens*	30.15	4.90
462	JGI	221851	Heat shock protein 90	*P. patens*	30.18	2.00
463	JGI	221852	Heat shock protein 91	*P. patens*	30.18	2.00
887	JGI	129876	β-Tubulin	*C. reinhardtii*	20.20	6.10
891	JGI	121199	β-Tubulin	*P. patens*	10.21	2.00

8 of these could be identified with high probability. Of the various spots detected, the putatively identified proteins are listed in Table 3. Some of the proteins involved in transport, protection, cytoskeleton, cell cycle and targeting and degradation were more expressed in the desiccated and rehydrated algae. Half of the highly up-regulated proteins corresponded only with two gene families. Three of the ten proteins were identified as the same Heat Shock Protein 90 (Hsp90). Other two, were putatively identified with β-tubulin proteins (55 kDa). The synthesis of all of these proteins was increased significantly after desiccation, but no differences were found neither between the samples dried slowly and rapidly nor before and after the rehydration (Fig. 4.3).

4.3.2. *mRNA quantification*

Cloning of the products of the Nested and Race-PCR allowed us to found five functional forms of the *Hsp90* gene and two of the *β-tubulin* gene.

To investigate the changes of gene expression at the mRNA level during a slow dehydration and rehydration, we performed RT-qPCR analysis (Fig. 4.4). All the genes analysed showed a similar pattern: first, one hour after the beginning of the dehydration a decrease in the mRNA amount respect the control (value 1 in the Fig. 4.4), and then an important increase during the dying process, arising the maximum value, around 1.5 and 4 folds the control value, when the samples were completely dried; after rehydration, the amount of mRNA decreased abruptly to values lower than the initials, followed by a recovery of the normal values along the time.

4.4. Discussion

4.4.1. *Changes in proteome during dehydration and rehydration*

Desiccation tolerance in plants is a phenomenon that is just beginning to be understood. Only a few proteomic studies have been carried out in plants to elucidate the mechanisms of desiccation tolerance. The responses vary widely among species and the results are difficult to compare because most studies have only focused on the effect of dehydration or on the effect of rehydration: in the resurrection plant *Xerophyta viscosa*, 41 proteins were up-regulated and 21 down-regulated during dehydration of the leaves (Ingle *et al.* 2007); and in *Boea hygrometrica* 78 proteins are up-regulated during dehydration, 10 during rehydration and only 9 are repressed (Jiang *et al.* 2007); dehydration and rehydration

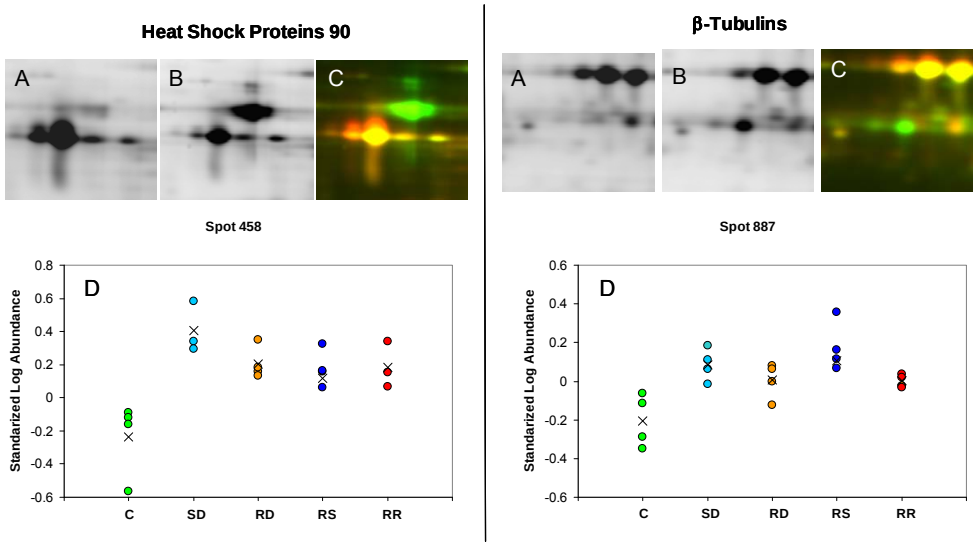


Figure 4.3 - Example of 2D-DIGE gel image of Heat Shock Protein 90 and □-Tubulin proteins: (A) Cy5 image, control; (B) Cy3 image, recovery after rapid drying; (C) two-color merged 2D-DIGE gel image, the green spots show that the protein expression during the recovery after rapid drying was greater than that of non-dehydrated samples, the yellow spots show not differences. (D) Standard Log Abundance of each sample used in each treatement, x symbol represent average value: C, Control; SD, slow drying; RS, recovery after slow drying; RD, rapid drying; RR, recovery after rapid drying.

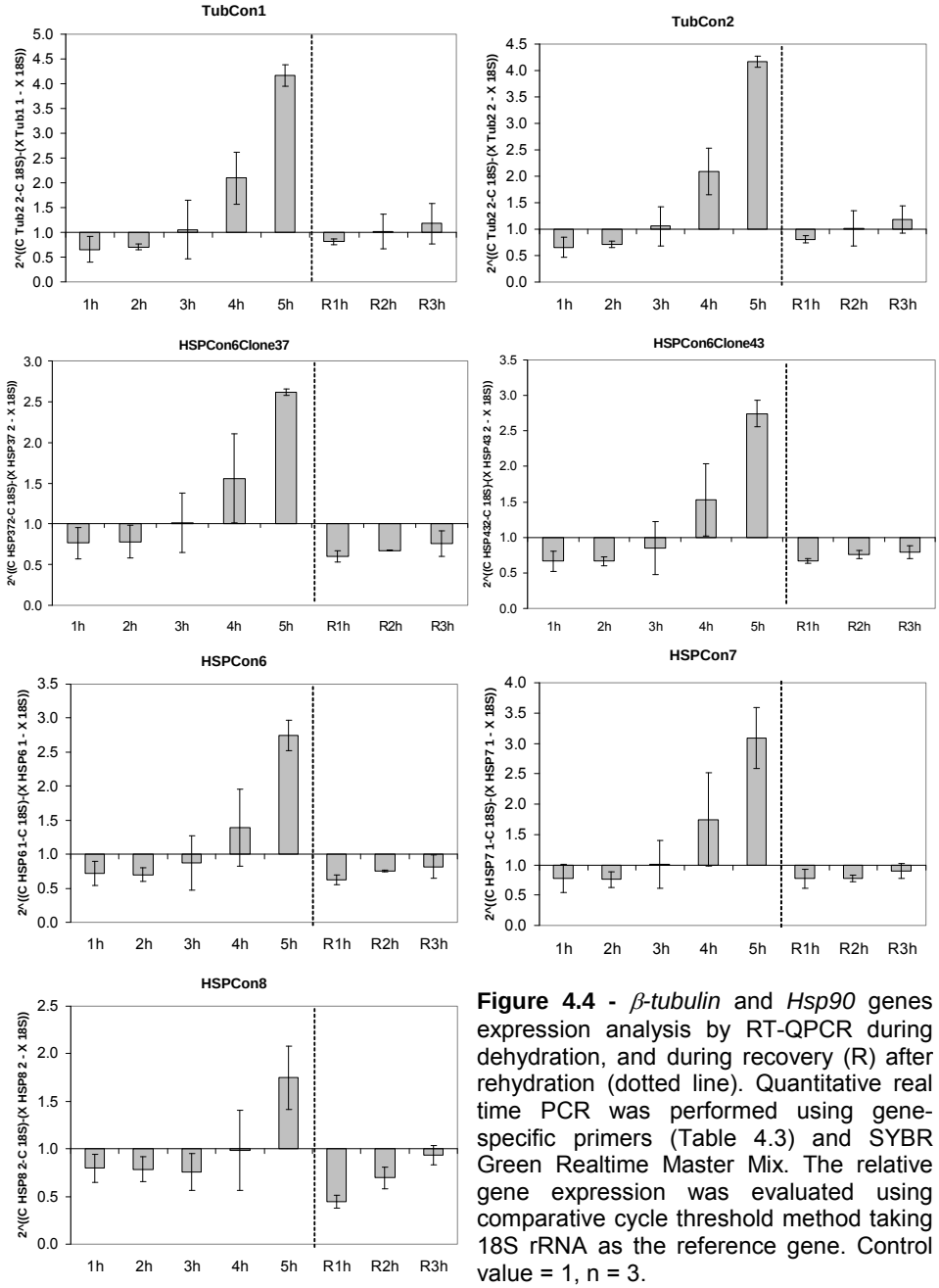


Figure 4.4 - *β-tubulin* and *Hsp90* genes expression analysis by RT-QPCR during dehydration, and during recovery (R) after rehydration (dotted line). Quantitative real time PCR was performed using gene-specific primers (Table 4.3) and SYBR Green Realtime Master Mix. The relative gene expression was evaluated using comparative cycle threshold method taking 18S rRNA as the reference gene. Control value = 1, n = 3.

of detached fronds of *Selaginella bryopteris* led to an increase of 21 proteins and a decrease of 27 proteins (Deeba *et al.* 2009); dehydration of the moss *Tortula ruralis* did not produce significant changes in protein abundance, but rehydration increased the synthesis of 74 protein and a decrease of 25 proteins (Oliver 1991); however, in another moss, *Physcomitrella patens*, dehydration up-regulated 46 proteins and down-regulated 25 (Wang *et al.* 2009). Our results with the lichen photobiont *T. erici* indicate that some 19 proteins were significantly up-regulated and 43 down-regulated. Similar changes in protein pattern were observed during dehydration and during rehydration, although certain differences were noticed depending on the drying rate. Proteins involved in both molecular protection and ultrastructural recovery, like Hsp90 and β-tubulins, were more abundant after slow drying. Although their synthesis was not statistically significant ($P>0.05$) between slow and rapid drying, this difference may partially explain the lower recovery capacity of the samples dried rapidly, described in Chapter 3. Furthermore, we observed that the tendency to recover the control protein levels after 3 h of rehydration was higher in the slowly dried than in the rapidly dried samples.

Thus, surprisingly, even after rapid drying there was an important up-regulation and down-regulation of proteins, which means that synthesis of the proteins involved in desiccation-tolerance mechanisms can begin in less than one hour after dehydration starts. Regulation of gene expression in response to desiccation stress may be ABA-dependent or ABA-independent in higher plants. Those genes that are activated by the accumulation of ABA are involved in slow response since ABA levels begin to increase hours after dehydration (Shinozaki & Yamaguchi-Shinozaki 1997; Yamaguchi-Shinozaki & Shinozaki 2006). Other genes are induced independently of ABA and are early-responsive to dehydration stress (ERDs) because they can be induced in only one hour, or less, after dehydration (Jensen *et al.* 1996). Interestingly, one group of ERDs genes expressed in response to dehydration stress in *Arabidopsis* encodes proteins identical to Hsp70 and Hsp90 (Kiyosue *et al.* 1994), one of the proteins that we found is up-regulated in *T. erici*. These genes can also be very rapidly activated under other kinds of stresses, e.g., in *Arabidopsis*, Hsp70s reached peak induction within 30 min of heat shock exposure while in spinach induction of Hsp70 genes has been detected as early as 5 min after a heat shock (Sung *et al.* 2001).

4.4.2. *Up-regulated proteins*

One of the spots analysed was similar to a putative cation transport protein of *Chlamydomonas reinhardtii*. Ion transporters and their regulatory systems carry out several crucial physiological roles, such as establishing and maintaining intracellular ion concentrations within the optimal range for normal cellular function. One of the main consequences of loss of protoplasmic water during dehydration is the high concentration of ions, which may inhibit metabolic functions (Hartung *et al.* 1998). It has been suggested that vacuoles play a vital role in maintaining ion homeostasis (Mundree *et al.* 2002). Maybe, vacuoles, or another organelle, carry out the same role in *T. erici* during dehydration, and accumulate the excess ions.

Another identified protein was a homologous protein of *Physcomitrella patens* with an F-box protein domain. The F-box is a conserved domain present in a large number of proteins with a bipartite structure. Through the F-box, these proteins are linked to the Skp1 protein and the core of SCFs (Skp1-cullin-F-box protein ligase) complexes. SCF complexes constitute a new class of E3 ligases. They function in combination with the E2 enzyme Cdc34 to ubiquinate G1 cyclins, Cdk inhibitors and many other proteins and mark them for degradation (Bai *et al.* 1996; Skowyra *et al.* 1997). The physiological roles of proteolytic enzymes are diverse, as they are necessary both for processing proteins from an inactive to active state and for recycling redundant/damaging polypeptides (Schwechheimer & Schwager 2004). It has been shown that protein degradation via the ubiquitin-proteoasome pathway plays a pivotal role in controlling cellular processes, such as cell cycle progression and transcriptional control in eukaryotic cells (Hershko & Ciechanover 1998).

The cell division cycle 48 protein (AtCdcd48 in Arabidopsis thaliana, Cdc48 in yeast, CDC-48 in *Caenorhabditis elegans*, p97 in mammal) is a highly abundant type II AAA-ATPase (Peters *et al.* 1990) involved in cell cycle control (Moir *et al.* 1982) and cell proliferation (Egerton & Samelson 1994). CDC48/97 is essential for cytokinesis, cell expansion and cellular differentiation in plants (Park *et al.* 2008). At the molecular level, CDC48/p97 is involved in many different cellular processes and its activity is modulated by

alternative adaptor proteins, which determine recruitment and processing of specific substrates. For example, in mammalian cells, CDC48/p97 requires the cofactor p47 to mediate endoplasmic reticulum (ER) and Golgi membrane assembly (Kondo *et al.* 1997; Yuang *et al.* 2001). It is a central factor for mobilizing and targeting ubiquitylated substrates to the 26S proteasome when it forms a complex with the cofactors Ufd and Npl4 (Meusser *et al.* 2005).

Microtubules constitute one of the major components of the cytoskeleton of eukariotic cells and are involved in many essential processes, including cell division, ciliary and flagellar motility and intracellular transport (Hyams & Lloyd 1993). The basic building block of the microtubule is the heterodimeric protein α/β-tubulin that assembles in a head-to-tail arrangement to form a linear protofilament. Except for yeast (Neff *et al.* 1983), all species examined thus far contain multiple genes encoding α- and β-tubulin, the principle subunits from which microtubules polymerize, and it has been shown that more than one α- and one β-tubulin gene can be expressed in a given cell (Hall *et al.* 1983; Havercroft & Cleveland 1984). In the literature, studies of the relationship between microtubule cytoskeleton and desiccation deal mainly with desiccation-sensitivity in recalcitrant seeds (Berjak & Pammenter 2000; Mycock *et al.* 2000; Rocha Faria *et al.* 2004) and descriptions of the disassembly of microtubules during maturation of moss spores (Brown & Lemmon 1980; Brown & Lemmon 1982; Brown & Lemmon 1987). However, recent works have reported that in the desiccation-tolerant moss *Physcomitrella patens* microtubule cytoskeleton is disassembled during dehydration (Wang *et al.* 2009), whereas in the moss *Polytrichum formosum* inhibition of β-tubulin polymerisation precluded the recovery of the normal cell ultrastructure upon rehydration (Pressel *et al.* 2006). In lichens, photobionts cells shrink during drying and eventually collapse (cytorrhysis) (Scheidegger *et al.* 1995; Honegger *et al.* 1996; Honegger 1998). The protoplast remains in close contact with the cell walls in the desiccated state (Honegger *et al.* 1996). During rehydration, the cytoplasm and organelles of the protoplast swell and cells recover their normal structure (Honegger 2006). Microtubule skeleton seems to play a key role in the recovery of the ultrastructure of *T. erici*.

Hsp90s proved to be the group of proteins whose synthesis increased most, as compared to the control. They are induced by a broad spectrum of stresses such as low and

high temperatures, strong light intensity, heavy metals, salt or dehydration (Timperio *et al.* 2008). This would indicate that the Hsps complex is one of the most powerful weapons that cells have developed to cope with environmental stresses. Some of the most important functions of Hsps include folding and transport of proteins, and therefore they are also called "molecular chaperones" (Vierling 1991). Most Hsps have strong cytoprotective effects, maintaining proteins in their functional conformation, preventing aggregation of non-native proteins, refolding of denaturated proteins to regain their functional conformation and removal of non-functional but potentially harmful polypeptides (arising from misfolding, denaturation or aggregation). Maintaining proteins in their functional conformations and preventing the aggregation of non-native proteins are particularly important for cell survival under stress. Thereby, Hsps ensure maintenance of homeostasis, protect cells and help them to return to equilibrium during stress recovery (Timperio *et al.* 2008). In *T. erici* we have only detected Hsp90 (83-99 kDa), which is one of the most abundant and evolutionarily conserved molecular chaperones (Young *et al.* 2001; Picard 2002; Wegele *et al.* 2004); however, Hsps include other families such as Hsp70 (68-80 kDa), Hsp60, and the small Hsps (25-28 kDa) (Lindquist & Craig 1988). Their expression varies broadly among species and may respond to different stresses (Timperio *et al.* 2008). Furthermore, some Hsps may also be constitutively expressed under non-stressful conditions, because they form part of the cell's own repair system; they are involved in the removal of old proteins and help the newly synthesized ones fold properly (Rachmilevitch *et al.* 2006).

4.4.3. *Down-regulated proteins*

During dehydration and rehydration we observed a decrease in the abundance of around 40 proteins. Degradation of proteins and an increase in proteolytic enzymes is a typical plant response against dehydration (Ingle *et al.* 2007; Deeba *et al.* 2009) or other environmental stresses (Yan *et al.* 2006). On one hand, it is possible that induction of proteolytic enzymes may play a role in the reallocation of resources for biosynthesis of novel proteins involved in drought resistance mechanisms. On the other hand, proteins down-expressed are likely to be involved in metabolic pathways that were active in a well-

hydrated state but inactivated during dehydration; e.g. photosynthetic proteins are down-regulated during dehydration, since they are inactive in desiccated plants and may produce photo-oxidative damage (Ingle *et al.* 2007; Wang *et al.* 2009; Deeba *et al.* 2009). Interestingly, we observed the up-regulation of two proteins: Cdc48 and the protein with an F-box domain, which are concerned with protein signalling and degradation.

4.4.4. β-tubulin *and* Hsp90 *gene expression*

The machinery leading to the expression of drought-stress genes conforms to the general cellular model (Ingram & Bartels 1996), with a complex signal transduction cascade that can be divided into the following basic steps: a) perception of stimulus; b) signal transduction, including amplification and integration of the signal; c) response reaction in the form of *de novo* gene expression or others: enzyme activity regulation, ion channel functional changes, etc. Signal transduction cascades from the sensing of water-stress signals to the expression of various genes are unknown (Shinozaki & Yamaguchi-Shinozaki 1997), although turgor change has been suggested as a possible physical signal. In *Saccharomyces cerevisiae*, Kamada *et al* (1995) suggest that heat-shock response is activated by an increase in membrane fluidity. The same model for the activation of a transduction pathway might be applied for a dehydration signal. We observed that five *Hsp90* and two *β-tubulin* genes were activated during dehydration and mRNA was accumulated until the cell was completely dried; indicating that synthesis of mRNA went on even after very low relative water content (RWC). As we have reported in Chapter 3, *T. erici* is able to maintain certain physiological activities, such as photosynthesis, at below 10 % RWC. Likewise, the moss *Tortula ruralis*, another poikilohydric desiccation tolerant organism, also accumulated mRNA throughout the drying process (Velten & Oliver 2001).

4.5 Resumen

Introducción

Los vegetales, como organismos sésiles, dependen de la plasticidad del proteoma tanto para remodelarse a ellas mismas durante el desarrollo, como para soportar las condiciones ambientales variables y para responder a los estreses bióticos y abióticos. Cuando se someten al estrés hídrico, los vegetales también cambian su expresión génica y su metabolismo. Con el fin de identificar los genes responsables de los mecanismos de tolerancia a la desecación, se han llevado a cabo experimentos basados en la expresión de RNA y que han examinado los cambios que ocurren durante la deshidratación y/o rehidratación en el transcriptoma de diversas plantas de resurrección *Xerophyta humillis* (Collett *et al.* 2003; Collett *et al.* 2004), *Craterostigma plantagineum* (Bartels *et al.* 1990; Bockel *et al.* 1998), *Sporobolus stapfianus* (Blomstedt *et al.* 1998; Neale *et al.* 2000), *Selaginella moelendorfii* (Weng *et al.* 2005; Iturriaga *et al.* 2006) y en el musgo tolerante a la desecación *Tortula ruralis* (Oliver *et al.* 2004). Sin embargo, la abundancia de ARNm solamente puede ser relacionada con una función putativa, ya que hoy en día todavía se cuestiona que haya una correlación directa entre ARNm y los niveles de proteínas. Por el contrario, los estudios de proteómica pueden revelar la porción del genoma que es activamente trasladada y que lleva a cabo las funciones enzimáticas, reguladoras y estructurales de la célula en un determinado momento. Los estudios de proteómica realizados en plantas de resurrección y musgos han mostrado que durante la deshidratación puede haber un incremento de los enzimas antioxidantes, proteínas chaperonas, enzimas del metabolismo energético, marcaje y degradación de proteínas y de proteínas implicadas en la señalización (Ingle et al. 2007; Jiang et al. 2007; Deeba et al. 2009; Wang et al. 2009). Así mismo, generalmente se produce una disminución en las proteínas relacionadas con la actividad fotosintética ya que el transporte de electrones de la fotosíntesis queda bloqueado durante la desecación y de esta forma la planta disminuye la formación de especies reactivas de oxígeno (Ingle *et al.* 2007; Wang *et al.* 2009; Deeba *et al.* 2009).

Por lo que nosotros sabemos, se han realizado muy pocos trabajos a nivel molecular acerca de la regulación de las respuestas durante la deshidratación y/o

rehidratación en fotobiontes liquénicos. Así pues, decimos llevar a cabo análisis proteómicos y de expresión genética de los cambios asociados con la desecación y la rehidratación en el fotobiontes aislado *T. erici.*

Materiales y métodos

Un cultivo axénico del fotobionte liquénicos *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) fue utilizado en nuestro estudio y cultivado en discos de acetato de celulosa como se describe en el capítulo 2.

Las algas fueron deshidratadas lentamente (DL) poniéndolas en un recipiente cerrado con una solución de nitrato de amonio (h.r. 66 %), con lo que las algas quedan secas en alrededor de 6 h. Las deshidrataciones rápidas (DR) se consiguieron con silicagel (h.r. 20 %) en alrededor de una hora. La recuperación tras una deshidratación lenta y rápida (RL y RR, respectivamente) fue estudiada con muestras que habían permanecido 24 h secas, y que posteriormente se rehidrataron y mantuvieron en condiciones de cultivo durante 3 h.

Para la identificación de aquellas proteínas que se acumulaban durante la deshidratación y la rehidratación, empleamos la técnica de la electroforesis en gel diferencial 2D (DIGE) junto con una identificación individual de las proteínas usando digestiones con tripsina (de geles teñidos con plata) y LC-MS/MS. Las genes que se seleccionaron para cuantificar su ARNm fueron los de las proteínas β-tubulinas y las Hsp90. Sin embargo, ambas forman parte de sendas familias multigénicas, por lo que antes de realizar la cuantificación de la expresión de estos genes con qRT-PCRs, utilzamos las técnicas GeneRacer y de clonaje para conocer el número de genes funcionales de cada familia y del extremo 3' de cada gen, respectivamente.

Resultados y discusión

Se observaron cambios similares en el patrón proteico de *T. erici* fueron observados durante la deshidratación y la rehidratación, aunque con ciertas diferencias dependiendo de la velocidad de deshidratación. Las proteínas implicadas en la protección

molecular y la recuperación de la ultraestructura, como las Hsp90 y las β-tubulinas, respectivamente, fueron más abundantes tras la deshidratación lenta. Aunque esta diferencia no fue estadísticamente significativa, su relativamente mayor síntesis en las algas DL podría explicar en parte la menor recuperación de las algas DR descrita en el capítulo 3. Así mismo, tras 3 horas de rehidratación, observamos que la tendencia a recuperar el patrón protéico inicial fue más importante en las muestras DL que en las DR. Así pues, incluso después de una deshidratación rápida se produjeron importantes cambios en la regulación de muchas proteínas lo que indica que la síntesis de las proteínas implicadas en los mecanismos de tolerancia a la desecación puede realizarse en menos de una hora tras el comienzo de la deshidratación.

Una de las proteínas con mayor incremento de su abundancia tras la deshidratación fue similar a una encargada del trasporte de cationes del alga *Chlamydomonas reinhardtii.* El transporte de iones y su regulación puede ser crucial durante la deshidratación, cuando la pérdida de agua provoca el aumento de su concentración (Hartung *et al.* 1998).

Otra proteína de las identificadas fue homóloga a una proteína con un dominio F-box del musgo *Physcomitrella patens*. Los dominios F-box se encuentran muy conservados y presentes en un gran número de proteínas con estructura bipartita. Estas proteínas son necesarias para el marcaje de proteínas y su posterior degradación (Bai *et al.* 1996; Skowyra *et al.* 1997).

También se identificó la proteína del ciclo de división celular 48 (Cdc48) que se encuentra implicada en el control del ciclo celular (Moir *et al.* 1982) y en la proliferación celular (Egerton & Samelson 1994). Es un factor central para la movilización y el marcaje de los sustratos ubiquitinizados para su degradación en el proteosoma 26S (Meusser *et al.* 2005).

Entre las proteínas que más incrementaron su síntesis se encontraron las β-tubulinas, las cuales son uno de los mayores componentes del citoesqueleto. En trabajos recientes, se ha mostrado que el citoesqueleto de microtúbulos juega un papel esencial en la recuperación de la ultraestructura celular durante la rehidratación en los musgos tolerantes a la desecación, *Physcomitrella patens* y *Polythichum formosum* (Pressel *et al.* 2006; Wang *et al.* 2009).

Así mismo, las Hsp90 fueron otras de las proteínas cuyo incremento en su abundacia fue de los más altos. Estas proteínas son inducidas por un amplio espectro de estreses como bajas y altas temperaturas, fuertes intensidades de luz, metales pesados, sales y deshidratación (Timperio *et al.* 2008). La mayoría de las Hsp tienen un fuerte efecto citoprotector, manteniendo las proteínas en su conformación funcional, previniendo la agreagación de proteínas no-nativas, replegando y desplegando proteínas desnaturalizadas para que recuperen su conformación funcional, y eliminando polipéptidos no funcionales pero poetencialmente peligrosos (Timperio *et al.* 2008).

La cuantificación del ARNm mostró que cinco y dos genes de Hsp90 y β-tubulinas, respectivamente, eran activados durante la deshidratación. El ARNm se acumuló hasta que las células se secaron completamente, indicando que la síntesis de ARNm se produjo incluso con contenidos hídricos muy bajos. Como hemos mostrado en el capítulo 3, *T. erici* es capaz de mantener ciertas actividades fisiológicas, como la fotosíntesis, con contenidos hídricos menores del 10 %.

5. ROLE OF NO IN THE REGULATION OF OXIDATIVE STRESS DURING REHYDRATION OF THE LICHENIC AND ISOLATED PHOTOBIONTS

Abstract

Lichens are symbiotic poiquilohydric organisms subject to repeated desiccation/rehydration cycles, and able to survive in frequently very dry environments. As lichen, both partners are able to colony extreme habitats but some authors suggest symbiosis enhances their resistance against abiotic stresses, such as high irradiance or desiccation. However, the biochemical interaction between fungal and algal partners that may be involved in triggering stress tolerance is not known. During rehydration there is a burst in the production of reactive oxygen species (ROS) together with the release of nitric oxide (NO) by the mycobiont. NO is a ubiquitous and multifaceted molecule involved in cell signalling and protection against abiotic stress, among other. In the present chapter, we investigated production and the antioxidant role of NO during rehydration of isolated photobionts and lichens. The production of ROS and NO was induced by rehydrating the organisms. NO was scavenged with c-PTIO. The production of ROS and NO in the lichen *Ramalina farinacea* and in the isolated photobiont *Trebouxia erici* were monitored by fluorescence and confocal microscopy using, respectively, $DCFH_2$-DA and 2,3-diaminonaphthalene as probes. NO release was quantified as NO_x end-products using the Skalar autoanalyzer. The effect of NO scavenging on the photosynthesis recovery of *R. farinacea* and *T. erici* after desiccation was measured by using a chlorophyll fluorometer PAM-2000. Rehydration of *R. farinacea* caused the release of NO together with a high production of ROS. The amount of NO detected decreased significantly with the addition of c-PTIO. In the isolated photobionts, scavenging of the self produced NO caused a decrease in the recovery of photosynthetic activity after dehydration**,** probably due to the higher levels of photo-oxidative stress. These data provide evidences of an important role for NO in antioxidant systems during the early stages of rehydration in the lichen *Ramalina farinacea,* including chlorophyll photostability of the photobionts of the genus *Trebouxia.* Our results also raise important questions about the evolutionary role of NO in the establishment of lichen symbiosis, due to its dual role as antioxidant and mediator in cell communication.

5.1. Introduction

Lichenization involves the evolution of a complex above-ground structure, which neither a fungus nor an alga can form alone. In this symbiosis, the lichen fungus benefits by obtaining its carbon nutrient from the photobiont, but the photobiont's gain from the association is less obvious, may be fungi facilitate inorganic nutrients through the haustoria (Nash 2008). In opposition, lichenization requires that the fungus give up saprophytic lifestyle below ground; and that the photobiont also cease its hidden life in bark, soil (Mukhtar *et al.* 1994), or small crevices in rocks (Ascaso *et al.* 1995) to live above-ground. Under this condition, both the mycobiont and the photobiont are exposed to much higher levels of solar radiation, and desiccation. As lichen, both partners are able to colony extreme habitats that would be unavailable by each one alone. The question of whether the extreme stress tolerance of the lichen is simply the sum of the traits of its symbiotic partners or whether it is enhanced by the process of lichenization, has not yet answer. However, some studies suggest that isolated photobionts are less tolerant to high light or desiccation stresses than the lichenized forms (Brock 1975; Lange *et al.* 1990; Kranner *et al.* 2005; Kosugi *et al.* 2009), although the biochemical interaction between fungal and algal partners that may be involved in triggering stress tolerance is not known.

One of the possible molecules that may be involved in the biochemical interaction is the nitric oxide (NO). Weissman *et al.* (2005) described the occurrence of intracellular oxidative stress during rehydration together with the release of great amounts of NO by the mycobiont of *Ramalina lacera*. The bioactive gas NO has multiple biological functions in a very broad range of organisms. These functions include signal transduction, intracellular signalling, cell death, transport, basic metabolism, reactive oxygen species (ROS) production and degradation (Courtois *et al.* 2008, Palmieri *et al.* 2008), among others (reviewed in Wilson *et al.* 2008). It is well-known that NO exerts both pro-oxidant and antioxidant effects, depending on the ambient redox status, the presence of other reactants, and the nature of the reaction (for a review of the antioxidant actions of NO, see Darley-Usmar *et al.*, 2000). In plants, ROS and reactive nitrogen species have been shown to be involved in the defensive response to biotic or abiotic stresses such as pathogens (Hong *et al.* 2008), draught (Neill *et al.* 2008), and air pollutants or UV-B radiation (Hérouart *et al.*

2002). In the latter study, the authors found support for the hypothesis that NO reactive species, together with the glutathione system, play a key role in the coordination of gene expression during legumen-*Rhyzobium* symbiosis. NO has been postulated as one of the first antioxidant mechanisms to have evolved in aerobic cells (Kröncke *et al.* 1997, Mallick *et al.* 2002). This idea builds on the work of Feelisch & Martin (1995), who 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). Although NO has not been observed in lichen photobionts with epifluorescence probes (Weissman *et al.* 2005; Picotto 2009), there are evidences that *Trebouxia sp. pl.* produce NO in low amounts (Catalá, not published).

As we have seen in the Chapters 3 and 4, desiccation tolerance in the photobiont *T. erici* is achieved by a complex system of constitutive and induced mechanisms. However, in a symbiotic relationship desiccation tolerance might be enhanced by other mechanisms like the NO released by the mycobiont. In the present work, we investigated production and the antioxidant role of NO during rehydration of isolated photobionts and lichens.

5.2. Materials and methods

5.2.1. *Biological material*

Ramalina farinacea (L.) Ach. is a fruticose, pendulous, epiphytic lichen species that is very common in Mediterranean sclerophyllous oak forests. For the present study, this lichen was collected in the air-dried state from *Quercus rotundifolia* Lam. at Sierra de El Toro (Castellón, Spain; 39º54'16"N, 0º48'22"W). Samples were frozen at –20ºC until the experiment, 1 month after collection.

The photobiont *R. farinacea* (*Trebouxia* sp.) was isolated following the protocol described in the Appendix 4. An axenic strain of the lichen photobiont *Trebouxia* sp. was grown in 3N Bold´s basal medium (BBM3N) containing 10 g casein and 20 g glucose per liter (Backor and Váczi 2002) with a 16:8 h light:dark photoperiod and at a temperature of

15ºC. The medium was changed every 2 weeks and the concentration of algae set at 10^5 cells/ml.

An axenic strain of the lichen photobiont *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) was cultured on sterile cellulose-acetate discs as is described in the Chapter 2 (Section 2.2.2.).

5.2.2. *Epifluorescence probes*

2,7-Dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was used as probe in the detection of ROS. $DCFH_2$-DA readily enters the cell, where it is hydrolyzed by esterases to dichlorodihydrofluorescein ($DCFH_2$), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF, λ_{exc} = 504 nm, λ_{em} = 524 nm) by the action of cellular free radicals. $DCFH_2$-DA is not appreciably oxidized to the fluorescent state without prior hydrolysis.

2,3-Diaminonaphthalene (DAN) reacts with the nitrosonium cation that forms spontaneously from NO to yield the fluorescent product 1H-naphthotriazole (λ_{exc} = 375 nm, λ_{em} = 425 nm). Since the selectivity of DAN for the nitrosonium cation is high, NO can be detected without the inhibition of its function (Kojima *et al.* 1997).

5.2.3. *Confocal microscopy*

Fragments of lichen thalli were rehydrated for 5 min with either deionized water or 200 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (c-PTIO). The excess solution was wiped off and the thalli were kept wet at room temperature. Prior to their observation by confocal laser scanning microscopy (TCS Leica SP Confocal Laser Scanner Microscope, Leica, Heidelberg, Germany) at the SCSIE (UVEG, Valencia), they were washed with deionized water and mounted on slides.

c-PTIO is a water-soluble and stable free radical that reacts stoichiometrically with NO. *In vivo*, c-PTIO inhibits the physiological effects mediated by NO.

For studies with the $DCFH_2$-DA probe, an Ar excitation laser (488 nm) was used. The magnification is indicated in each figure.

5.2.4. *Fluorescence microscopy*

Fragments of lichen thalli were rehydrated for 5 min with either deionized water or 200 µM c-PTIO, and the corresponding fluorescence probe (10 µM $DCFH_2$-DA or/and 200 µM DAN). The samples were then placed in a freezing microtome (CM 1325; Leica, Germany) and cut in sections of 30 microns. Prior to their observation by fluorescence microscopy (OLYMPUS Provis AX 70 fluorescence microscope), the slices were washed with deionized water and mounted on slides.

The probe DCF was excited with blue light using a 460-490 nm excitation filter (Olympus fluorescence filter cube U-MWB2), and the emitted fluorescence analyzed through a 520 nm barrier filter (520IF). DAN was excited with ultraviolet light using a 330-385 nm excitation filter (Olympus fluorescence filter cube U-MWU2), and the emitted fluorescence analyzed through a 420 nm barrier filter. UPLSAPO 40X2 and 100XO objectives were used. All images were acquired with a digital camera (Olympus Camedia C-2000 Z camera).

5.2.5. *Effect of NO scavenging in photosynthesis*

21 days *T. erici* algal discs were removed from the culture medium and dried in a closed container with a saturated solution of ammonium nitrate (R.H. 66%). The samples remained in the dried state for 24 h and were then rehydrated with distilled water or 200 µM c-PTIO. The samples were returned to the agar culture medium and maintained under culture conditions for 24 h.

Thalli of *R. farinacea* were rehydrated for 5 min with distilled water or 200 µM c-PTIO. The excess solution was wiped off and the thalli were put into Petri plates and kept at 30 mmol m-2 s-1 (12/12 light/dark photoperiod) and 17 ºC for 24 h.

In vivo chlorophyll *a* fluorescence was measured with a modulated light fluorometer (PAM-2000, Walz, Effeltrich, Germany). Slow induction fluorescence kinetics were carried out following the protocol described in the Chapter 2 (Section 2.2.3.).

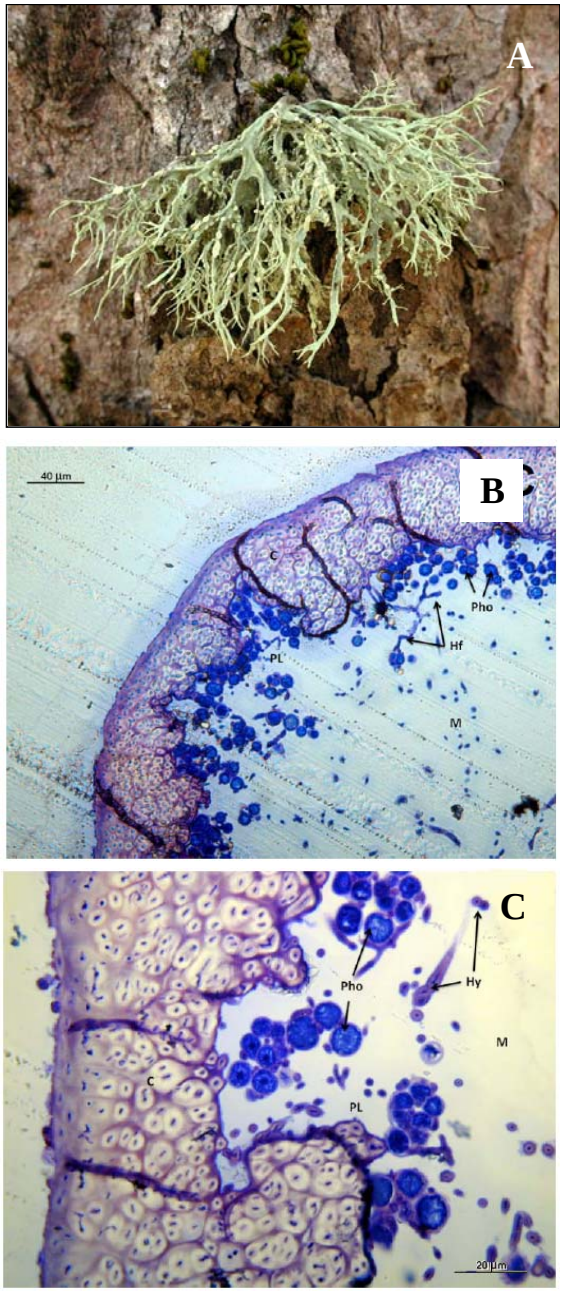


Figure 5.1 - Thallus of *Ramalina farinacea*: **B**, **C** ultramicrotome survey sections (10 µm) stained with toluidine blue (magnification 200× and 1000×, respectively). C cortex, PL photobiont layer, Pho photobiont, M medulla, Hy fungal hyphae

5.2.6. *NO end-products determination*

Air oxidation of NO in an aqueous environment results in the near exclusive generation of NO_2^-, which is further oxidized to NO_3^- (Fukuto *et al.* 2000).

To estimate NO generation, NO oxidation end-products (nitrate and nitrite) were measured in the soluble fraction of the samples using a Skalar autoanalyzer, model SAN++. The automated determination of nitrate and nitrite is based on the cadmium reduction method: the sample is passed through a column containing granulated copper-cadmium to reduce nitrate to nitrite. The nitrite (that originally present plus that obtained from the reduction of nitrate) concentration is determined by its diazotization with sulfanilamide followed by coupling with N-(1-naphthyl)ethylenediamine dihydrochloride to form a highly colored azo dye, the absorbance of which is measured at 540 nm. This is the most commonly used method to analyze NO production and is known as the Griess reaction (Nagano 1999).

5.2.7. *Statistics*

Every experiment was repeated four times and we used one-way ANOVA in combination with LSD test to compare the means of rehydration treatements.

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5.3. Results

Ultramicrotome micrographs showing the general anatomy of *Ramalina farinacea* are presented in Fig. 5.1. The photobiont layer is located in the medulla and is surrounded by dispersed fungal hyphae, which become densely packed in the cortex of the lichen.

5.3.1. *ROS generation during lichen rehydration*

Although several works have described an extracellular oxidative burst during rehydration in some lichen species, virtually nothing is known about intracellular ROS production and its relationship to abiotic stress. In order to determine whether intracellular

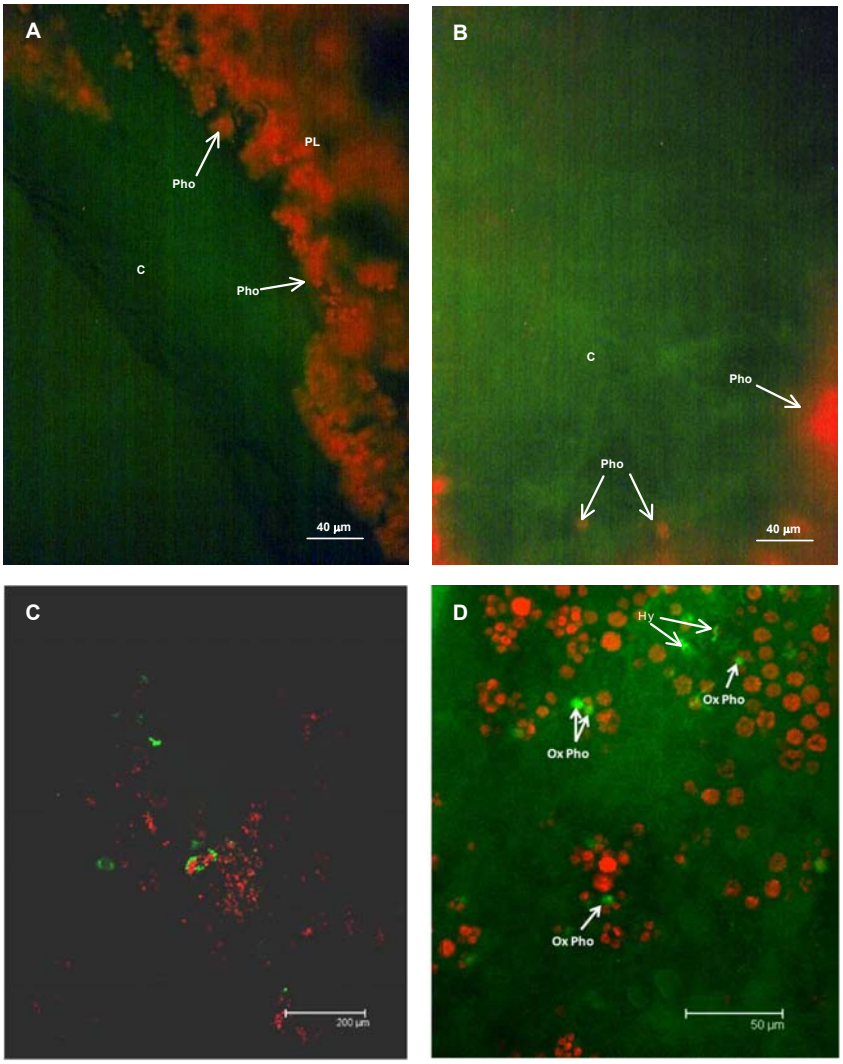

Figure 5.2 - Thalli of *Ramalina farinacea* rehydrated with deionized water and 10 µM $DCFH_2$-DA. **A, B** ROS content, as revealed by the green fluorescence emission of DCF under a fluorescence microscope after rehydration with water or c-PTIO 200 µM, respectively; **C** overlay of confocal microscopy images reveals ROS distribution around some of the photobionts (green fluorescence); **D** overlay of confocal microscopy images of ROS content of *R. farinacea* thalli that had been rehydrated with c-PTIO 200 µM to inhibit NO action, arrows point to photobionts photobleached by the confocal laser during the observation (oxPho). Red fluorescence is due to the photobiont's chlorophyll in all cases. C cortex, M medulla, PL photobiont layer, Pho photobiont, oxPho photobleached photobiont, Hy fungal hyphae

ROS release follows the rehydration of *R. farinacea* thalli, 10 μM of the fluorescent probe $DFCH_2$-DA was added to the deionized water used for rehydration. The samples were observed by fluorescence and confocal microscopy 2-3 h after rehydration.

The presence of 2',7'- dichlorofluorescin (DCF), the fluorescent oxidation product of $DCFH_2$, indicated the intracellular production of free radicals during lichen rehydration (Fig. 5.2 A). DCF was especially concentrated in the lichen cortex. No significant green autofluorescence was detected in the absence of the probe (image not shown). Confocal microscopy showed discrete points of green fluorescence around several large photobionts (Fig. 5.2 C), probably due to mycobiont hyphae tips.

5.3.2. *NO release during lichen rehydration*

The release of NO in a lichen species was recently demonstrated for the first time.

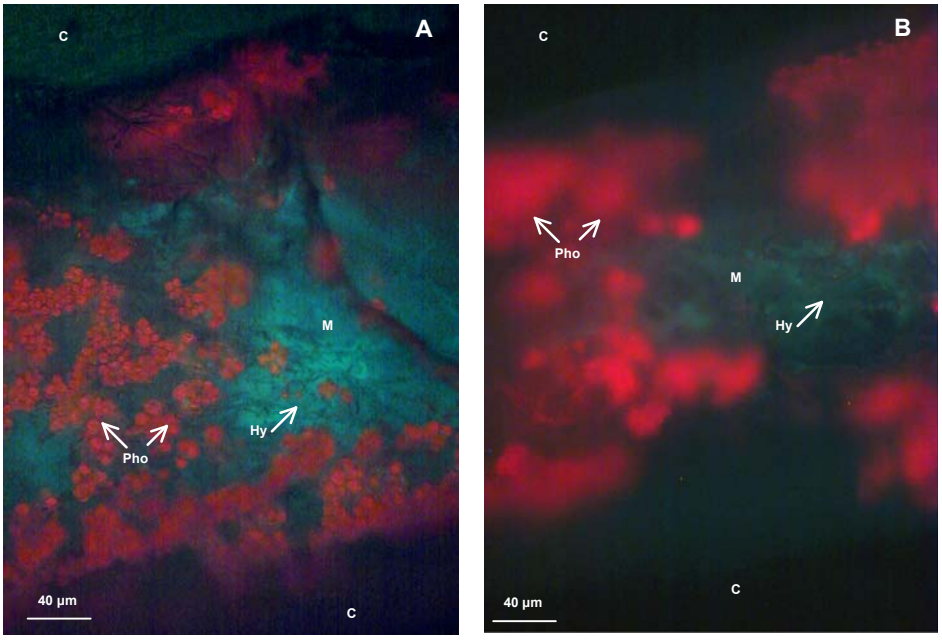

Figure 5.3 - Fluorescence microscopy of thalli of *Ramalina farinacea* rehydrated with deionized water (A) or c-PTIO 200 μM (B) and 200 μM DAN. Red fluorescence is due to the photobiont's chlorophyll in all cases. C cortex, M medulla, Pho photobiont, Hy fungal hyphae

In order to confirm these results in another lichen species, *R. farinacea,* two approaches were used: fluorescence visualization of the released NO and quantification of the NO end-products. Accordingly, thalli were rehydrated in deionized water containing 200 µM DAN for the visualization of NO release and in deionized water alone for the quantification of NO end-products.

Microscopic analysis of DAN fluorescence evidenced the production of NO, which was intimately associated with the fungal hyphae. Staining was especially intense in the medulla (Fig. 5.3).

NO end-products (NO_x) were quantified by the classical method of Griess. NO_x levels (Fig. 5.4 A) were initially 3.69 ± 0.493 µg NO_x/g lichen, and then increased over 2 h to reach a maximum of 5.56 ± 1.41 µg NO_x/g lichen. By 4 h, NO_x levels had decreased to slightly below the initial levels, reaching a minimum of 3.12 ± 0.412 µg NO_x/g lichen, after which the levels remained constant for up to 24 h.

5.3.3. *Effect of NO scavenging during lichen rehydration on ROS production*

To study the role of NO during rehydration, *R. farinacea* thalli were rehydrated with 200 µM of the membrane-permeable compound c-PTIO, which specifically reacts with NO to inhibit its biological actions.

NO scavenging with c-PTIO decreased DAN fluorescence emission (Fig. 5.3 B). It also produced a remarkable increase in ROS production in both the cortex and the medulla

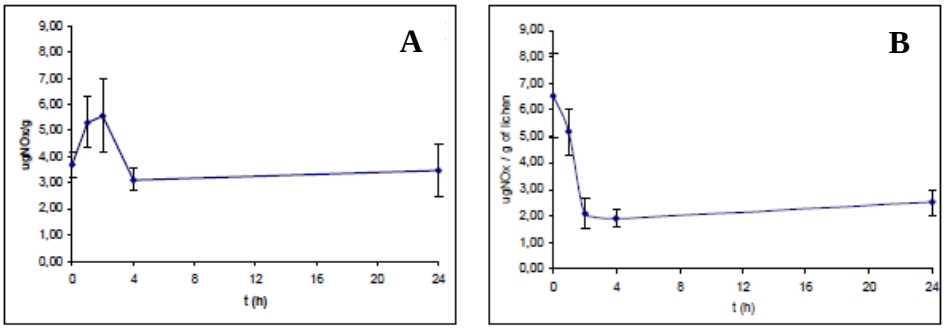

Figure 5.4 - MDA content of *Ramalina farinacea* thalli rehydrated with: **A** deionized water, **B** c-PTIO (200 µM) to inhibit NO action.

(Fig. 5.2 B,C). The confocal laser beam produced an oxidative burst in the photobionts, leading to chlorophyll photo-oxidation and DCF fluorescence onset within seconds (Fig. 5.2 D).

NO end-products (NO_x, quantified by the classical method of Griess, decreased during the first two hours after rehydration with c-PTIO 200 mM. NO_x levels (Fig. 5.4 B) were initially 6.65 ± 1.513 µg NO_x/g lichen, and then decreased over 2 h to reach a value of 2.21 ± 0.72 µg NO_x/g lichen, after which the levels remained constant for up to 24 h.

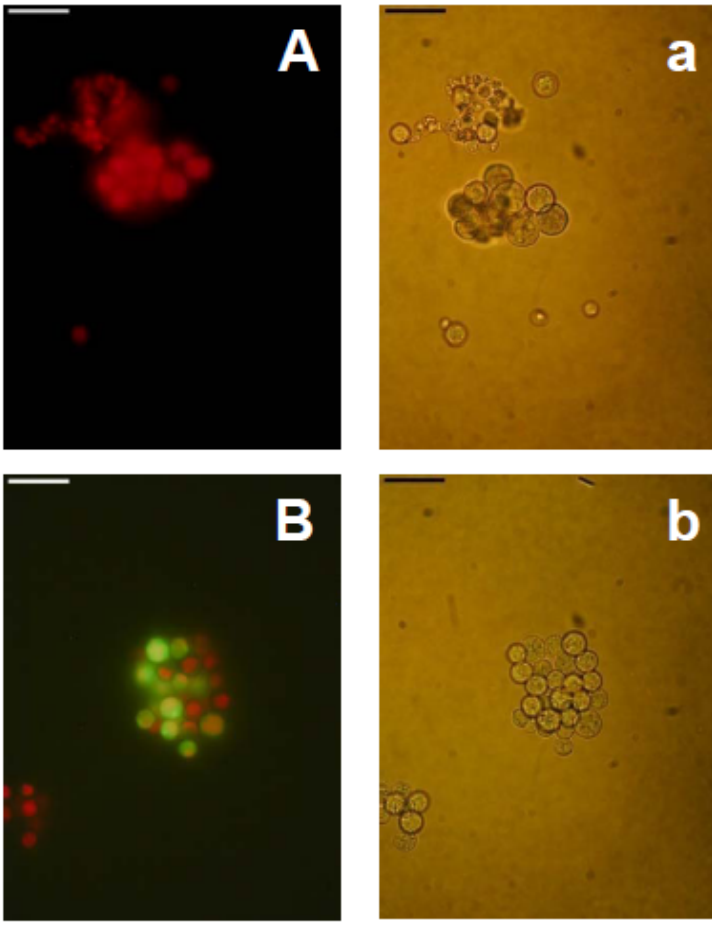

Figure 5.5 - ROS content of isolated *Trebouxia* sp. Capital letters identify the fluorescence image; the lower-case letter indicates the corresponding bright-field images: A-a control; B-b algae treated with 200 µM c-PTIO. Magnification 1000×. Bar 20 µm

5.3.4. *Microscopy studies of isolated algae*

Confocal studies showed that NO deprivation caused photo-oxidative damage in the photobiont (Fig. 5.2 D). NO is known to reduce photo-oxidative stress in some species of green algae. A specific role for NO in the prevention of photo-oxidation in *Trebouxia* algae was confirmed in the following studies.

A suspension of axenically cultured *Trebouxia* sp., the photobiont isolated from *Ramalina farinacea*, was treated with 200 μM c-PTIO in the presence of both $DCFH_2$-DA and DAN. The images of control cells are presented in the Fig. 5.5 A. NO inhibition by c-PTIO resulted in chlorophyll bleaching in some algae (Fig. 5.5 B). DAN fluorescence could not be detected by this method but the oxidative burst caused by c-PTIO provided indirect evidence of endogenous NO production in the algae.

5.3.5. *Photosynthetic studies on isolated algae*

To confirm that the visualized alterations in chlorophyll fluorescence were linked to alterations in the photosynthetic activity of the algae during NO deprivation, axenic cultures of *Trebouxia erici*, a well-characterized photobiont of the *Trebouxia* genus, were studied. The cells were cultured on cellulose-acetate discs, desiccated for 24 h, and rehydrated with culture medium containing c-PTIO. Measurements were made in cells that had been maintained in culture conditions for 24 h. The significant decrease of F_v/F_m and Φ_{PSII} indicated that NO scavenging induces photo-inhibition of PSII (Fig. 5.6). The degree of quinone A (Q_A) oxidation was determined as qP, which depends on the activation state of photosystem I (PSI) and the Calvin cycle (Maxwell *et al.* 1994). After the dehydration/rehydration cycle, no differences were observed in q_P, indicating that photoinhibition was produced before Q_A.

The same treatments and measurements were carried out in whole thalli of *Ramalina farinacea* but no alterations in photosynthesis at 24 h were observed.

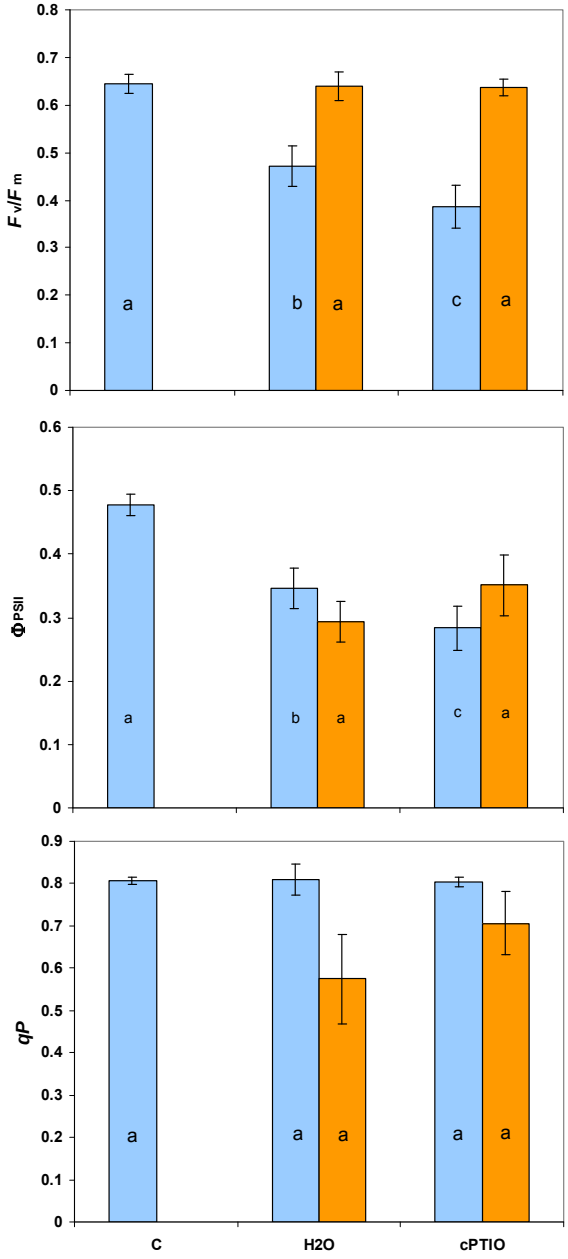

Figure 5.6 - Photosynthetic parameters of axenic cultures of *Trebouxia erici* (blue) desiccated for 24 h and then rehydrated with destilled water (H_2O) or 200 µM c-PTIO. The algae were incubated under normal culture conditions for 24 h before chlorophyll *a* fluorescence measurement. Control algae were not desiccated but instead maintained under normal culture conditions. Thalli of *Ramalina farinacea* (orange) were rehydrated with the same solutions and kept in culture conditions for 24 h. F_v/F_m, maximum photochemical efficiency of photosystem II (PSII); Φ_{PSII}, photochemical efficiency in light; qP, photochemical component of fluorescence relaxation. Different letters show significant differences between treatments. LSD test (Pv<0.05), *n* = 4.

5.4. Discussion

NO is a well-studied critical signaling molecule involved in abiotic stress responses (Neill *et al.* 2008) and plant defense (Hong *et al.* 2008). Our results demonstrated that, in addition to its utility for quantification methods, DAN is an excellent fluorescence microscopy probe for the histophysiological characterization of NO production in lichen. However, fluorescence microscopy is not suitable for NO quantification. Instead, the quantification of NO end-products is best-achieved with the Griess reaction, is a well-established method used to estimate NO production (Nagano 1999). The results of this approach complement qualitative data obtained with fluorescence microscopy.

The use of $DCFH_2$ for the anatomical localization of intracellular ROS production is also well-established. This probe is oxidized by a wide range of oxidants, mainly $^{\bullet}OH$, $CO_3^{\bullet-}$, $NO_2^{\bullet}$, and thyl radicals (such as $GS^{\bullet}$). The ability of ROS production to induce oxidative stress depends on the balance between cellular pro-oxidants and antioxidants, with an imbalance between the two resulting in oxidative damage. Thus, studies of ROS release using probes such as $DCFH_2$ only determine the levels of pro-oxidant species but do not indicate the degree of oxidative stress.

Images showed that rehydration is accompanied by ROS and NO generation and thus confirmed the results of Weissman *et al.* (2005). The quantification of NO end-products showed that released NO reaches a maximum 1–2 h post-rehydration. Our microscopy studies revealed that the production of ROS and NO is closely related to lichen morphology: ROS was mainly associated with the hyphae of the cortex whereas NO was clearly localized to the medullar hyphae of the mycobiont. However, this pattern may vary with the lichen morphology; Picotto *et al.* (2009) reported than in foliose lichens, such as, *Flavoparmelia caperata, Parmotrema perlatum* or *Parmelia quercina*, NO release was mainly produced between the upper-cortex and the algal layer. Confocal microscopy confirmed that the medulla is free of intracellular ROS, which were seen only in a few punctate zones around several large photobionts (Fig. 5.2 C). Since ROS are now recognized as key signaling molecules in yeast and in plants (Hérouart *et al.* 2002, Dominy 2008, Neill *et al.* 2008, Herrero *et al.* 2008), these areas could constitute points of

communication between the fungus and algae and are perhaps related to the mutual up-regulation of protective systems, as suggested by Kranner *et al.* (2005). Further investigations are needed to clarify this point.

NO scavenging during lichen rehydration resulted in increased ROS production. This finding suggest that NO is involved in antioxidant defences. In plants and in animals, NO is known to modulate the toxic potential of ROS and to limit lipid peroxidation, acting as a chain-breaking antioxidant to scavenge peroxyl radicals (Kröncke *et al.* 1997, Darley-Usmar *et al.* 2000, Miranda *et al.* 2000).

The incidence of the confocal laser on the algae of NO-deprived rehydrating thalli caused a rapid photo-oxidative burst. Furthermore, isolated photobionts showed evidence of oxidative destruction of chlorophyll even in the absence of the photo-stress caused by a confocal laser. NO has been shown to ameliorate ROS toxicity in the chlorophycean alga *Scenedesmus obliquus*, probably by preventing the photo-inhibition that leads to photo-oxidation and pigment bleaching (Mallick *et al.* 2002). Our studies on the physiology of photosynthesis show that the inhibition of NO action by 200 μM c-PTIO altered the photosynthetic activity of the photobionts. Recently, it has been observed that *T. erici* is able to produce NO in the low-nanogram range (Catalá, personal communication). These results suggest that NO is involved in PSII stabilization during algal rehydration. Several authors have demonstrated that in higher plants NO reversibly binds to PSII (Petrouleas and Diner. 1990, Diner and Petrouleas. 1990, Goussias *et al.* 1995, Sanakis *et al.* 1997, Sanakis *et al.* 1999) and modulates electron transfer and quenching processes (Wodala *et al.* 2008). The fact that the same dose of c-PTIO than that used for photobionts, did not alter photosynthetic activity in the photobionts of intact lichens suggests that the mycobiont is involved in stabilizing the photobiont's chlorophyll. Assays with higher doses of c-PTIO and specific inhibitors of fungal NO synthases are needed to confirm this possibility.

These data provide evidences of an important role for NO in antioxidant systems during the early stages of rehydration in the lichen *Ramalina farinacea,* including chlorophyll photostability of the photobionts of the genus *Trebouxia.* Our results also raise important questions about the evolutionary role of NO in the establishment of lichen symbiosis, due to its dual role as antioxidant and mediator in cell communication.

Future research should be directed at functional (NO donors, NO synthase inhibitors, exposure to SO_2, $Cu^{+/2+}$, etc.), ultrastructural (sites of NO synthesis, immunohistochemistry), and cell communication (co-culture of isolated symbionts, NO donors, c-PTIO) studies of NO, with the aim of clarifying the role of this multifaceted molecule.

5.5 Resumen

Introducción

Con la formación de la simbiosis liquénica tanto fotobionte como micobionte son capaces de colonizar hábitats extremos que por sí solos no podrían. La cuestión de si la tolerancia a los estreses extremos de los líquenes es simplemente la suma de la de ambos simbiontes, o si se ve incrementada por el proceso de la liquenización, todavía sigue sin respuesta. Algunos estudios sugieren que los fotobiontes aislados son menos tolerantes al estrés lumínico e hídrico que las formas liquenizadas (Brock 1975; Lange *et al.* 1990; Kranner *et al.* 2005; Kosugi *et al.* 2009), sin embargo, todavía no se conoce cual es la interacción bioquímica entre el hongo y el alga que sería responsable del aumento de la tolerancia al estrés.

Una de las posibles moléculas que podrían estar implicadas en la interacción bioquímica entre los simbiontes liquenicos es el óxido nítrico (ON). Weissman *et al.* (2005) observaron que durante la rehidratación del liquen *Ramalina lacera* se producía un gran estrés oxidativo y a la vez la liberación de grandes cantidades de ON por el micobionte. El gas bioctivo ON tiene múltiples funciones biológicas en un amplio rango de organismos. Estas funciones incluyen, entre otras, transducción de señales, señalización celular, muerte celular, transporte, metabolismo básico y producción y degradación de especies reactivas de oxígeno (EROs) (Courtois *et al.* 2008, Palmieri *et al.* 2008; Wilson *et al.* 2008). Es bien conocido que el ON puede producir efectos tanto oxidantes como antioxidantes dependiendo del estato redox ambiental, la presencia de otros reactivos, y de la naturaleza de la reacción (Darley-Usmar *et al.*, 2000). Se ha demostrado que el ON se encuentra implicado en la respuesta al estrés abiótico en algas, tales como *Chlorella pyrenoidosa* Pringsheim, en la cual reduce el daño producido por el estrés fotooxidativo (Chen *et al.* 2003).

Como hemos visto en los capítulos 3 y 4, la tolerancia a la desecación del fotobionte *T. erici* es alcanzada por un complejo sistema de mecanismos constitutivos e inducidos. Sin embargo, en la relación simbióntica la tolerancia a la desecación podría ser además incrementada por otros mecanismos, como el ON liberado por el micobionte. En el

presente estudio, nosotros investigamos la producción y el papel antioxidante del ON durante la rehidratación de líquenes y de fotobiontes aislados.

Materiales y métodos

Los talos *Ramalina farinacea* (L.) fueron recolectados sobre *Quercus rotundifolia* Lam. en la Sierra de El Toro (Castellón, Spain; 39º54'16"N, 0º48'22"W). Un cultivo axénico del fotobionte liquénicos *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) fue utilizado en nuestro estudio y cultivado sobre discos de acetato de celulosa como se describe en el capítulo 2.

Para la detección de EROs fue utilizada la sonda 2,7-Diclorodihidrofluoresceina diacetato ($DCFH_2$-DA). Esta molécula, que en estado reducido no es fluorescente, cuando es oxidada por la acción de los radicales libres celulares se convierte en la fluorescente diclorofluoresceina (DCF, λ_{exc} = 504 nm, λ_{em} = 524 nm). El 2,3-diaminonaftaleno (DAN) reacciona con el cation nitrosonio que se forma espontáneamente del ON para dar el producto fluorescente 1H-naftotriazol (λ_{exc} = 375 nm, λ_{em} = 425 nm). Para la observación de estas sondas fluorescentes se utilizó un microscopio de barrido con laser (TCS Leica SP Confocal Laser Scanner Microscope) y un microscopio de fluorescencia (OLYMPUS Provis AX 70 fluorescence microscope).

El c-PTIO es un radical estable y soluble en agua que reacciona estoiquiometricamente con el ON e inhibe su función fisiológica. La estimación de la generación de ON se realizó cuantificando con un autoanalizador Skalar (modelo SAN++) los productos finales de la oxidación del ON (nitratos y nitritos) en la fracción soluble de las muestras.

Discos de algas crecidos durante 21 días fueron separadas del medio de cultivo y deshidratadas poniéndolas en un recipiente cerrado con una solución saturada de amonio de nitrato (h.r. 66 %). Las muestras permanecieron secas durante 24 h y después fueron rehidratadas con agua destilada o c-PTIO 200 μM. A continuación fueron devueltas al medio de cultivo y mantenidas en condiciones de cultivo durante 24 h. Los talos de *R. farinacea* fueron rehidratados sumergiéndolos en agua destilada o en c-PTIO 200 μM. Los talos se mantuvieron durante 24 h a 30 μmol m^{-2} s^{-1} (fotoperíodo 12/12) y 17 ºC.

La fluorescencia de la clorofila *a* fue medida *in vivo* con un fluorímetro de luz modulada PAM-2000. Las cinéticas de inducción de la fluorescencia fueron llevadas a cabo como se describe en el capítulo 2.

Resultados y discusión

Las imágenes de microscopía mostraron que la rehidratación de los talos de *R. farinacea* iba acompañada de la generación de EROs y ON, confirmando los resultados obtenidos por Weissman *et al.* (2005). La cuantificación de los productos finales del ON mostró que la liberación del ON alcanzó un máximo alrededor de 1-2 h después de la rehidratación. Nuestros análisis de microscopía revelaron que la producción de EROs y ON va íntimamente ligada a la morfología del liquen: las EROs se encontraron principalmente relacionada con las hifas del córtex, mientras que el ON se localizó claramente en las hifas de la médula. Este patrón puede variar con la morfología del liquen; Picotto (2009) observó que en los líquenes foliosos, tales como, *Flavoparmelia caperata*, *Parmotrema perlatum* o *Parmelia quercina*, el ON liberado fue principalmente localizado entre el cortex superior y la capa algal. La microscopía confocal confirmó que en *R. farinacea* la médula se encuentra libre de EROs intracelulares, y solamente se observaron en zonas puntuales alrededor de algunos fotobiontes de gran tamaño. Las EROs están actualmente reconocidas como esenciales en la comunicación molecular en levaduras y plantas (Hérouart *et al.* 2002, Dominy 2008, Neill *et al.* 2008, Herrero *et al.* 2008), por lo tanto, estas zonas podrían constituir puntos de comunicación entre el alga y el hongo , y podrían estar relacionadas con la mutuo incremento de los sistemas protectivos como sugieren Kranner *et al.* (2005).

El secuestro del ON durante la rehidratación de los líquenes produjo un incremento en la producción de las EROs. Se ha demostrado que el ON alivia la toxicidad de las EROs en el alga clorofícea *Scenedesmus obliquus*, probablemente previniendo la fotoinhibición (Mallick *et al.* 2002). Nuestros estudios sobre la fotosíntesis mostraron que la inhibición de la acción del ON con 200 μM c-PTIO disminuyó la capacidad de recuperación de la actividad fotosintética tras la desecación. Estos resultados sugieren que el ON está implicado en la estabilización del fotosistema II durante la rehidratación del fotobiontes. Diversos autores han demostrado que en las plantas superiores el ON se une

reversiblemente al PSII (Petrouleas and Diner. 1990, Diner and Petrouleas. 1990, Goussias *et al.* 1995, Sanakis *et al.* 1997, Sanakis *et al.* 1999) y modula la transferencia de electrones y los procesos de disipación de energía (Wodala *et al.* 2008).

Todos estos resultados sugieren que el ON ejerce un importante papel en el sistema antioxidante durante las primeras fases de la rehidratación en el liquen *R. farinacea*, incluyendo la protección de las clorofilas de los fotobiontes del género *Trebouxia*. De estos resultados también surgen nuevas cuestiones acerca del papel evolutivo del ON en el establecimiento de la simbiosis liquénica, debido a su doble función como oxidante y como mediador en la comunicación celular.

6. DESICCATION TOLERANCE OF LICHEN PHOTOBIONTS IS AGE-DEPENDENT

Abstract

Lichens are symbiotic poikilohydric organisms, able to survive in a desiccated state. Both symbionts, mycobiont and photobiont, are desiccation tolerant, but is usually supposed that lichenization increases the endurance of symbiotic partners under desiccation. In many lichens, recovery of photosynthesis after desiccation is not homogeneous and is age-dependent along the thallus, however this relationship has not been yet analyzed in free photobionts. The objective of this work was to study whether ageing can influence the recovery of isolated photobionts. Cells of the photobiont *Trebouxia erici* (UTEX 911) were inoculated in the centre of acetate-cellulose discs placed on agar Trebouxia Medium. After 21 d of culture algal cells were subjected to fast (60 min) or slow (5 - 6 h) desiccation treatments and after rehydration spatial and temporal changes of photosynthesis were recorded with an imaging-PAM fluorimeter. The capacity to recover photosynthesis activity after drying was higher at the margins of the colonies than in the centres. The Tetrazolium Test showed that metabolic activity was also greater at marginal areas. Microscopy analysis revealed larger cell sizes in the centre than in margins of the discs, while the number of cells in division was scarce in the central area but high in the margins. These results indicate that most of the cells at the centre of the discs had become senescent whereas cells in the marginal areas were young and maintained a high growth rate. In conclusion, the capacity of photobionts to recover photosynthetic activity after desiccation lowers with cell ageing. Culture ageing might be a partial explanation to the lower capacity to recover after desiccation of the isolated photobiont with respect to the isolated ones.

6.1. Introduction

Lichens are associations between fungi (mycobionts) and certain groups of cyanobacteria or unicellular green algae (photobionts). This symbiotic association forms poikilohydric organisms, most of which are capable of surviving in a desiccated state over long periods. Both symbionts are known to be desiccation tolerant; however, some controversial results of experiments with isolated photobionts suggest that desiccation tolerance of photobionts is increased by symbiosis. Brock (1975) reported that photosynthesis stopped earlier in the isolated alga than in the lichen. Lange *et al.* (1990) also observed the same effect but the difference was quite small and concluded that the mycobiont did not increase desiccation tolerance of the photobiont. Kranner *et al.* (2005) found that the protective mechanisms in the lichen were more effective, by orders of magnitude, than those of its isolated photobiont and mycobiont. Kosugi *et al.* (2009) concluded that symbiosis increases desiccation tolerance of the photobionts by increasing resistance to photoinhibition in the dry state. These conclusions may lead to the mistaken belief that normal cell activity is always fully recovered in lichen thallus. However, in many lichens, recovery of photosynthesis is not homogeneous and seems to be age-dependent along the thallus. Fruticose lichens, such as *Cladina stellaris, Cladonia rangiferina* and *Cetraria cucullata*, have the highest photosynthetic rates in the tips of the branch, where the youngest tissues occur, and decline along the thallus with ageing (Moser & Nash 1978; Lechowicz 1983; Moser *et al.* 1983). Barták *et al.* (2000) used an imaging fluorometre to analyse the photosynthetic activity of three foliose lichens with different growing patterns. The highest photosynthetic rates corresponded with the highest metabolism and growing areas: marginal thallus parts in *Hypogymia physodes*, central part and close-umbilicus spots in *Lasallia pustulata*, and irregularly-distributed zones within the thallus in *Umbilicaria hirsuta*.

In placodioid, foliose and fruticose lichen thalli, which grow in length at lobe or branch tips, two different phases of the photobiont cell cycle have been distinguished: Phase 1, the cells grow till a certain size and divide actively into endospores, which mainly occurs at the growing margins and tips of the thalli; Phase 2, the cells enlarge becoming senescent and dying, it happens more frequently in the mature regions (Hill 1981; Hill

1989; Amstrong & Smith 1998). Thus, there are few changes in living photobiont cell number per unit area, or in the thickness of the algal layer, in the older thallus regions (Hill 1992; Schofield *et al.* 2003).

In a broad sense, aging of plants refers to the process of slow, progressive and sequential alterations that a plant or a part of a plant undergoes during its development until death, which is characterized by a decline in physiological processes and vigour (Partridge & Barton 1993; Bond 2000). Rates of photosynthesis vary with foliage age; this has been known for a long time (Freeland 1952) and demonstrated in a wide variety of species (Jurik 1986; Warren & Adams 2001). Generally, rates of photosynthesis increase as expansion proceeds, approximately reach a maximum when expansion ceases, and decline thereafter. Likewise, their capacity to resist abiotic or biotic stresses also declines with age, thereby increasing the plant's likelihood of dying (Coleman 1986; Stark *et al.* 2004; Ohe *et al.* 2005).

These changes in photosynthesis during ageing also occur in single cells, such as cultures of plant cells or unicellular algal cultures (Winokur 1949; Peters *et al.* 2000). During the first few days of culture, photosynthetic activity increases and then declines with increasing age of the culture.

The aim of this work is to study ageing of photobiont cultures. As a second objective we investigate whether aging can contribute to the lower desiccation tolerance observed in isolated photobionts as compared with lichenized ones. We analyze the changes in photosynthetic activity and in the response to two hydric water-stress intensities as the photobiont *T. erici* ages. Spatial and temporal changes of photosynthesis in colonies growing on agar medium were recorded with an imaging-PAM fluorimeter. Microscopic analyses were carried out to determine the cell cycle phase in the areas demonstrating different photosynthetic activity. We also discuss the correlation between the results obtained in the isolated photobiont and the growth and photosynthetic patterns observed in lichens.

6.2. Material and methods

6.2.1. *Biological material*

An axenic strain of the lichen photobiont *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) was cultured on sterile cellulose-acetate discs as is described in the Chapter 2 (Section 2.2.2.).

Thalli of *Flavoparmelia caperata* and *Parmotrema perlatum* were collected in Sierra de Espadán (Castellón, Spain, 39º54'16"N, 0º48'22"W) and in Colunga (Asturias, Spain, 3°28'45" N, 5°13'39"W), respectively. The material was left to dry out in open air, in the shade, for one day and stored at -20ºC. Previously to experimentation, lichen thalli were immersed in distilled water for 5 min, shacked and maintained into Petri plates at 20ºC under a 12 h photoperiod of 30 μmol m^{-2} s^{-1} white light for 24 h.

6.2.2. *Desiccation and rehydration treatments*

The analysis of the photosynthesis recovery was carried out with 21 days *T. erici* cultures. Slow drying (SD) was achieved by placing algal disks into a closed container with a saturated solution of ammonium nitrate (R.H. 66%), and maintained under culturing conditions. Rapid dehydration (RD) was achieved in the same conditions but changing the ammonium nitrate by silicagel (R.H. 20%). The algae remained dried during 24 h and then rehydrated with distilled water. Samples were returned to the agar culture media and maintained in culture conditions until fluorescence measurements.

6.2.3. Fluorescence measurements

An Imaging-PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany) was used to investigate the spatial-temporal heterogeneity of Chl *a* fluorescence parameters in both cultures of *T. erici* and in lichen thalli.

All lights were placed in a ring arrangement and directed at a fixed angle and distance onto the disc area. Two outer LED-rings (a total of 96 LEDs) provided the

measuring light, actinic light and saturating pulse with a peak wavelength at 470 nm. The inner LEDring (total of 16 LEDs) provided the pulse-modulated light for assessment of PAR-absorptivity at 650 nm and a 780 nm LED. The charge-coupled device (CCD) camera has a resolution of 640 × 480 pixel. Pixel value images of the fluorescence parameters were displayed with help of a false colour code ranging from black (0.000) through red, yellow, green, blue to pink (ending at 1.000) (Berger *et al.* 2004). All measurements were carried out with maximal distance between camera and algal discs (26 × 34 mm area). Samples were dark adapted for 30 min prior to measurement. The minimum (dark) fluorescence F_o was obtained by applying measuring light pulses at low frequency (1 Hz). The maximum fluorescence F_m was determined by applying a saturating blue pulse (10 Hz). The maximum fluorescence F_m was determined by applying a saturating blue pulse (10 Hz) (see Calatayud et al. 2006). The maximum quantum yield of PSII photochemistry (F_v/F_m), was determinate as $F_m - F_o/F_m$. Then, actinic illumination (154 μmol photons m–2 s–1) was switched on and saturating pulses were applied at 60 s intervals for 10 min in order to determine the maximum fluorescence yield during the actinic illumination (F'_m), and the chlorophyll fluorescence yield during the actinic illumination (F_s). For each interval, saturation pulse images and values of various Chl fluorescence parameters were captured. The quantum efficiency of PSII photochemistry, Φ_{PSII}, was calculated according to Genty et al. (1989) by the formula: $(F'_m - F_s)/F'_m$. The phochemical quenching (q_P) is non-linearly related to the fraction of open PSII reaction centres and was calculated as $(F'_m - F_s)/(F'_m - F'_o)$ (Schreiber et al. 1998). To determine F'_o correctly, it is necessary to switch off the actinic light and quickly reoxidise the PSII acceptor side with the help of far-red light, but this is not feasible with Imaging-PAM as far-red light would penetrate the CCD-detector and cause serious disturbances to fluorescence images (see http://www.walz.com). The value of F'_o was estimated using the approximation of Oxborough and Baker (1997), $F'_o = F_o/(F_v/F_m + F_o/F'_m)$. Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, according to the equation NPQ= (Fm-F'm)/F'm (Bilger & Bjorkman 1990).

A good homogeneity of intensity of excited light was obtained for the whole disc area. Chl *a* fluorescence determinations for the study of the dehydration in the *T. erici* cultures were obtained from five discs. Eight areas of interest (AOI) were selected, four in

the central part of the disc and four in the outer zones (Fig. 6.1) in order to evaluate spatial heterogeneity.

6.2.4. Tetrazolium test

Discs of *T. erici*, which had been slowly or rapidly dried and desiccated for 24 h, were immersed in 0.6 % 2,3,5-triphenyltetrazolium chloride (TTC) in 0.05M phosphate buffer (pH 6.8) containing 0.005% Triton X 100. Samples were incubated in the dark for 18-20 h (Backor & Fahselt 2005).

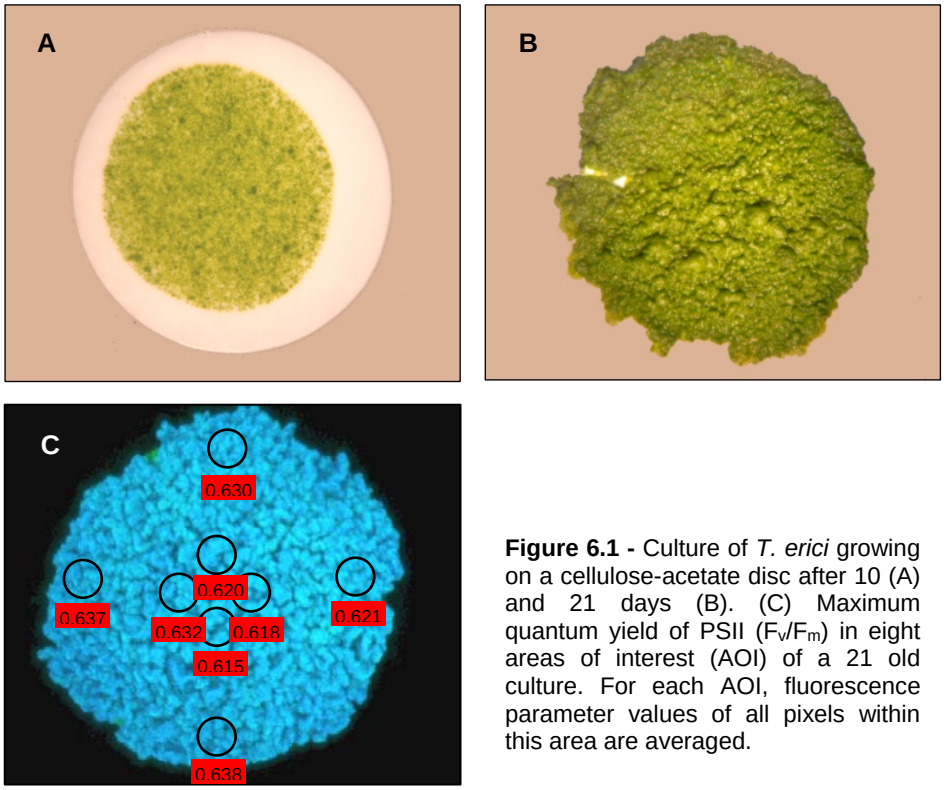

Figure 6.1 - Culture of *T. erici* growing on a cellulose-acetate disc after 10 (A) and 21 days (B). (C) Maximum quantum yield of PSII (F_v/F_m) in eight areas of interest (AOI) of a 21 old culture. For each AOI, fluorescence parameter values of all pixels within this area are averaged.

6.2.5. Microscopical and image measurements

Lichen thalli and algal discs were fixed with FAA (formyl acetic alcohol). After washing with a 0.1 M phosphate buffer (pH 7.4), they were dehydrated by means of an ethanol series and embedded in LR-White medium grade acrylic resin (London Resin Co.). The infiltration was carried out overnight at a temperature of 60 ºC. Slicing of the resulting blocks was done with a Sorvall MT 5000 ultramicrotome (Knifemaker, Reichert-Jung) provided with special glasscutters (45º) (Leica 6.4 mm Glass Strips). This microtome allowed the obtention of semi-thin sections (1.5 μm). Samples were stained with toluidine blue (1%). Sections were observed and photographed with an Olympus Provis AX 70 brightfield microscope fitted with an Olympus Camedia C-2000 Z camera. Eight images were randomly taken from sections of the margin and the centre of the algal discs. An image was also made of a stage micrometer for size calibration. In order to determine spatial differences in the relative diameter of the cells the digital images were analysed by ImageJ software (http://rsb.info.nih.gov/ij/). The number of dividing cells per image was also assessed. Dividing cells can be distinguished from nondividing cells because they form distinctive clusters of 4, 8, 16 or 32 cells.

6.2.6. Statistical analysis

Prior to calculate means and standard error of the data obtained, we checked normal probability plot, (Statgraphics Plus® (4.11) for windows) according to Lazár and Naus (1998). When the data adjusted to a normal distribution, analysis of variance (ANOVA) was performed, while, not normal distributed data were analyzed by the Mann Whitney test.

Table 6.1 - Chlorophyll *a* fluorescence parameters in 21 days control samples after steady-state kinetics. F_v/F_m, maximal quantum efficiency; Φ_{PSII}, effective quantum yield of photochemical conversion in PSII; q_P, photochemical quenching; NPQ, quantum yield of regulated energy dissipation in PSII. The four values of the margin and the centre AOI were averaged for each sample. Values are means of 5 samples. For comparison of means, ANOVA followed by LSD test, calculated at 95% confidence level, were performed. Values followed by the same letter indicate no significant differences.

	F_v/F_m	Φ_{PSII}	NPQ	q_P
Margin	0.632 a	0.525 a	0.364 a	0.942 a
Centre	0.621 a	0.507 b	0.349 a	0.927 a

6.3. Results

6.3.1. *Chlorophyll* a *fluorescence measurements in T. erici*

No significant differences were observed in chlorophyll fluorescence parameters, like F_v/F_m, q_P and NPQ. Only the effective quantum yield of photochemical energy conversion in PSII (Φ_{PSII}) was significantly higher at the margin than in the centre ($P<0.05$) (Table 6.1).

However, the response to a desiccation/rehydration cycle was heterogeneous in the algal colony and dependent of the drying rate and the position in the disc (Fig. 6.2A); recovery of photosynthesis was lower after a rapid drying than after a slow drying, and higher in the marginal areas of the disc that in the centre. Photosynthetic activity of the marginal cells remained almost unaltered after slow drying and similar to the non-dehydrated samples; while in the centre of the disc, photosynthesis did not recover after rehydration and even decreased a 65 % during the first 24 h after rehydration, and then started to recover (Fig. 6.2B). After rapid dehydration, the photosynthetic activity dropped quickly and reached its minimum during the first eight hours after rehydration, 9 % and 30 % of the initial F_v/F_m in the centre and at the margin, respectively (Fig. 6.2B). F_v/F_m reached zero in some areas of the centre, indicating that some cells were unable to recover from the severe stress caused by the rapid rehydration and died (Fig. 6.2A, 24h). However, the marginal cells were more resistant and most of them started to recover their photosynthetic activity after 48 h (Fig. 6.2B).

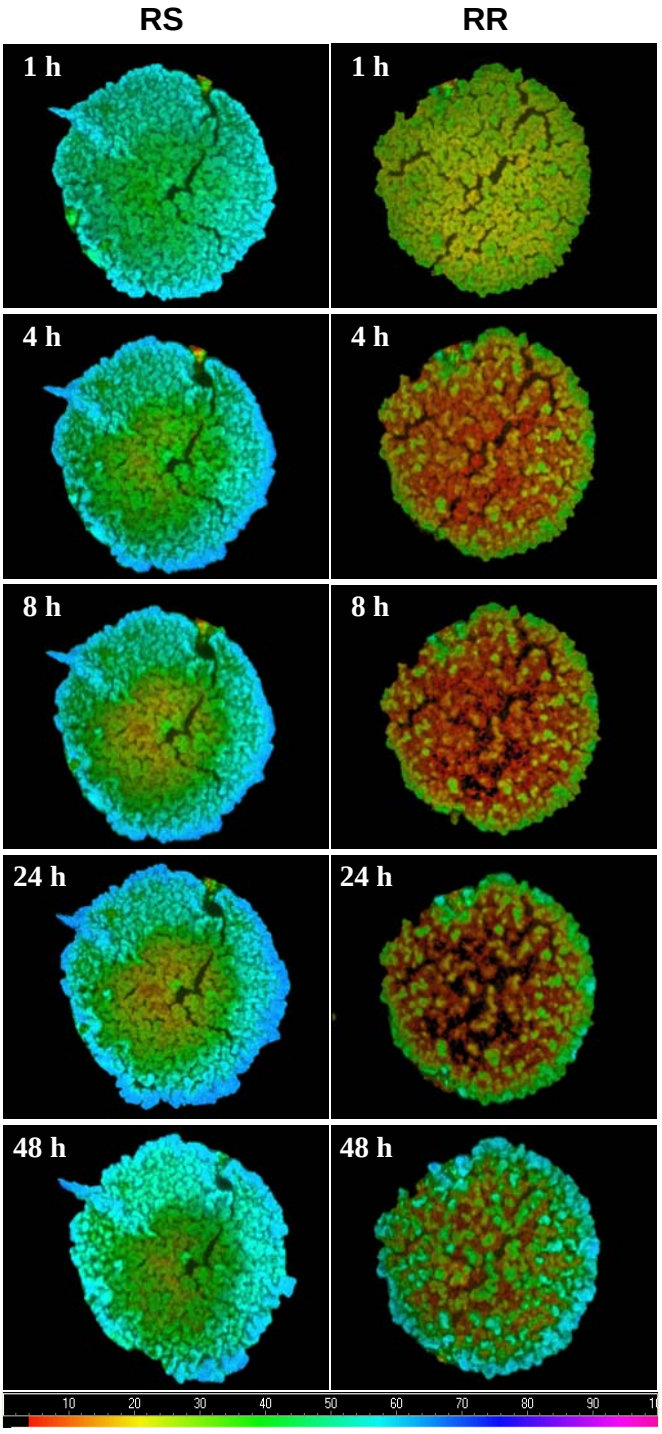

Figure 6.2A - Recovery of maximum quantum yield (F_v/F_m) after slow drying (RS) and rapid drying (RR). Culture discs of *T. erici* desiccated for 24 h were rehydrated and fluorescence measurements carried out after 1h, 4h, 8h, 24h and 48h. The colour scale in the bottom indicates the corresponding F_v/F_m value (x 10^{-2}).

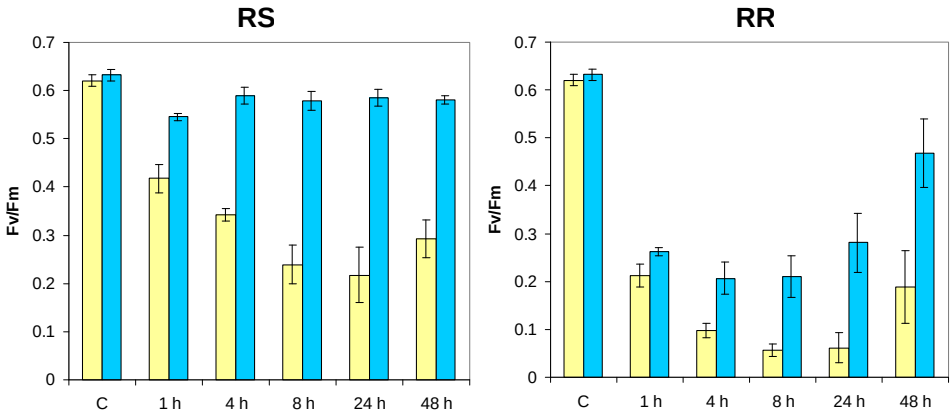

Figure 6.2B - Recovery of maximum quantum yield (F_v/F_m) after slow drying (RS) and rapid drying (RR). Culture discs of *T. erici* desiccated for 24 h were rehydrated and kept under culture conditions during the indicated times. Results are expressed as mean ± SD (n=5).

6.3.2. *Cell vitality*

An indicator of metabolic activity or vitality is the ability to reduce 2,3,5-triphenyltetrazolium chloride (TTC) to triphenyl formazan (TPF). The conversion from a colourless water soluble compound (TTC) to a water-insoluble red product (triphenyl

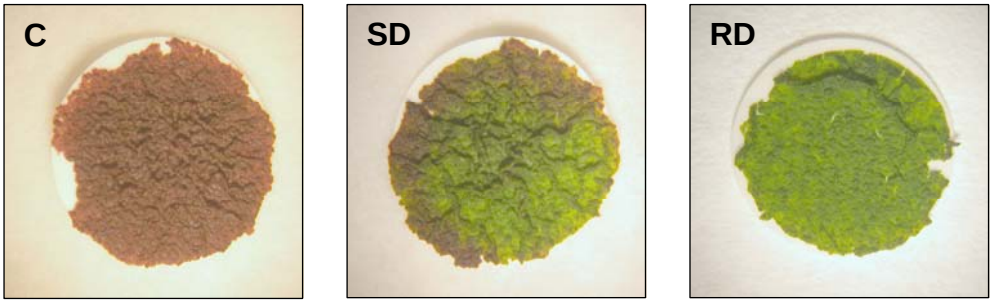

Figure 6.3 - Discs of *T. erici* were slowly (SD) and rapidly dryed (RD) incubated in 0.6% tetrazolium salt during 20 h. C, non-dehydrated samples.

formazan, TPF) is driven by dehydrogenases. TTC reduction has long been used as an

estimate of the viability of seeds and other plant tissues. The results obtained in *T. erici* culture showed the same pattern as for photosynthesis. Control cells reduced the tetrazolium salt very efficiently (Fig 6.3). After slow drying, only cells located at the margin of the disc produced a clear tetrazolium reduction (Fig. 6.3). Rapid dehydration caused greater damage in all areas of disc and TPF production was only barely visible (Fig. 6.3).

6.3.3. *Microscopic and image analysis*

During the first two weeks of culture, the *T. erici* colony grew inside the area where the algae were inoculated (Fig. 6.1A), and gradually occupied the whole acetate-cellulose disc. After 21 days, the colony grew beyond the disc limits (Fig. 6.1B). At the margin the cells spread freely and tightly to the disc, while overpopulation produced folds at the centre (Fig. 6.4). Photosynthetic activity was similar in both marginal and central areas (Fig. 6.1C).. Figure 4 shows the relative cell size in the central and marginal areas of a representative *T. erici* disc. The relative diameter was significantly higher in the centre, 4.50 μm, than in the marginal cells, 3.70 μm ($P<0.001$). Thirty five percent of cells in the centre had a diameter over 5 μm, while in the marginal area less than 20 % reached this size. Here it must be pointed out, that the data obtained from 1.5 μm sections did not fit to a normal distribution and were negatively biased, because the probability of cutting a cell at its maximum diameter is low and the apparent diameter observed is always lower than the real one. Cells in division were significantly more abundant at the disc margin (10.87 per image) than in the centre (3.00 per image) ($P=0.003$). These results indicate that most of the cells at the margin of the discs were in cell-cycle Phase 1, while those in the centre were in Phase 2.

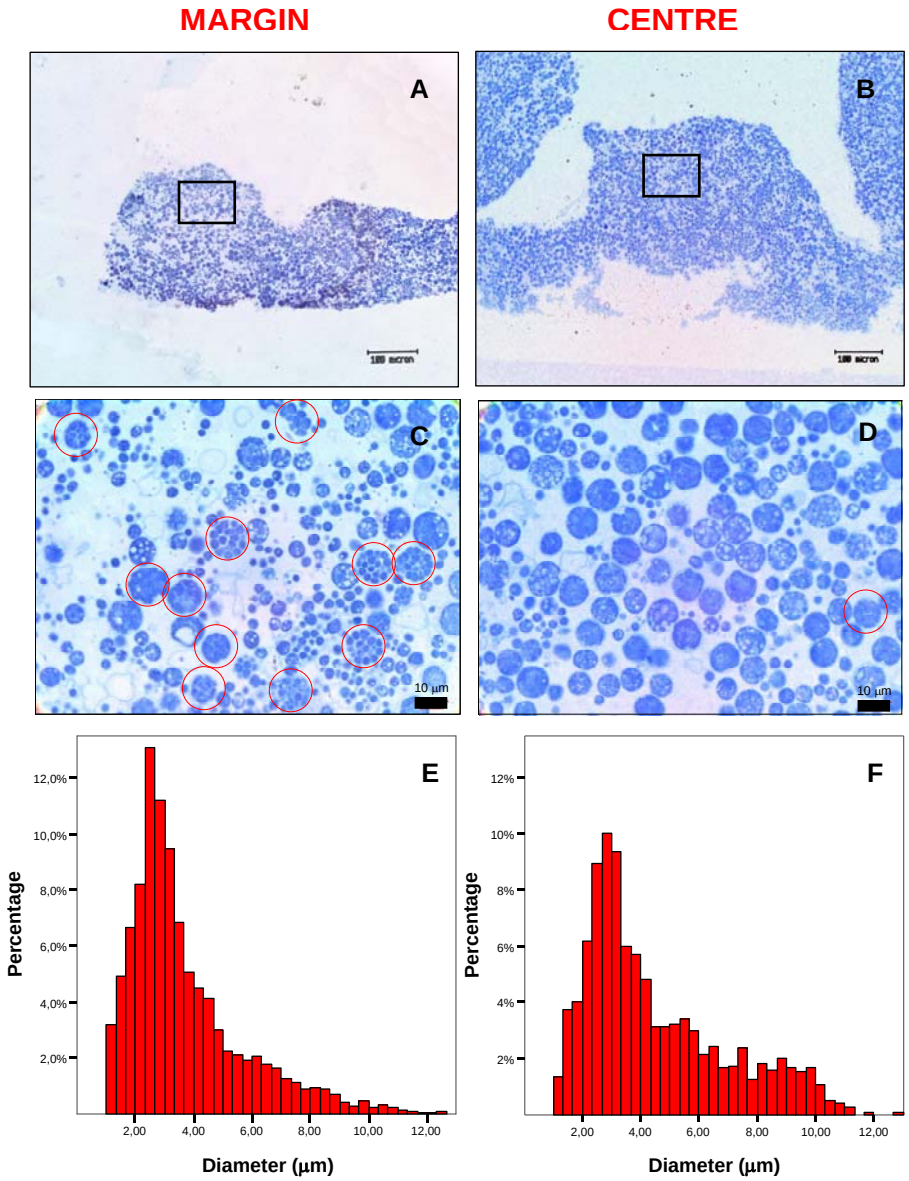

Figure 6.4 - Marginal (A, C) and central (B, D) semi-thin section images of a 21 d old *T. erici* disc. A and B, 100X image. C and D, 1000X image of the area indicated by a square in the 100X image; clusters of dividing cells designed by red circles. E and F, relative cell diameter assessed from eight 1000X images of the margin and central regions, respectively. E, n = 2134; F, n = 1462.

6.3.4. *Chlorophyll* a *fluorescence measurements and microscopic observations in lichens*

Flavoparmelia caperata revealed a heterogeneous photosynthetic pattern (Fig. 6.5 A, B). The maximum quantum yield was higher in the marginal area of the thallus and decreased gradually towards the centre. We observed dividing cells within the first 2 mm from the thallus margin, but none were detected in the older parts of the thallus (Fig 6.5 C,D). Conversely, *Parmotrema perlatum* displayed homogeneous photosynthetic activity along the thallus, no differences in F_v/F_m were observed in any area (Fig. 6.5 F). Likewise,

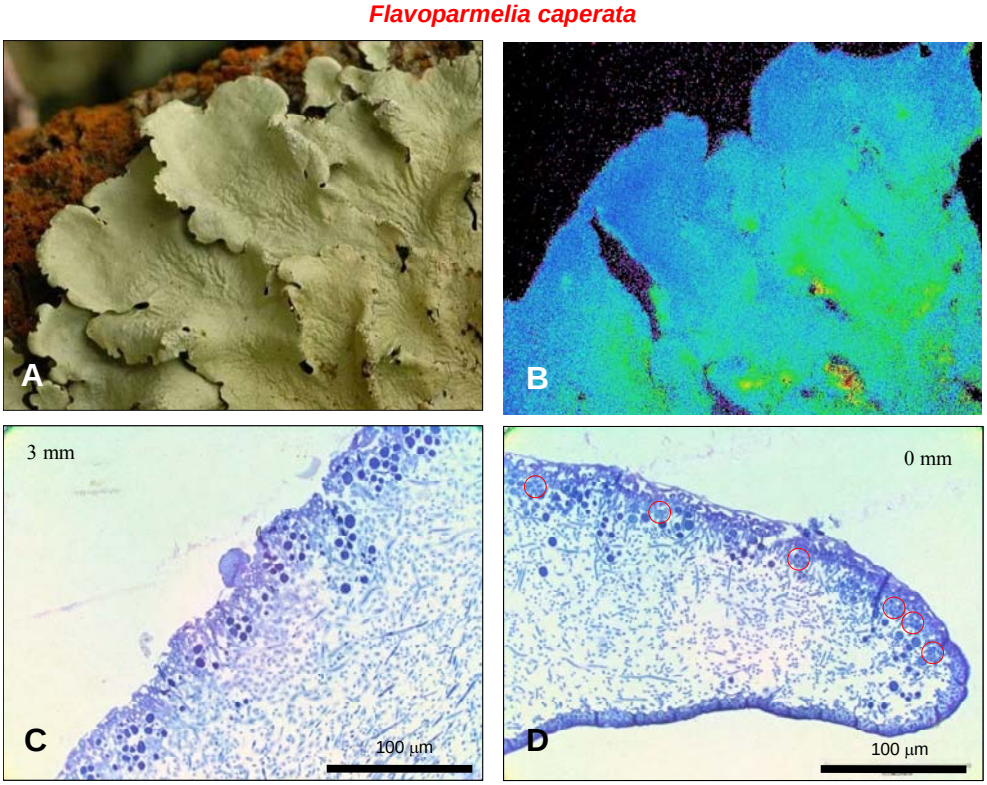

Figure 6.5 - A, images of *Flavoparmelia caperata* thallus. B, maximum quantum yield (F_v/F_m) 1 h after thalli rehydration. C, semi-thin section image of an area 3 mm distant from the thallus margin. D , semi-thin section image of the thallus margin; clusters of dividing cells designed by red circles.

we observed a homogeneous distribution of division cells along the thallus, algal cell clusters appeared both at the margin and in the centre of the thallus (Fig. 6.5 G,H).

6.4. Discussion

Animal cell cultures lose viability after continuous cell division. A major reason for this phenomenon is telomere shortening due to gradual inactivation of telomerase (Bodnar *et al.* 1998; Dumont *et al.* 2001). Consequently, in animal cell cultures, cell division may

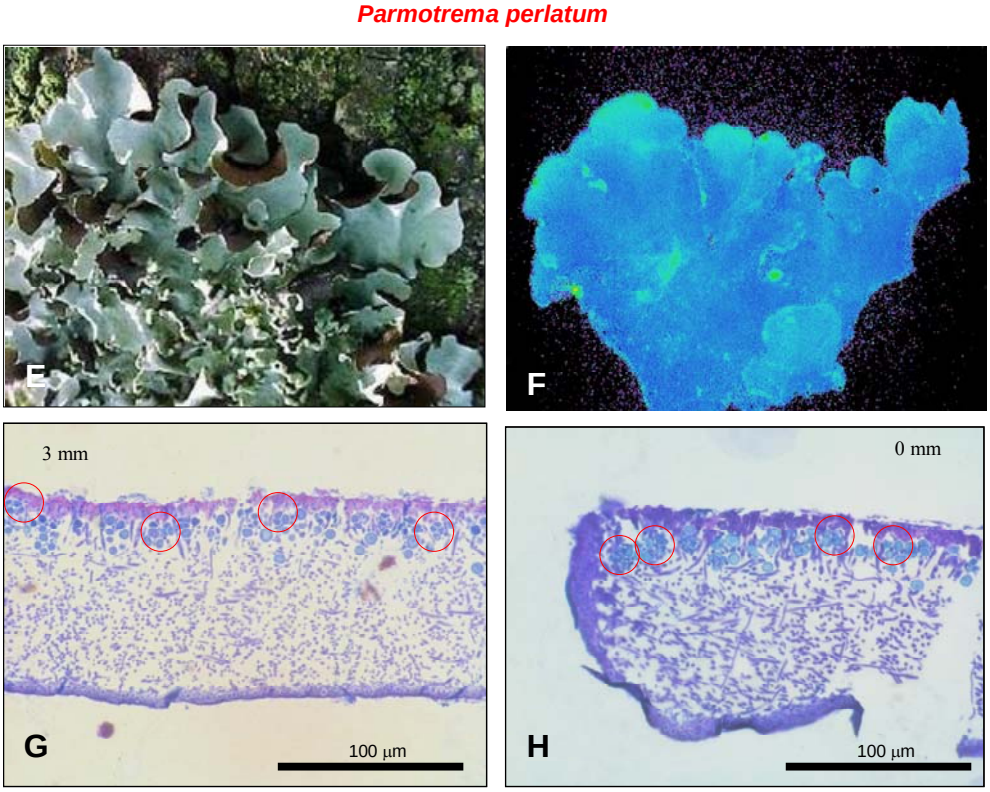

Figure 6.5 (continuation).- E, images of *Parmotrema perlatum* thallus. F, maximum quantum yield (F_v/F_m) 1 h after thalli rehydration. G, semi-thin section image of an area 3 mm distant from the thallus margin. H, semi-thin section image of the thallus margin; clusters of dividing cells designed by red circles.

promote ageing. By contrast suspension-cultured plant cells apparently maintain their viability upon division and can be maintained indefinitely. In contrast to animal cells, division of plant cells apparently does not depend on a constant telomere length (Shippen & McKnight 1998). Plant cells rejuvenate upon division. Differentiated plant cells are known to have the ability to dedifferentiate and resume cell division, which is the basic prerequisite for all methods of vegetative propagation. However, this capacity declines with the age of the explant. Cultures of the isolated photobiont *T. erici* grew on the discs placed on the agar medium forming apparently homogeneous circular colonies. At the margin of the colony most of the cells grew up to a certain size and then divided actively into aplanospores; however, in the centre, cells enlarged and the frequency of cell division was very low. Similar increase in cell size and loss of reproduction capacity with time was also observed in liquid cultures (chap. 2, section 2.3.1.). These results suggest that cell crowding at the centre of the discs induce aging of the culture. According to Hill (1989), the cells in the margin and in the centre correspond to Phase 1 and Phase 2 of the photobiont cell cycle, respectively. At the centre of the colony, cells accumulated forming folds (Fig. 6.4B), where the cells on the upper surface were separated from the culture medium. Thus, it is possible that cells at the top of these folds receive fewer nutrients causing the cells to enter into Phase 2 and age. An alternative, but not exclusive explanation, is that *T. erici* cells release a growth inhibitor, which could regulate cell accumulation. Autoinhibition of growth in old-age cultures of the unicellular alga *Chlorella vulgaris* is reported to be due to the release and accumulation of chlorellin, a mixture of unsaturated fatty acids peroxides (Scutt 1964; Ikawa 1984; Ikawa 1997).

Before drying, photosynthetic activity was similar in both marginal and central areas. No significant differences in chlorophyll fluorescence parameters, like F_v/F_m, q_P and NPQ, were observed. However, the effective quantum yield of photochemical energy conversion in PSII (Φ_{PSII}) was significantly higher at the margin than in the centre, indicating that the photosynthetic rate is lower in older cells. After drying and rehydration, the marginal areas of the colonies displayed a high capacity for photosynthetic and metabolic recovery, whereas recovery of the central areas after slow dehydration was poor and death occurred after rapid drying. The reason for this heterogeneity in desiccation tolerance may, again, be related to ageing of the cells. Ageing has been classically defined

as the accumulation of changes in plant development responsible for slow, progressive and sequential alterations that accompany the plants as they age (Harman 1981; Harman 1991). This does not automatically lead to death, but lowers their physiological activity and resistance against stress, thereby increasing the likelihood of death (Thompson 1988). The negative effect that ageing has on resistance to dehydration stress has also been reported in other desiccation-tolerant plants. In mosses, leaves lose their photosynthetic capacity and their ability to withstand desiccation as they age (Egunyomi 1979; Alpert 2000; Stark & Delgadillo 2003), with the older leaves normally having a higher net carbon loss (Callaghan *et al.* 1978), greater membrane damage after desiccation (Beckett 2001), lower regeneration capacity (Stark *et al.* 2004) and greater vulnerability to biotic stresses like fungal attack (Stark *et al.* 2004). In resurrection plants, like the angiosperms *Sporobolus stapfianus* and *Eragrostis nindensis*, older leaves are desiccation-sensitive whereas younger leaves are desiccation-tolerant (Vander Willigen *et al.* 2003; Martinelli *et al.* 2007). Both lichen species studied in our work, *Flavoparmelia caperata* and *Parmotrema perlatum*, showed the same relationship between photosynthetic recovery after rehydration and ageing. In the lichen F. caperata, showed an heterogeneous photosynthetic pattern with the highest photosynthetic activity in the margins of the thallus, where were the youngest algal cells. On the contrary, in P. perlatum division cells were founded spread along the thallus which was correlated with a homogeneus photosynthetic pattern. Thus, like in other lichens, the highest photosynthetic rates were observed in the thallus areas with division cells and young cells (Moser & Nash 1978; Lechowicz 1983; Moser *et al.* 1983; Barták *et al.* 2000; Schofield *et al.* 2003). Likewise, the older regions of thalli are less resistant to other types of stress, such as high irradiations (Barták *et al.* 2004).

Physical factors may also contribute to the different desiccation-resistance between young and old cells. Mechanical damage associated with loss of turgor is proposed to be one of the major causes of irreversible desiccation-induced damage in plants. A relatively large surface area-to-volume ratio is suggested to be important to the mechanical stabilization of dehydrated cells, smaller cells are more resistant to collapse (cytorrhysis) under water stress (Iljin 1957; Cutler *et al.* 1977). Angiosperm desiccation-tolerant plants use two different approaches to minimize this mechanical stress: water replacement with vacuoles containing non-aqueous contents or a reduction in cellular volume (Gaff 1971;

Farrant 2000). Agrawal and Signh (1999) also reported that the akinete of *Pithophora oedogonia* were more sensitive to water stress than the zoospores of *Cladophora glomerata* probably due to their large size. Therefore, the smaller cells observed at the margin of the *T. erici* colonies may be mechanically more stable than the larger ones in the centre in desiccated state.

In conclusion, the capacity of photobionts to recover photosynthetic activity after desiccation is inversely related to cell ageing, suggesting that desiccation resistance is age-dependent. This relationship may be a partial explanation of the results discussed in Chapters 3 and 5; *T. erici* cultures did not recover their full photosynthetic activity after dehydration and rehydration because of ageing. Culture ageing might also explain the controversial observations of Kosugi *et al.* (2009) and Kranner *et al.* (2005), who found that cultures of isolated photobionts partially lost their capacity to recover after desiccation, while lichen photobionts were not affected by desiccation. The age of the photobiont culture is not considered in the study by Kosugi *et al.* (2009), but Kranner *et al.* (2005) carried out experiments with two-month-old cultures, hence a fraction of the cells would have aged, which might interfere with the results and lead to an underestimation of the recovery capacity of the isolated photobiont.

6.5. Resumen

Introducción

La mayoría de los líquenes son tolerantes a la desecación, y como es lógico, ambos simbiontes también lo son. Sin embargo, algunos resultados obtenidos en experimentos con fotobiontes aislados son controvertidos y sugieren que la tolerancia ala desecación se incrementa con la simbiosis. Brock (1975) observó que, durante la deshidratación, la fotosíntesis se detenía con un contenido hídrico mayor en el fotobionte aislado que en el liquenizado. Lange *et al.* (1990) también observan el mismo efecto pero con diferencias más pequeñas, por lo que concluyen que la simbiosis no incrementa la tolerancia a la desecación del alga. Kranner *et al.* (2005) encontraron que los mecanismos de protección son mucho más efectivos en el fotobionte liquenizado que en el aislado. Kosugi *et al.* (2009) concluyen también que la simbiosis incrementa la tolerancia a la desecación aumentando la resistencia a la fotoinhibición en el estado seco.

Todas estas conclusiones pueden conducirnos a la idea equivocada de que en los líquenes la actividad celular se recupera completamente tras la rehidratación. Sin embargo, en muchos líquenes, la recuperación de la fotosíntesis no es homogénea a lo largo del talo y parece ser dependiente de la edad. Las tasas fotosintéticas más altas corresponden con las áreas que tienen una actividad metabólica más alta y mayor tasa de crecimiento (Moser & Nash 1978; Lechowicz 1983; Moser *et al.* 1983; Barták *et al.* 2000). En los líquenes placoidales, foliosos y fruticulosos en los cuales el crecimiento se produce en el margen de los lóbulos o en la punta de las ramas se pueden distinguir fotobiontes en dos fases diferentes del ciclo celular: células en Fase 1, que se encuentran en los márgenes de los lóbulos y en las puntas de los talos y se caracterizan por ser células que crecen hasta un cierto tamaño y que después se dividen activamente; en la Fase 2, las células crecen más que las anteriores pero no se dividen, se vuelven senescentes y mueren, lo cual sucede en las partes más viejas del talo (Hill 1981; Hill 1989; Amstrong & Smith 1998).

El objetivo del presente capítulo es estudiar las diferentes fases del ciclo celular de *T. erici* y analizar si el envejecimiento celular puede estar relacionado con la capacidad de recuperación de los fotobiontes aislados.

Materiales y métodos

Un cultivo axénico del fotobionte liquénicos *Trebouxia erici* Ahmadjian (SAG 32.85 = UTEX 911) fue utilizado en nuestro estudio y cultivado en discos de acetato de celulosa como se describe en el capítulo 2. Los talos de *Flavoparmelia caperata* y *Parmotrema perlatum* fueron recolectados en la Sierra de Espadán (Castellón, España) y en Colunga (Asturias, España), respectivamente.

El análisis de la recuperación de la actividad fotosintética tras la desecación se llevo a cabo con discos de algas de 21 días. Las algas fueron deshidratadas lentamente (DL) poniéndolas en un recipiente cerrado con una solución de nitrato de amonio (h.r. 66 %), con lo que las algas quedan secas en alrededor de 6 h. Las deshidrataciones rápidas (DR) se consiguieron con silicagel (h.r. 20 %) y tardaron alrededor de una hora. Un fluorímetro Imaging-PAM fue utilizado para analizar los cambios espacio-temporales de los parámetros de la fluorescencia de la clorofila a tanto en talos de líquenes como en cultivos de *T. erici*.

Los discos de *T. erici* desecados fueron sumergidos en cloruro de 2,3,5-trifeniltetrazolio (CTT) al 0.6 % y se incubaron en oscuridad durante 18-20 h (Backor & Fahselt 2005).

Los talos liquénicos y los discos de algas fueron fijados con alcohol formil acético, deshidratados con series de etanol e infiltrados con resina acrílica de medio grado LR-White. Ocho imágenes fueron tomadas de secciones semifinas del margen y del centro de los talos y de los discos. Las imágenes fueron analizadas con ayuda del programa Image-J.

Resultados y discusión

Los cultivos del fotobionte *T. erici* crecieron sobre los discos puestos en medio con agar formando colonias circulares aparentemente homogéneas. Sin embargo, el análisis de las imágenes mostró que mientras en los márgenes del disco las células crecían hasta un cierto tamaño y después se dividían formando aplanosporas, en el centro las células alcanzaban mayores tamaños y la tasa de división era muy baja. Según la clasificación

propuesta por Hill (1989), las células en el margen estarían en Fase 1 y las del centro en Fase 2 del ciclo celular del fotobionte. Estos resultados sugieren que la superpoblación en el centro de los discos induce el envejecimiento de las algas. En el centro de la colonia, las células se acumulan formando pliegues, por lo que las células de la parte superior quedan muy separadas del medio de cultivo, así pues, es posible que estas células reciban menos nutrientes y se produzca su entrada en la Fase 2 y el envejecimiento celular. Una explicación alternativa, pero no exclusiva, es que las células de *T. erici* liberen algún tipo de inhibidor del crecimiento, el cual podría liberarse para regular la acumulación de células y evitar competición por los recursos. La autoinhibición del crecimiento ha sido observada en otras algas verdes, como en el caso de *Chlorella vulgaris*, la cual libera ácidos grasos insaturados para controlar su crecimiento poblacional (Scutt 1964; Ikawa *et al.* 1984, Ikawa *et al.* 1997).

En las algas hidratadas, la actividad fotosintética era homogénea en todo el disco de *T. erici*. Sin embargo, después de la desecación las células del margen del disco mostraron una mayor capacidad de recuperación de la actividad fotosintética y metabólica que las del centro, donde tras la deshidratación rápida muchas células incluso murieron. La razón para esta heterogeneidad de la tolerancia a la desecación parece estar, de nuevo, relacionada con el envejecimiento de las células. El envejecimiento no conduce direnctamente a la muerte, pero disminuye la actividad fisiológica y la resistencia frente al estrés incrementando la posibilidad de morir (Thompson 1988). Ambos líquenes utlizados en nuestro estudio mostraron también una clara relación entre el envejecimiento celular y la actividad fotosintética en el talo. En *Flavoparmelia caperata* las algas se dividen en los márgenes de los talos y envejecen hacia su interior, y también muestra un patrón fotosintético heterogéneo, teniendo una mayor actividad fotosintética en los márgenes. Mientras que en *Parmotrema perlatum* los fotobiontes se dividen activamente por todo el talo, lo que va correlacionado con un patrón fotosintético homogéneo.

Los factores físicos también podrían estar relacionados con la diferente tolerancia a la desecación entre células jóvenes y viejas. El daño mecánico asociado con la pérdida de turgor es una de las mayores causas de daños irreversibles en plantas (Iljin 1957; Cutler *et al.* 1977). Una mayor proporción entre el área de la superficie y el volumen facilita la estabilización mecánica de las células, por lo que las células del margen del disco, de menor

tamaño que las del centro, serían más resistentes al colapso bajo estrés hídrico.

En conclusión, la capacidad de los fotobiontes para recuperar la actividad fotosintética tras la desecación está inversamente relacionada con el envejecimiento celular, lo cual sugiere que la tolerancia a la desecación es dependiente de la edad. Esta relación puede explicar, en parte, porqué, en los experimentos llevados a cabo en los capítulos 3 y 5, los cultivos de *T. erici* no recuperaron completamente la actividad fotosintética y metabólica tras el ciclo de deshidratación/rehidratación.

7. FINAL REMARKS

The lichen photobiont *Trebouxia erici* Ahmadjian is desiccation tolerant even when it is cultured isolated from its mycobiont partner. It can be classified into the group of the "fully desiccation-tolerant plants", like most bryophytes and some small pteridophytes. A dehydration/ rehydration cycle produced injuries that were dependent on the drying rate and the duration of desiccation. Long desiccations produced greater injuries and reduced the capacity to recover. After rapid drying (around 60 min) membrane damage was higher and recovery of metabolic and photosynthetic activity lower than after slow drying (4 – 5 h) (chaps. 3 and 6). Photosynthesis was never recovered totally neither after rapid nor slow drying. The inhibition of the photosynthetic electron transport was mainly caused by a decrease in the photochemical reactions associated to PSII, whereas PSI remains apparently fully active after desiccation (chap. 3). Further studies should analyze the effect of longer desiccation periods and consecutive desiccation cycles in order to know better the grade of desiccation resistance of *T. erici*.

The ability of *T. erici* to survive even rapid dehydrations may be explained, on one hand, by protective mechanisms that are constitutively expressed. Classical mechanisms involved in the protection of the cell against oxidative stress were present in hydrated algae although were not enhanced during dehydration/rehydration: 1) antioxidant enzymatic activities, like superoxide dismutase or peroxidase, decreased during desiccation and the early rehydration recovery; 2) the xanthophyll cycle was not activated, as far as the ratio of xanthophyll deepoxidation remained constant after dehydration and rehydration (chap. 3). Likewise, in *T. erici*, dehydrins were also constitutively expressed, since no significantly different amounts of them were found before and after desiccation (chap. 3). Dehydrins are in the group II of the Late Embryogenesis Abundant (LEA) proteins, which are thought to play a primary role in desiccation tolerance. LEA proteins can replace the water molecules removed from the water shell of macromolecules during desiccation in order to prevent the hydrophobic interactions between membranes and proteins. Further studies focused on gene expression, protein characterization, comparative studies among species with different grade of desiccation resistance, should be carried out with the aim of determining the importance of these proteins in lichen desiccation tolerance. On the other hand, we

observed that proteins involved in protection and repair mechanisms were rapidly synthesized during drying; which might also be contributing to the capacity of *T. erici* to resist even rapid dehydrations.

However, the full activation of some protective and repair mechanisms required minimal time, which may explain the higher recovery capacity showed by the slowly dried samples. In *T. erici*, a slow drying time was required for activating the conformational change in the photosystem II which allows converting absorbed light energy into heat in dried stated, thereby protecting them against photoinactivation (chap. 3). Thus, rapidly dried samples were more exposed to photoinhibition than those slowly dried. Likewise, proteins involved in both molecular protection and ultrastructural recovery, were more abundant, around 20 % higher, after slow drying than after rapid drying (chap. 4). Although the difference was not statistically significant, it may be also contributing to the higher recovery capacity of the samples dried slowly described in chapters 3 and 6.

In lichens, full recovery of photosynthetic activity occurs within minutes after rehydration, but, we observed that the isolated photobiont *T. erici* did not reach the previous photosynthetic activity even after 48 h (chap. 3 and 5). Microscopy studies showed that a fraction of the cells in the algal discs were aged (chap. 6). Photobiont capacity to recover photosynthesis activity after desiccation was inversely related with cell ageing, suggesting that desiccation tolerance is age-dependent. Thus, the fraction of aged cells aged interferes with the results and leads to an underestimation of the recovery capacity of the isolated photobiont.

Proteomic analysis showed that desiccation caused up-regulation of around 19 proteins and down-regulation of 43 proteins in *T. erici* (chap. 4). Among the proteins up-regulated during drying were found:

1) a putative cation transport protein, that might contribute to maintain intracellular ion concentrations within the optimal range during the dehydration process.

2) a protein with an F-box domain, which function is to recognize proteins for ubiqutination and further degradation.

3) a cell division cycle 48 protein, which is essential for cytokinesis, cell expansion and cellular differentiation in plants, and is involved in many different cellular

processes, such as to mediate endoplasmic reticulum and Golgi membrane assembly, or to mobilize and target ubiquitylated substrates to the 26S proteosome.

4) β-tubulin, which constitute one of the major components of the cytoskeleton of eukariotic cells and seems to play a key role in the recovery of the ultrastructure of cells in desiccation tolerant plants.

5) a 90 kDa Heat shock protein (Hsp90), member of a family of proteins with strong cytoprotective effects, maintaining proteins in their functional conformation, preventing aggregation of non-native proteins, mediating correct folding of proteins to reach their functional conformation and removal of non-functional polypeptides.

These results provide interesting data that increase the knowledge about the ways that lichens may use to survive continuous cycles of desiccation/rehydration, opening new windows for the study of desiccation tolerance in lichens.

Two protein families showed greater changes in abundance than the others: Hsp90s and β-tubulins. We analyzed the expression of their correspondent genes by RT-qPCR and observed that five *Hsp90* and two *β-tubulin* genes were activated during dehydration. mRNA was accumulated until the cell was completely dried; indicating that synthesis of mRNA went on even after very low relative water content (RWC) (chap. 4). As we report in chapter 3, *T. erici* is able to maintain certain physiological activities, such as photosynthesis below 10 % RWC. It is important to determine, in future studies (e.g. with inhibitors), the key role that these proteins seems to have in the photobiont desiccation tolerance.

Although, lichen photobionts have their own mechanisms to resist dehydration, in a symbiotic relationship desiccation tolerance might be enhanced by other mechanisms. Our studies with the lichen *Ramalina farinacea* and with isolated photobionts showed, on one hand, that the bioactive gas nitrogen oxide (NO) is released, mainly by the mycobiont, during thallus rehydration. Moreover, our results suggested that NO has an important role contributing to the antioxidant machinery during the early stages of rehydration. Thus, desiccation tolerance of lichenized photobionts might be enhanced by the NO released by the mycobiont. To clarify the role of this multifaceted molecule, future research should be directed at functional (NO donors, NO synthase inhibitors), ultrastructural (sites of NO

synthesis, immunohistochemistry), and cell communication (co-culture of isolated symbionts, NO donors, c-PTIO) mechanisms.

The results presented in this thesis demonstrate that desiccation tolerance of *T. erici* is achieved by a complex system of constitutive and induced mechanisms involved in photooxidative protection, protein and membrane protection, ultrastructure cell recovery, protein targeting and degradation, ion transport, and probably others unknown Moreover, some factors such as drying rate, desiccation duration, lichenization and ageing may alter the recovery capacity of *T. erici* after desiccation.

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Appendix 1. Culture chamber irradiance.

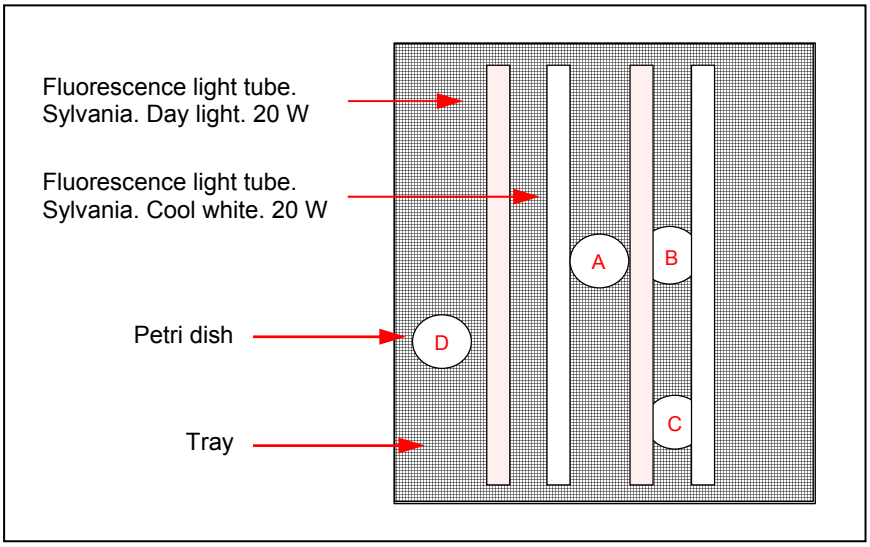


Figure 1.- Diagram of a culture chamber tray.

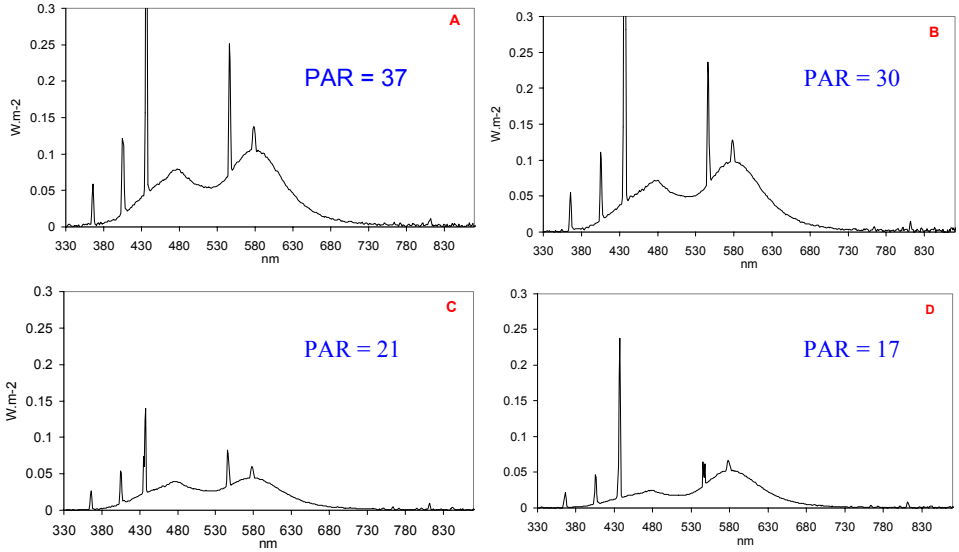


Figure 2.- Light spectrum and PAR intensity at four different points of a culture chamber tray.

Appendix 2. Pigment chromatogram

Method Information

Comments	Protocolo para la columna de 4um + la de 5um. 40 °C para ajustar la presion
Modified User	System
Locked	No
Method Id	1511
Method Version	6
Edit User	

W600 Instrument Setup

Type	W600	%B	20.0	Silk On	Off
Instrument Status	On	%C	0.0	Vacuum Degas On	Off
Channel Name	600 PRESS	%D	0.0	Switch 1	Off
Description		High Limit	5000	Switch 2	Off
Use channel monitor	On	Low Limit	0	Switch 3	Off
Monitor parameter	Pressure	Sparge Rate	40	Switch 4	Off
Chart Parameter	%A	Sparge A	On	Use Events	Off
Head Volume	100	Sparge B	On	Solvent A	metanol 100%
Pump Type	600E	Sparge C	On	Solvent B	acetatoamonico 0.1M
Pump Mode	Gradient	Sparge D	Off	Solvent C	acetona 100%
Flow	0.80	Setpoint	35.0	Solvent D	
%A	80.0	High temp limit	40.0		

W600 Gradient Table

	Time	Flow	%A	%B	%C	%D	Curve
1		0.80	80.0	20.0	0.0	0.0	
2	5.00	0.80	80.0	10.0	10.0	0.0	6
3	45.00	1.25	80.0	5.0	15.0	0.0	6
4	50.00	1.50	80.0	0.0	20.0	0.0	6
5	65.00	0.80	80.0	0.0	20.0	0.0	6
6	67.00	0.80	80.0	20.0	0.0	0.0	6
7	95.00	0.80	80.0	20.0	0.0	0.0	6

996 PDA Instrument Setup

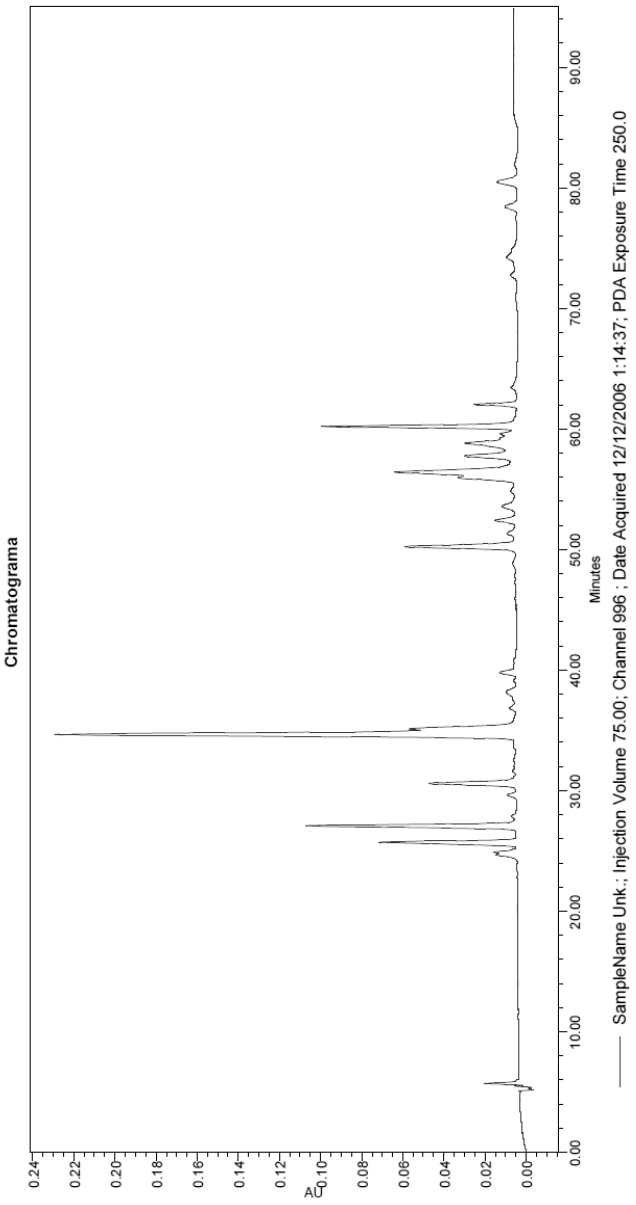
Type	996 PDA	Filter Response	1	Channel 2 Bandwidth	4.8
Instrument Status	On	Digital Filter Response	1.0	Channel 2 Wavelength	254.0
Channel Name	996	Channel 1 Mode	Off	Channel 2 Offset	0.000
Start Wavelength	350.0	Channel 1 Bandwidth	4.8	Channel 2 Ratio Wavelength	254.0
End Wavelength	800.0	Channel 1 Wavelength	254.0	Channel 2 Threshold	0.001
Sampling Rate	1.0	Channel 1 Offset	0.000	Channel 2 Minimum Ratio	0.001
Resolution	1.2	Channel 1 Ratio Wavelength	254.0	Channel 2 Maximum Ratio	100.000
Auto Exposure	Yes	Channel 1 Threshold	0.001	Channel 2 Filter Type	Hamming
Lamp On	Yes	Channel 1 Minimum Ratio	0.001	Channel 2 Filter Response	0
Interpolate 656 nm	Yes	Channel 1 Maximum Ratio	100.000	Event Enable	Off
Channel 1 Enable	Off	Channel 1 Filter Type	Hamming		
Channel 2 Enable	Off	Channel 1 Filter Response	0		
Exposure Time	15.00	Channel 2 Mode	Off		

W715 Instrument Setup

Type	W715
Instrument Status	On

Revision History

This method contains 2 items in the revision history.


Chromatograma
AU
Minutes
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
90.00
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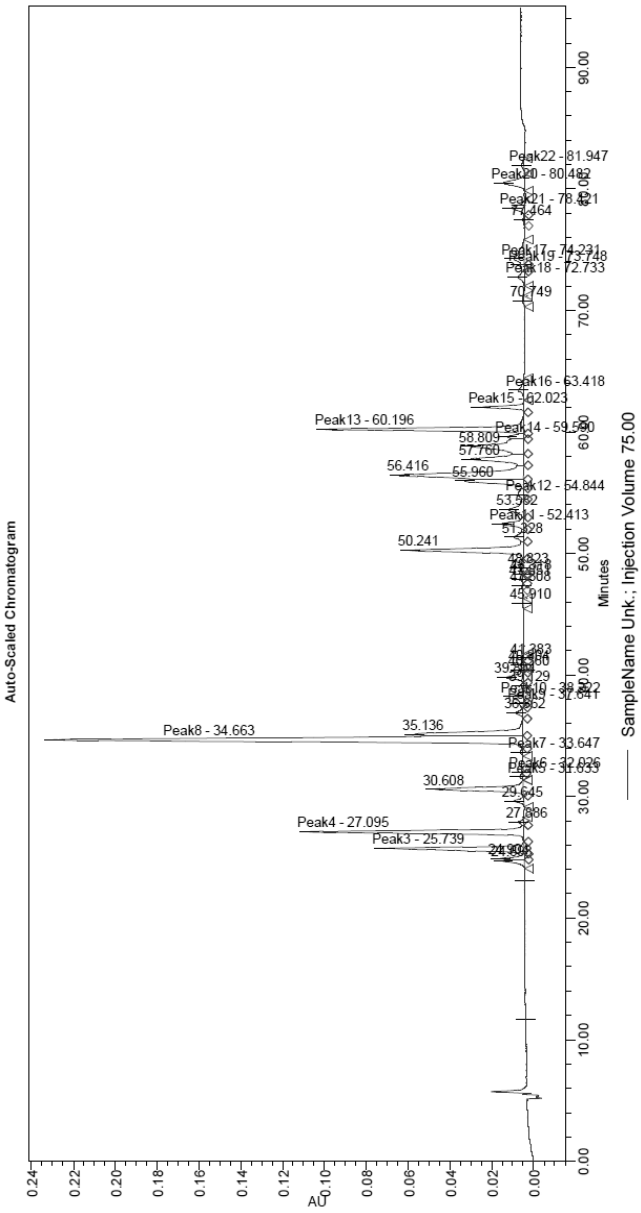
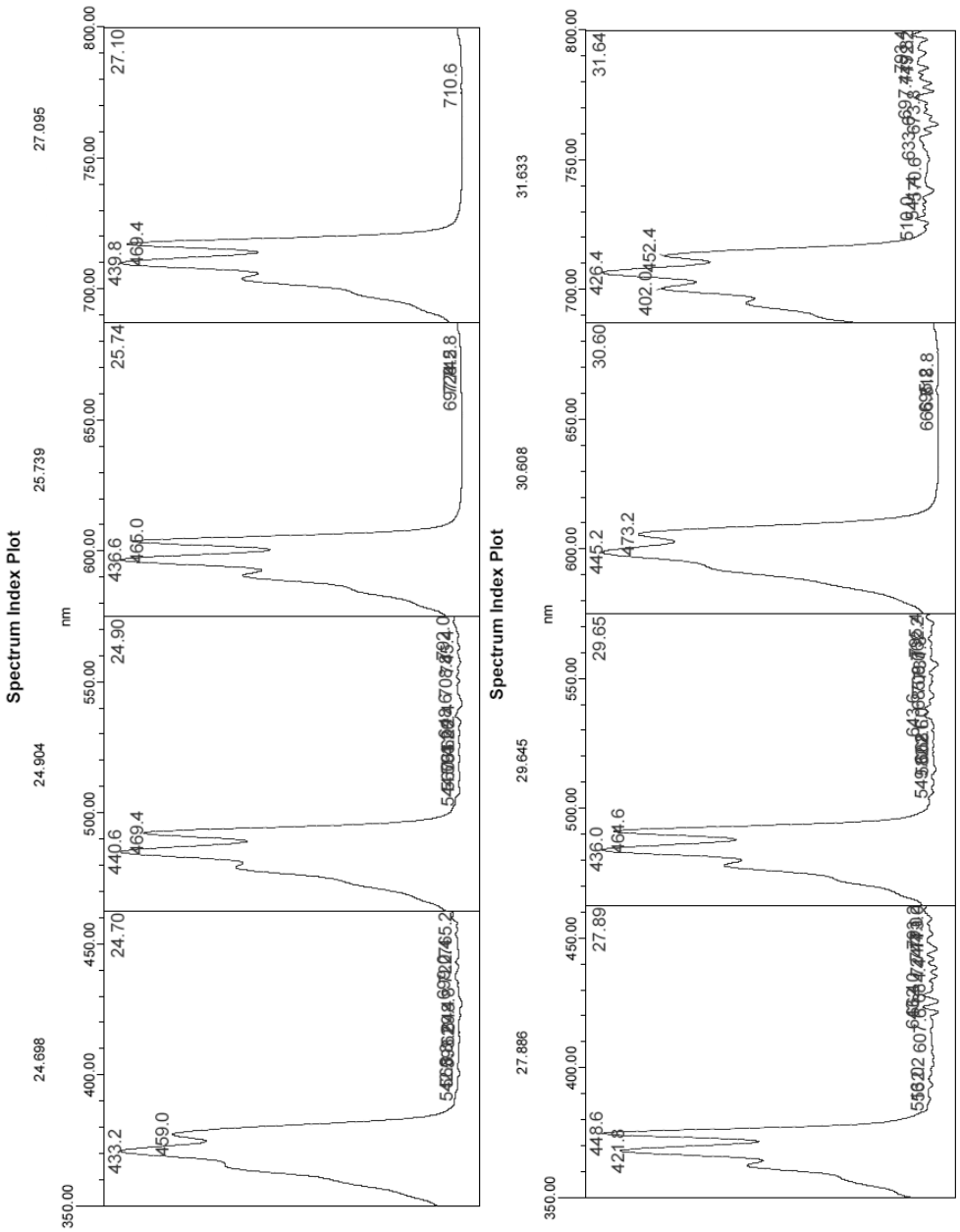

Auto-Scaled Chromatogram
Peak22 - 81.947
Peak20 - 80.482
Peak21 - 78.421
Peak17 - 74.231
Peak18 - 72.733
70.749
Peak16 - 63.418
Peak15 - 62.023
Peak13 - 60.196
Peak14 - 59.590
58.809
57.760
56.416
55.960
Peak12 - 54.844
53.582
Peak11 - 52.413
50.241
48.910
35.136
Peak8 - 34.663
Peak7 - 33.647
30.608
29.645
Peak4 - 27.095
Peak3 - 25.739
AU
Minutes
SampleName Unk.; Injection Volume 75.00

Tabla de Resultados

	RT	PDA Match1 Spect. Name	Area	Height	Purity1 Angle	Purity1 Threshold	% Area
1	11.681						
2	23.051						
3	24.698		170382	10231	3.358	2.171	0.86
4	24.904		157157	11557	3.641	2.065	0.79
5	25.739	Neoxantina	1163745	66979	0.341	1.239	5.85
6	27.095	Violaxantina	1793424	102741	0.300	1.189	9.02
7	27.886		46565	2514	4.698	4.426	0.23
8	29.645		94719	4711	3.710	3.949	0.48
9	30.608	Antheraxantina	850293	42155	1.691	1.380	4.27
10	31.633		22748	1324	8.877	6.045	0.11
11	32.026		6100	410	13.187	9.647	0.03
12	33.647		26315	1223	8.731	10.065	0.13
13	34.663	Luteina	4892894	224531	0.553	1.062	24.60
14	35.136	Zeaxantina	1038892	51894	1.040	1.271	5.22
15	36.862		84106	3234	4.380	4.801	0.42
16	37.641		31839	1712	4.111	6.092	0.16
17	38.222		168379	4841	3.876	3.290	0.85
18	39.129		24581	1197	9.696	9.674	0.12
19	39.804		186775	8176	7.078	2.459	0.94
20	40.360		30489	1474	4.729	6.754	0.15
21	40.804		32522	1451	5.590	7.643	0.16
22	41.383		11196	541	14.861	18.192	0.06
23	45.910	Clorofila b	14309	578	10.277	13.754	0.07
24	47.308	Clorofila b	15005	632	11.860	13.450	0.08
25	47.851	Clorofila b	18419	731	9.534	11.192	0.09
26	48.318		2484	333			0.01
27	48.823	Clorofila b	65985	1669	10.176	6.050	0.33
28	50.241	Clorofila b	1176438	54253	0.869	1.189	5.91
29	51.328	Clorofila a	129995	4434	5.231	3.480	0.65
30	52.413	Clorofila b	249351	10380	3.239	1.784	1.25
31	53.582	Clorofila a	254870	7062	6.103	2.621	1.28
32	54.844	Clorofila a	96797	2720	3.416	1.921	0.49
33	55.960	Clorofila a	610396	28269	2.487	1.306	3.07
34	56.416	Clorofila a	1721199	59438	0.846	1.161	8.65
35	57.760	Clorofila a	681737	25305	2.590	1.341	3.43
36	58.809	Clorofila a	897709	25253	5.184	1.202	4.51
37	59.590	Clorofila a	155477	7758	3.129	1.640	0.78
38	60.196	Clorofila a	1607930	94771	0.335	1.176	8.08
39	62.023	Clorofila a	303196	20676	0.608	1.644	1.52
40	63.418	Clorofila a	93358	2773	5.820	5.327	0.47

Tabla de Resultados

	RT	PDA Match1 Spect. Name	Area	Height	Purity1 Angle	Purity1 Threshold	% Area
41	70.749		17380	638	26.483	18.519	0.09
42	72.733		96720	2990	12.394	5.388	0.49
43	73.748		47516	1647	19.169	6.530	0.24
44	74.231		268204	4925	6.455	3.510	1.35
45	77.464	Feofitina	18104	541	1.844	2.580	0.09
46	78.421	Beta-caroteno	174700	5874	6.411	3.165	0.88
47	80.482		305334	9805	2.946	2.103	1.53
48	81.947		37724	1156	9.150	11.313	0.19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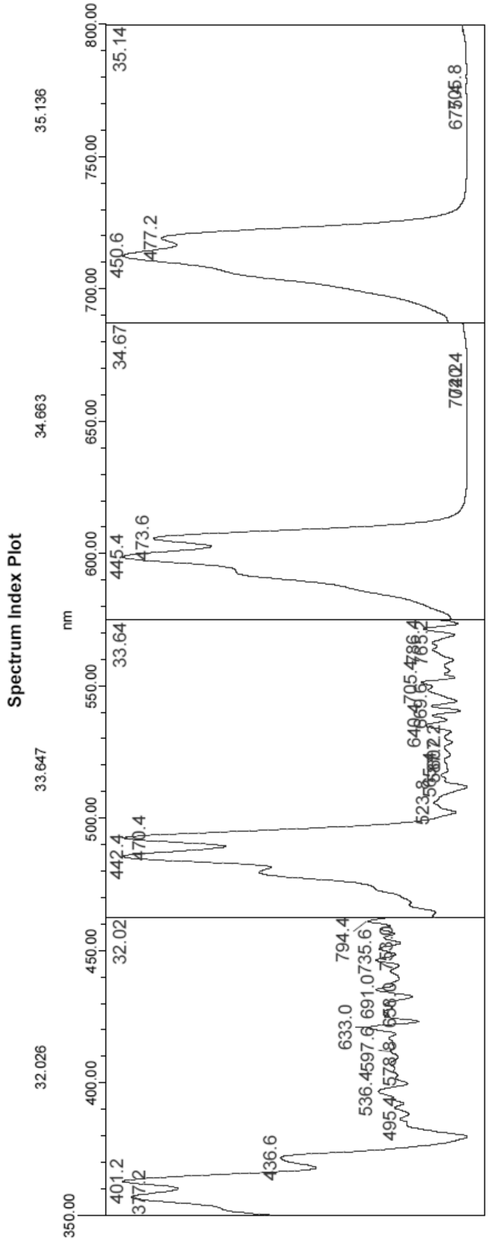
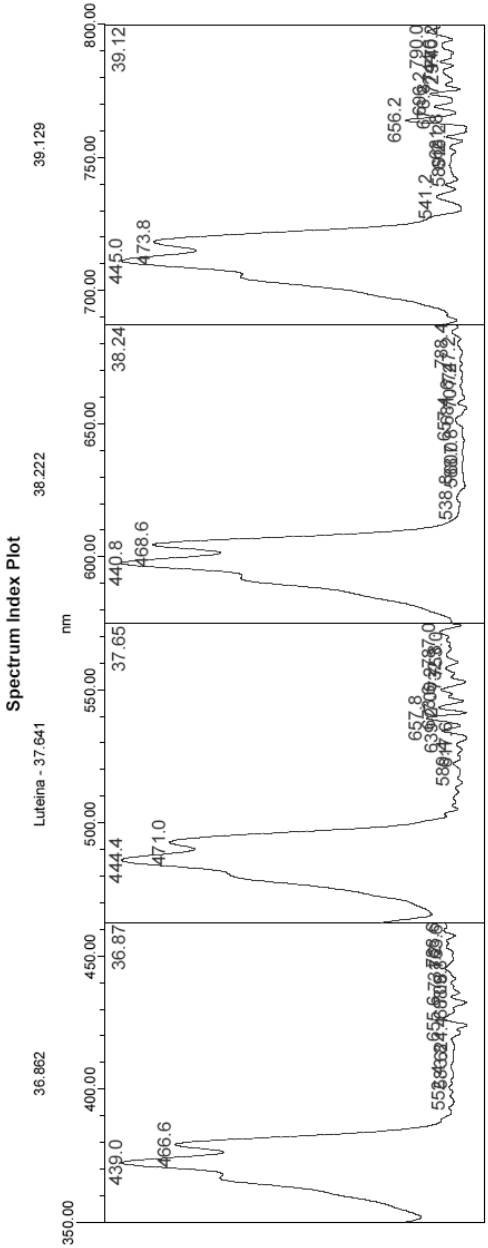
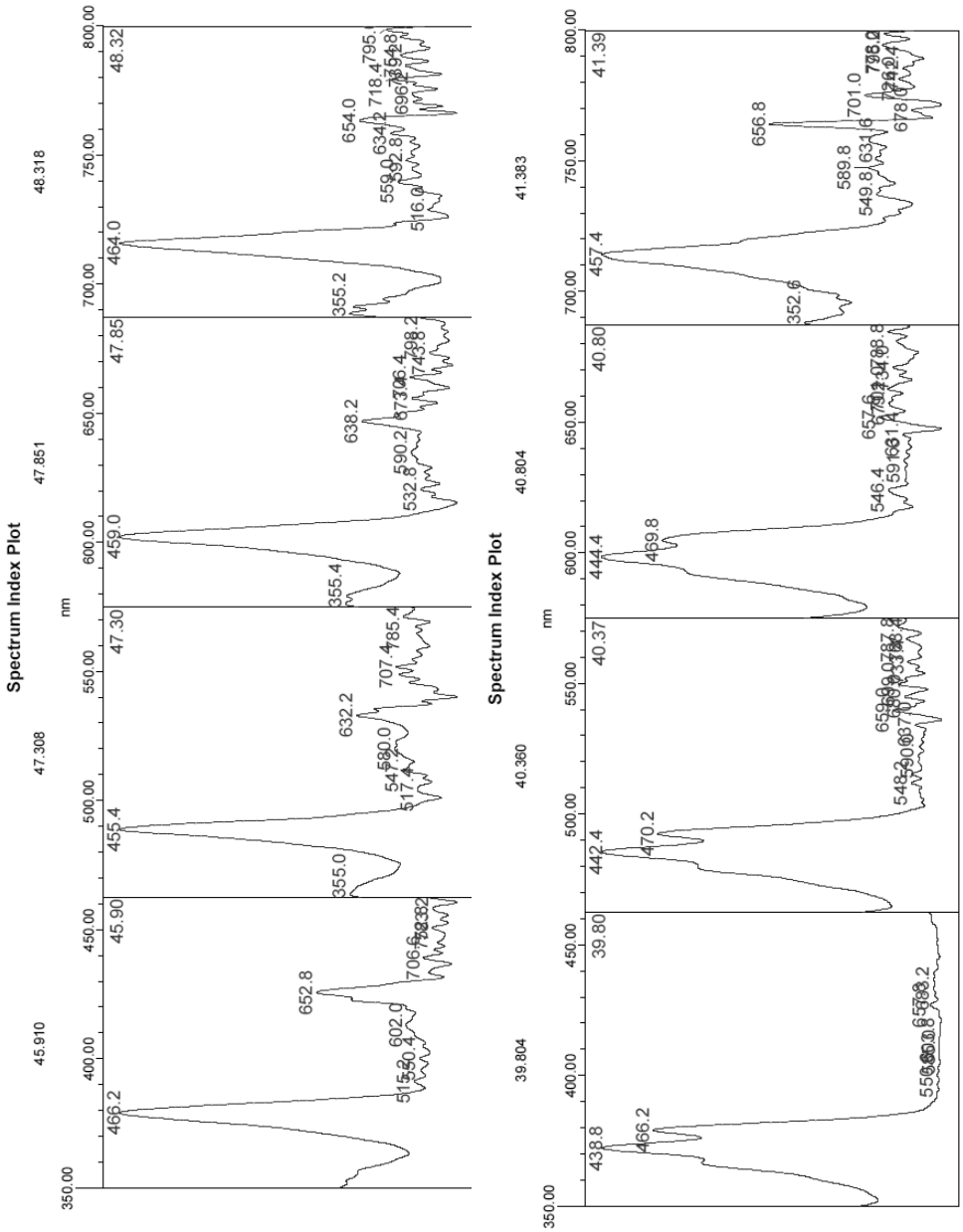
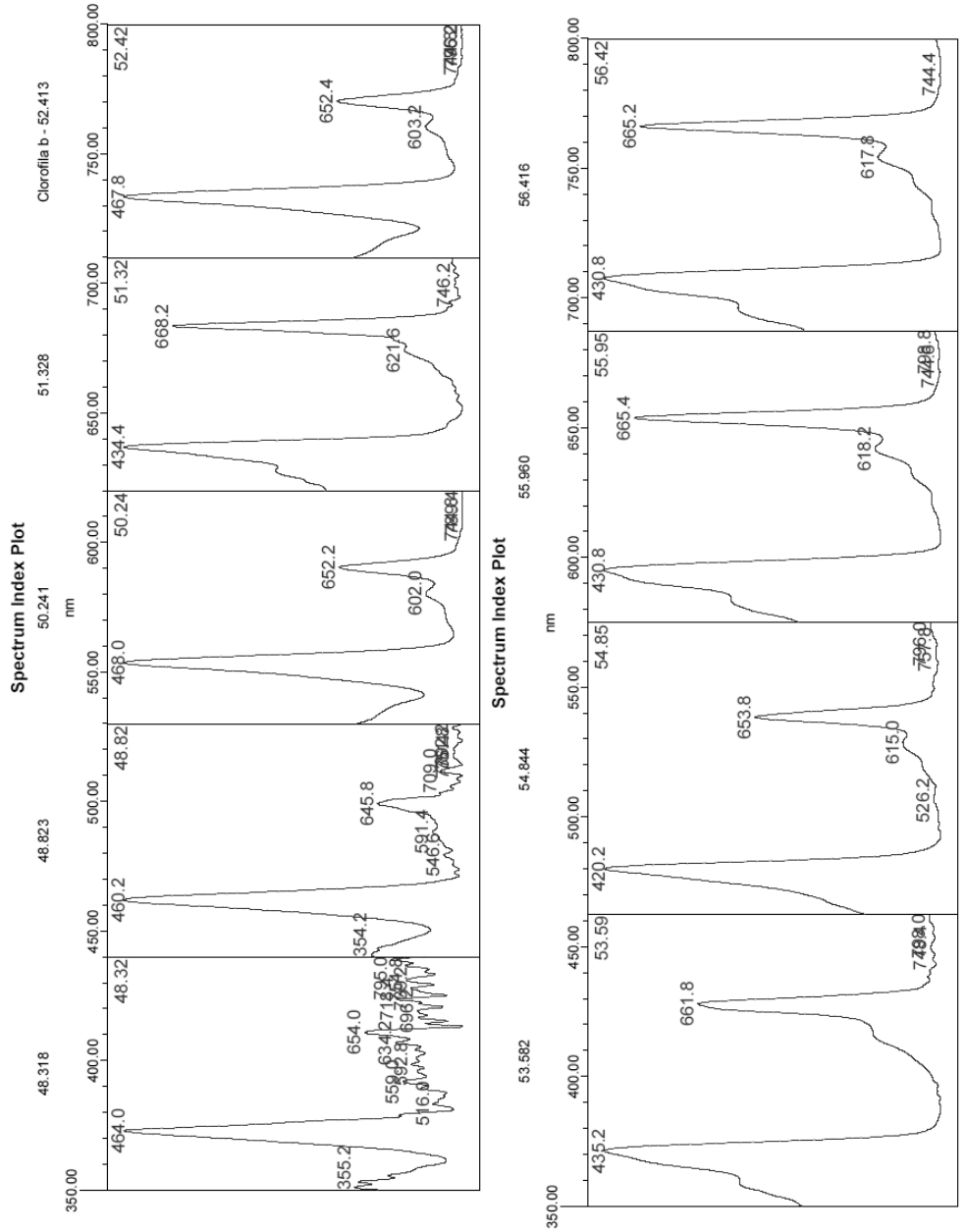
24.698
24.904
25.739
27.095
nm
350.00
400.00
450.00
500.00
550.00
600.00
650.00
700.00
750.00
800.00
433.2
459.0
24.70
440.6
469.4
24.90
436.6
466.0
25.74
439.8
468.4
27.10
710.6
Spectrum Index Plot
27.886
29.645
30.608
31.633
nm
350.00
400.00
450.00
500.00
550.00
600.00
650.00
700.00
750.00
800.00
448.6
421.8
27.89
436.0
464.6
29.65
445.2
473.2
30.60
426.4
402.0
452.4
31.64


Spectrum Index Plot
nm
32.026
33.647
34.663
35.136


Spectrum Index Plot
nm
Luteina - 37.641
36.862
38.222
39.129


Spectrum Index Plot
nm
Spectrum Index Plot
nm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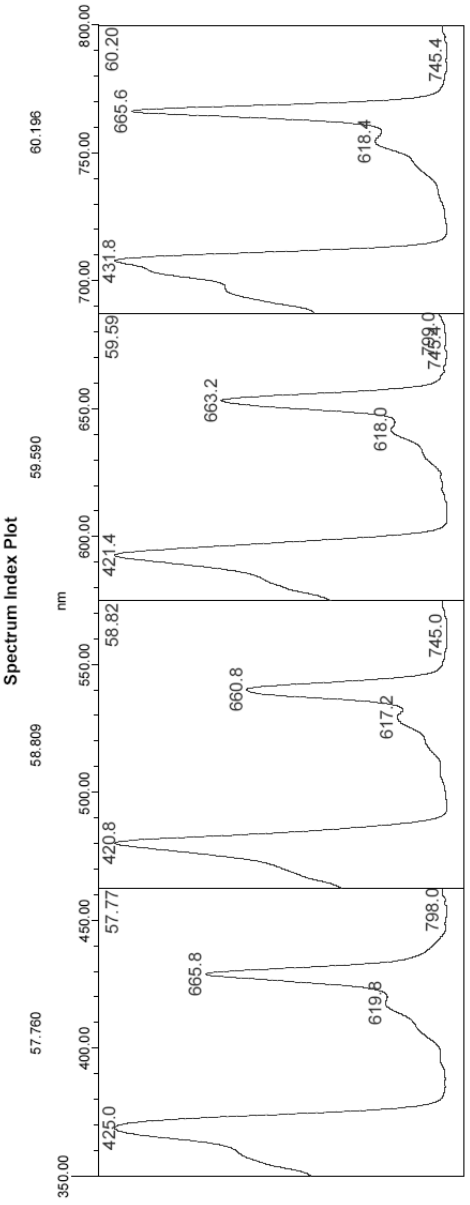
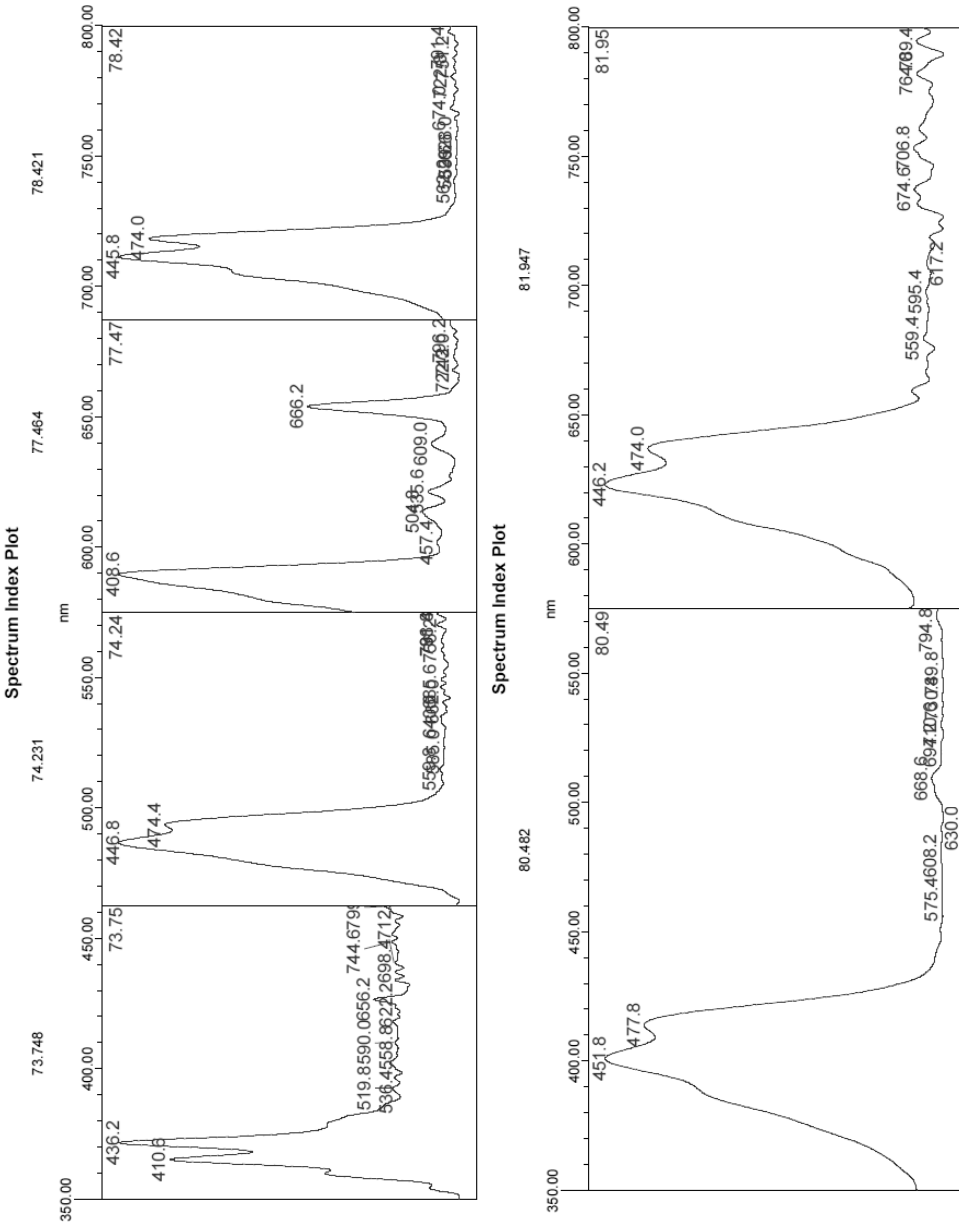
48.318
48.823
50.241
51.328
Clorofila b - 52.413
nm
350.00
400.00
450.00
500.00
550.00
600.00
650.00
700.00
750.00
800.00
464.0
355.2
654.0
460.2
354.2
645.8
468.0
652.2
602.0
434.4
668.2
621.6
746.2
467.8
652.4
603.2
Spectrum Index Plot
53.582
54.844
55.960
56.416
nm
435.2
661.8
420.2
653.8
615.0
526.2
430.8
665.4
618.2
665.2
617.8
744.4

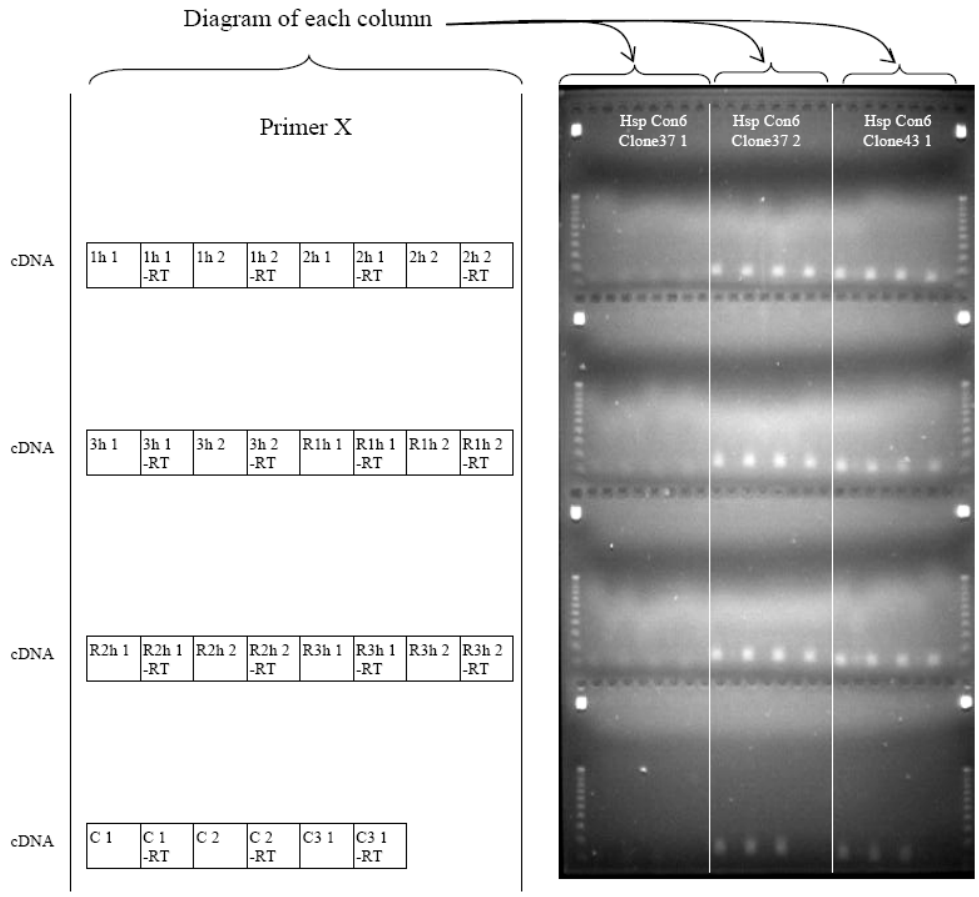

Spectrum Index Plot
57.760
58.809
59.590
60.196
nm
350.00
400.00
450.00
500.00
550.00
600.00
650.00
700.00
750.00
800.00
425.0
665.8
619.8
798.0
420.8
660.8
617.2
745.0
421.4
663.2
618.0
745.4
431.8
665.6
618.4

Spectrum Index Plot
Clorofila a - 62.023
63.418
70.749
72.733
nm
350.00
400.00
450.00
500.00
550.00
600.00
650.00
700.00
750.00
800.00
431.2
666.2
618.2
429.8
668.0
620.8
451.4
481.6
568.0
698.6
676.4
440.4
473.2
660.0

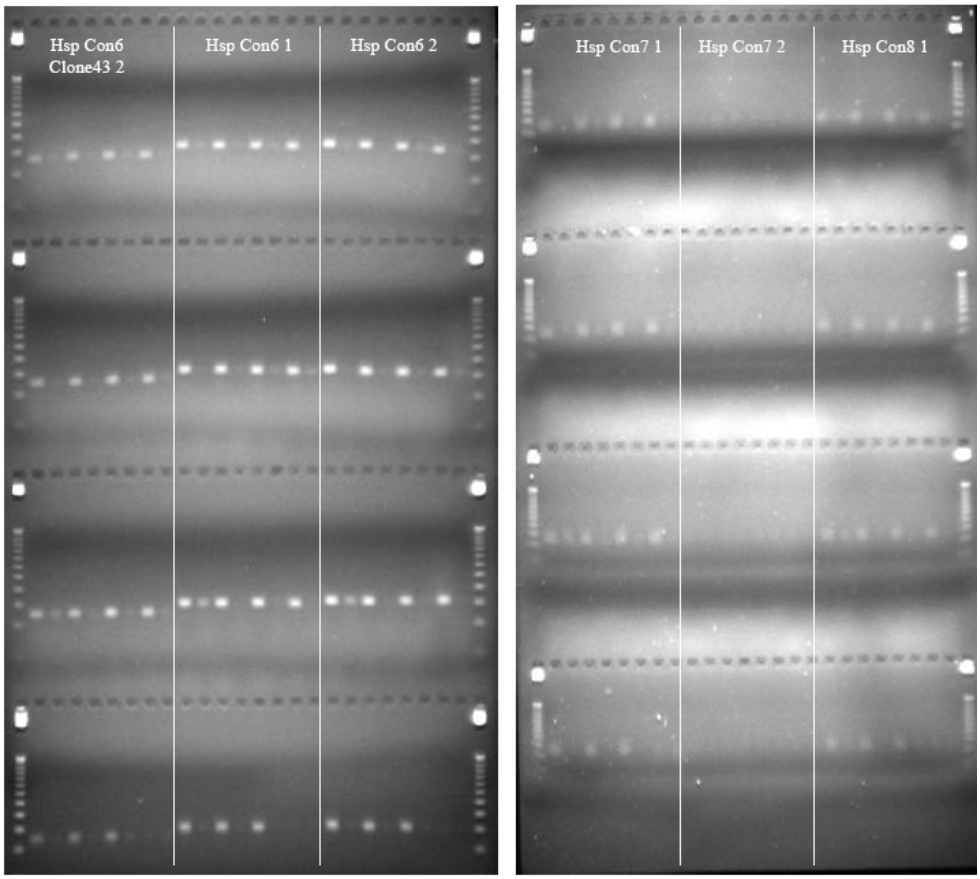

Spectrum Index Plot
nm
73.748
74.231
77.464
78.421
436.2
410.6
519.8
590.0
656.2
744.6
446.8
474.4
408.6
666.2
609.0
445.8
474.0
Spectrum Index Plot
80.482
80.49
81.947
81.95
451.8
477.8
575.4
608.2
630.0
794.8
446.2
474.0
559.4
595.4
617.2
674.6
706.8

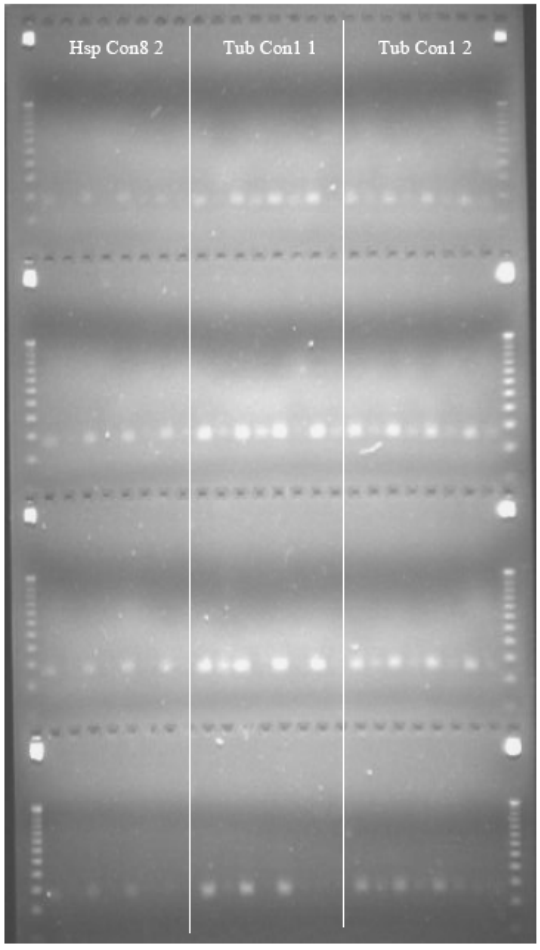
Appendix 3.- RT-PCR products and dissociation curves

Figure 1.- Two sets of primers were designed for each gene. Primers were checked in a PCR amplification with cDNA of some dried and rehydrated samples. The best primer of each set was selected for RT-PCR.



C = Control (non-dehydrated)
1h, 2h and 3h, 4h = time after starting dehydration
R1h, R2h and R3h = time after rehydrating samples desiccated for 24h
-RT = negative controls, cDNA made without reverse transcriptase


Hsp Con6
Clone43 2
Hsp Con6 1
Hsp Con6 2
Hsp Con7 1
Hsp Con7 2
Hsp Con8 1


Hsp Con8 2
Tub Con1 1
Tub Con1 2

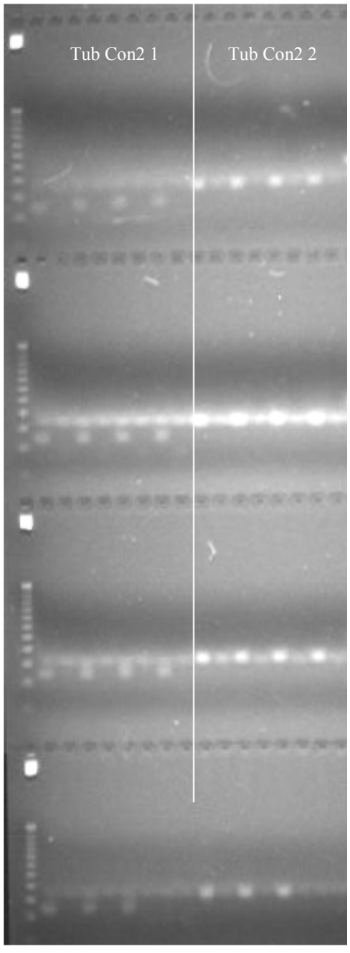
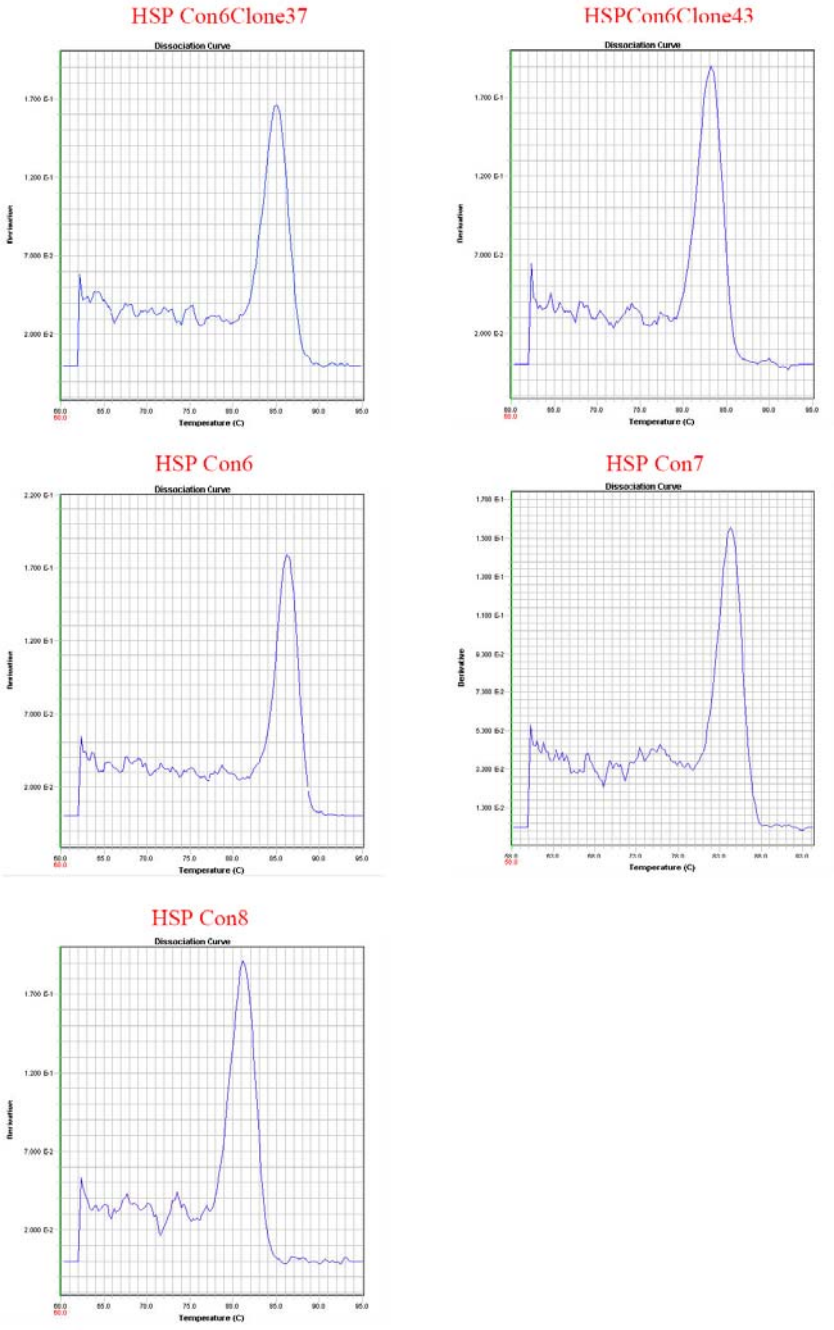
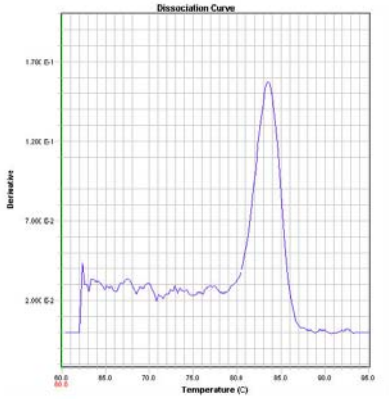

Tub Con2 1
Tub Con2 2

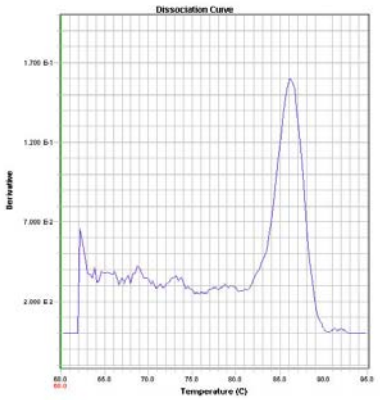
Figure 2.- Dissociation curve of RT-PCR products carried out with different primers.



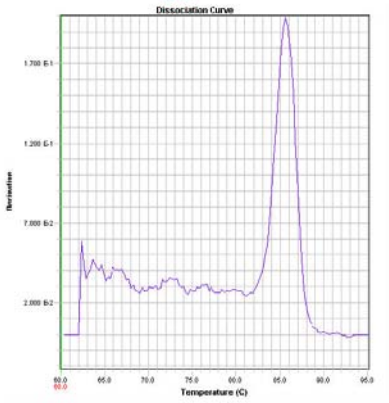
Tub Con1



Tub Con2



18S



Appendix 4. Photobiont isolation.

Material and methods

Lichen material

Ramalina farinacea (L.) Ach. is a fruticose, pendulous, epiphytic lichen species that is very common in the Mediterranean sclerophyllous oak forest. It was collected in the air-dry state from *Quercus rotundifolia* Lam. at Sierra de El Toro (Castellón, Spain; 39º54'16"N, 0º48'22"W) and samples were frozen at –20ºC until photobiont cells isolation, 1 month after collection.

Isolation method

Two variations of the method were performed depending on the amount of lichen talli used. In the first one, that we called macromethod (M), 2 g of dry weight thalli (DW). In the second one, named micromethod (m), 25 mg (DW) of *Ramalina farinacea* thallus were used.

Prior to isolation of the phycobionts cells, lichen thalli were washed with running tap water, and then washed in sterile distilled water. The fragments of thalli were homogenised with a mortar and pestle (M), or in an eppendorf with a pellet pestle (m), in 20 ml (M), or 1 ml (m), of isotonic buffer (0.3 M sorbitol, 50 mM HEPES pH 7.5), filtered through muslin and centrifuged at 490 x *g* in a bench-top centrifuge for 5 min. The pellet was resuspended in 1 ml (M) or 200 µl (m) of isotonic buffer and loaded on 40 ml (M), or 1.5 ml (m), of 80 % Percoll ® in isotonic buffer.

After centrifugation at 10000 x *g* for 10 min (non-stop), four different fractions appeared: A) a 2-3 ml dark green layer at the top of the tube when using the macromethod, yet almost undetectable in the micromethod, containing the less dense particles such as dead cells, remains of cell walls, subcellular particles, etc.; B) a larger and more diffuse layer with a high concentration of non-aggregated algal cells almost free of fungal hyphae; C) a thick layer appeared at the bottom of the tube, composed of fragments of hyphae, aggregates of algal cells and fungal hyphae, and some single algal cells; D) a pellet with small fragments of thallus and large fragments of fungal hyphae (Fig. 1).

Five millilitres (M), or 400 μl (m), of the "B" layer was recovered, diluted 2-fold in sterilized water and centrifuged at 1000 x *g* for 10 min. The supernatant was discarded; the pellet resuspended in 2 ml of sterilized water and a drop of Tween 20 was added. The resulting suspension was sonicated at 40 KHz (Elma Transsonic Digital 470 T, 140% ultrasound power) for 1 min and centrifuged at 490 x *g* for 5 min. This treatment was repeated 5 times, except when described otherwise.

The final pellet was resuspended in 1 ml of distilled water and the cellular density was assessed using a Neubauer hematocytometer. The suspension was diluted 10 times and 50 μl of this suspension were spread on sterile 1.5 % agar 3xN Bold's Basal Media (3NBBM) in five Petri dishes using the streaking technique, under sterile conditions. The cultures of *Ramalina farinacea* were maintained under conditions of 15 μmol m^{-2} s^{-1} (PPFD) in a 12/12 h light-dark cycle, at 14ºC.

The number of algal colonies growing on each plate was counted after 45 days. Ten non-contaminated colonies were selected under the stereo-microscope and transplanted, using a sterile toothpick, onto Petri dishes with 1.5 % agar 3NBBM plus glucose (20 g l^{-1}) and casein (10 g l^{-1}).

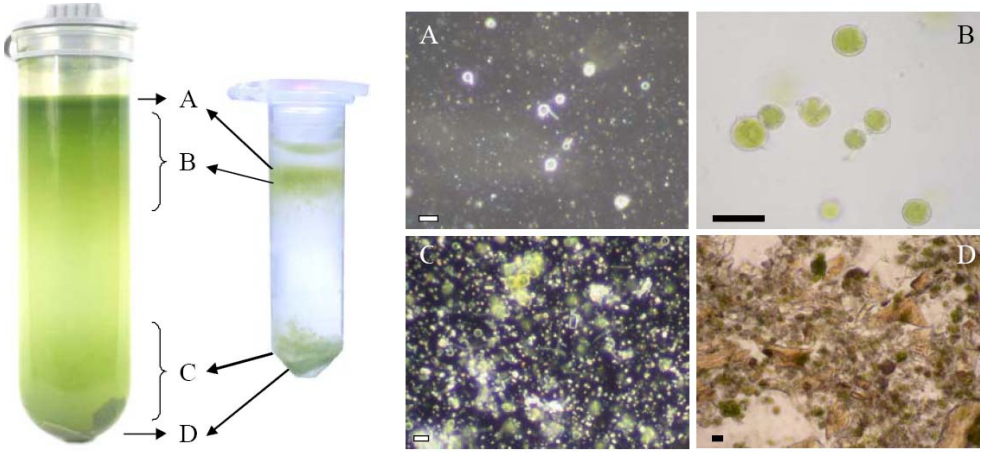


Figure1.- Layers that appeared after centrifugation of the extract of *Ramalina farinacea* thalli at 10,000 x g for 10 min on 40 ml (macromethod), or 1.5 ml (micromethod) of 80 % Percoll® in isotonic buffer. A-D, each optical micrograph refers to the corresponding layer; A and C phase contrast microscopy. Scale = 15 μm. * Not real size.