CHANGES IN METABOLIC PROFILE AND DEFENSE SYSTEMS IN LICHEN MICROALGAE WITH DIFFERENT DESICCATION TOLERANCE

Cotutelle Doctoral Thesis presented by

ALINE FORGATTI HELL

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Cotutelle thesis presented in partial fulfilment of the requirements for the degree of Doctor in Biotechnoscience at Universidade Federal do ABC.

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CONTENTS

ABSTRACT

Most lichens and their photobionts are considered desiccation tolerant, however, the mechanisms involved in their incredible ability to survive the water loss and resume active metabolism are still poorly studied. It is believed that the desiccation tolerance (DT) of lichenized photobionts may be, at least partially, associated with constitutive and induced species-specific mechanisms of protection, related with the activation of the antioxidant system and the synthesis of protective molecules. Thus, the objective of this PhD Thesis was to analyze the composition, metabolic and defense system alterations of two species of lichenized microalgae, with different hydric behaviors: *Trebouxia* sp. (TR9) and *Coccomyxa simplex* (Csol) and relate them with the DT of each species. For this, analyzes of metabolic profile, antioxidant enzymes, characterization of polyols, and quantification of nitric oxide (NO) and transcripts levels of antioxidant and sugar alcohols-related genes were performed. Initially, microalgae cultures were subjected to a single cycle of desiccation-rehydration (D/R), under relative humidity (RH) of 25%-30%. Under these conditions, the relative water content and the water potential indicated that each specie present a DT strategy adjusted to the water regime of their natural habitat. The metabolic profile analysis indicated that TR9 constitutively accumulated higher amounts of polyols, while Csol induced the synthesis of these compounds, which seemed to play an important role in the DT of both species. In a second approach, DT was tested by subjecting the species to different RH conditions and consecutive D/R cycles. The results showed that the RH close to that of their natural habitats (RH 25% for TR9 and RH 60% for Csol) is crucial for maintaining the photosynthetic rates. Some key antioxidant enzyme activities and antioxidant genes (transcript levels) were induced by the subsequent D/R cycles in a species-specif way, probably due to the increase in the formation of reactive oxygen species (ROS). In addition, the analysis of the transcript levels related to the synthesis of *myo*-inositol and raffinose also demonstrated, in general, an induction during the consecutive D/R cycles, especially in Csol. Correlation analyzes also suggest that NO can act as signaling factor, assiting to modulate metabolic pathways ofpolyols production. In conclusion, among the experimental evidences presented in this PhD Thesis, it is highlighted the species-specific induction of the antioxidant system and synthesis of polyols by exposition to cyclic desiccation, which strongly suggest a process of metabolic "priming" performed by lichenized microalgae to cope with the oxidative and osmotic stress during the sudden changes in water content to which they are normally subjected.

Key-words: *Coccomyxa***,** *Trebouxia*, desiccation tolerance, oxidative stress, priming, osmoprotectants

RESUMEN

La mayoría de los líquenes y sus fotobiontes son considerados tolerantes a la desecación, sin embargo, los mecanismos involucrados en su increíble capacidad de sobrevivir a la pérdida de agua y retomar el metabolismo aún están poco estudiados. Se cree que la tolerancia a la desecación (DT) de los fotobiontes liquenizados puede estar asociada con mecanismos de protección constitutivos e inducidos especies-dependientes, relacionados, al menos en parte, con la activación del sistema antioxidante y la síntesis de moléculas protectoras. Por lo tanto, el objetivo de esta tesis Doctoral fue analizar la composición y las alteraciones metabólicas y del sistema de defensa de dos especies de microalgas liquenizadas, con diferentes comportamientos hídricos: *Trebouxia* sp. (TR9) y *Coccomyxa simplex* (Csol) y relacionarlos con la DT de cada especie. Para ello, se realizaron análisis de perfil metabólico, enzimas antioxidantes, caracterización de polioles, cuantificación de óxido nítrico (NO) y análisis de transcriptos de genes antioxidantes y relacionados con la síntesis de polioles. Inicialmente, los cultivos de microalgas fueron sometidos a un solo ciclo de desecación-rehidratación (D/R), bajo una humedad relativa (HR) del 25% al 30%. En estas condiciones, el contenido relativo de agua y el potencial hídrico indicaron que las especies presentan una estrategia de DT ajustada al régimen hídrico de su hábitat natural. El análisis del perfil metabólico indicó que TR9 acumuló constitutivamente mayores cantidades de polioles, mientras que Csol indujo la síntesis de estos compuestos, que presentan un importante papel en la DT de ambas especies. En un segundo enfoque, se evaluó la DT sometiendo a la especie a diferentes condiciones de HR y ciclos consecutivos de D/R. Los resultados mostraron que la HR durante la desehidratación cercana a la de sus hábitats naturales (25% para TR9 y 60% para Csol) es crucial para mantener la tasa fotosintética al rehidratarse. Algunas enzimas y genes antioxidantes (niveles de transcriptos) clave fueron inducidos por ciclos D/R consecutivos, probablemente debido al aumento en la formación de especies reactivas de oxígeno. Además, el análisis de los niveles de transcriptos relacionadas con la síntesis de *myo*-inositol y rafinosa también demostró, en general, la inducción durante los consecutivos ciclos D/R, especialmente en Csol. Los análisis de correlación también sugieren que el NO puede actuar como un factor de señalización, ayudando a modular las rutas metabólicas para la producción de polioles. En conclusión, entre las evidencias experimentales presentadas en esta Tesis Doctoral, se destaca la inducción especie-específica del sistema antioxidante y de la síntesis de polioles por exposición a la desecación cíclica, lo que sugiere un proceso de "priming" del metabolismo de defensa contra el estrés oxidativo y osmótico realizado por las microalgas liquénicas durante los frecuentes cambios en el contenido hídrico a los que están normalmente expuestas.

Palabras-clave: *Coccomyxa***,** *Trebouxia*, tolerancia a la dessecación, estrés oxidativo, priming, osmoprotectores

RESUMO

A maioria dos líquens e seus fotobiontes são considerados tolerantes à dessecação, no entanto, os mecanismos envolvidos em sua incrível capacidade de sobrevivência à perda de água e retomada do metabolismo ainda são pouco estudados. Acredita-se que a tolerância à dessecação (DT) de fotobiontes liquenizados possa estar, ao menos parcialmente, associada à mecanismos espécie-dependentes de proteção constitutivos e induzidos, relacionados com a ativação do sistema antioxidante e à síntese de moléculas protetoras. Dessa maneira, o objetivo dessa tese de doutorado foi analisar a composição e alterações metabólicas e do sistema de defesa de duas espécies de microalgas liquenizadas, com diferentes comportamentos hídricos: *Trebouxia* sp. (TR9) e *Coccomyxa simplex* (Csol) e relacioná-los à DT de cada espécie. Para isso, análises de perfil metabólico, enzimas antioxidantes, caracterização de polióis, quantificação de óxido nítrico (NO) e níveis de transcritos de genes relacionados à antioxidantes e açúcares álcoois foram realizadas. Inicialmente, culturas de microalgas foram submetidas à um único ciclo de dessecação-reidratação (D/R), sob umidade relativa do ar (UR) de 25%-30%. Sob essas condições, o teor relativo de água e o potencial hídrico indicaram que as espécies apresentam estratégia de DT ajustada ao regime hídrico de seu habitat natural. A análise de perfil metabólico indicou que TR9 acumulou constitutivamente maiores quantidades de polióis, enquanto Csol induziu a síntese desses compostos, que parecem desempenhar um importante papel na DT de ambas as espécies. Em uma segunda abordagem, a DT foi testada submetendo as espécies à diferentes condições de UR e consecutivos ciclos de D/R. Os resultados demonstraram que durante a dessecação a UR próxima à de seus habitats naturais (UR 25% para TR9 e UR 60% para Csol) é crucial para o mantenimento das taxas fotossintéticas ao reidratar-se. Algumas atividades de enzimas antioxidantes e genes antioxidantes (níveis de transcritos) chaves foram induzidas pelos subsequentes ciclos de D/R de maneira espécie-específica, provavelmente devido ao aumento da formação de espécies reativas de oxigênio. Além disso, a análise dos níveis de transcritos relacionados à síntese de *myo*-inositol e rafinose também demonstrou, de maneira geral, a indução durante os consecutivos ciclos de D/R, especialmente em Csol. Análises de correlações sugerem, ainda, que o NO possa atuar como um fator de sinalização, auxiliando na modulação das rotas metabólicas para produção de polióis. Em conclusão, dentre as evidências experimentais apresentadas nesta tese de doutorado, destacam-se, a indução espécie-específica do sistema antioxidante e síntese de polióis pela exposição à ciclos de dessecação, o que sugere um processo de "priming" metabólico realizado pelas microalgas liquenizadas para lidar com os estresses oxidativos e osmóticos durante as frequentes alterações no conteúdo de água ao qual estão normalmente submetidas.

Palavras-chave: *Coccomyxa***,** *Trebouxia*, tolerância à dessecação, estresse oxidativo, priming, osmoprotetores

1. INTRODUCTION

1.1 The tolerance to desiccation in lichen-forming microalgae

Water is essential to all organisms on Earth, and therefore their removal from cells can represent severe and even lethal damage, which makes the lack of water in the environment one of the greatest challenges to the survival of terrestrial animals and plants (Alpert, 2006). Most land plants are sensitive to desiccation; therefore, water loss can lead to structural and metabolic damage (Le and McQueen-Mason, 2006). However, some organisms are able to survive under severe conditions of water loss for prolonged periods and tolerate the uptake of water after rehydration. These organisms are classified as desiccation tolerant.

In those terms, desiccation tolerance (DT) can be defined as the ability of an organism to survive severe losses of its cellular water content (for periods that varies among the group) and completely recover its metabolic competence after rehydration (Farrant et al., 2007, Porembski, 2011). In the plant kingdom, DT is a phenomenon commonly found in reproductive tissues, such as spores, seeds and pollen grains, and also in sporophytes of bryophytes and lichens. However, it is absent in gymnosperms and rare in vegetative tissues of pteridophytes and angiosperms, except in the “resurrection plants” (Farrant et al., 2007, Porembski and Bartholott, 2000).

In "resurrection" angiosperms and pteridophytes, desiccation is relatively slower compared to lichens and bryophytes, allowing the development of a complex series of protection mechanisms, which minimize the repair systems necessity during subsequent rehydration (Vicre et al., 2004, Farrant et al., 2007). This category of desiccation-tolerance, present in vascular plants, is called as “inducible”, which means that resurrection plants require a minimum dehydration time (e.g. days or weeks) for the activation of tolerance mechanisms. These are focused mainly on protective strategies than on the repair after rehydration (Oliver et al., 2000). Thus, the mechanisms of DT differ between non-vascular and vascular plants (Morse et al., 2011).

Non-vascular plants are not able to prevent the loss of water from the tissues to the environment, undergoing abrupt changes in their hydration degree (Oliver et al., 2010). In these plants, also known as true desiccation tolerant or poikilohydrics, desiccation occurs very rapidly (e.g. minutes or hours), therefore it is considered that the survival to this state is mainly based on the presence of constitutive mechanisms and the induction of mechanisms of repair of possible damages, only after rehydration (Farrant et al., 2012, Oliver et al., 1998). Lichens are poikilohydric organisms owing to their lack of structures to prevent or actively

control the water exchange with the environment. Therefore, they are submitted to daily dehydration and rehydration cycles, which involves the inactivation and reactivation, respectively, of their metabolism and photosynthetic activity (Del Prado and Sancho, 2007). Moreover, almost all species of lichens can survive in a state of suspended animation (anabiosis) until the availability of water in the environment increases, allowing them to rehydrate and restore their normal metabolism (Kranner and Lutzoni, 1999).

In addition, lichens and algae are categorized as homoiochlorphyllous organisms, which means, they do not changes chlorophyll content during desiccation (Farrant, 1999) and rapid changes in water content may alter the normal operation of chloroplasts and mitochondria, increasing the reactive oxygen species (ROS) formation (Navrot et al., 2007, Jardim-Messeder et al., 2015, Inupakutika et al. 2016). For instance, during the desiccation period light can excite the photosynthetic pigments and induce the generation of ROS. In the same way, during the rehydration process, a rapid and important increase of ROS can occur (Minibayeva and Beckett, 2001, Weissman et al., 2005, Catalá et al., 2010, Casano et al., 2014). When the steady state level of ROS surpasses certain cytological threshold ultimately result in oxidative stress (Scheibe and Beck, 2011, Inupakutika et al., 2016). The generation of ROS and their destructive effects have been addressed in studies on desiccation tolerance in lichens (Kranner et al., 2008, Kranner and Birtic, 2005) and appear to be associated with the oxidative damage of nucleic acids, proteins, lipids (Holzinger and Karsten, 2013) and membranes (Nishizawa-Yokoi, 2008).

Lichens have developed important protective mechanisms to cope with oxidative stress associated with D/R and other stresses. One of these mechanisms, denominated antioxidant system, is based on antioxidant enzymes such as glutathione reductase (GR), superoxide dismutases (SOD), peroxidases (including ascorbate peroxidase; APx), catalases (CAT), and non-enzymatic antioxidants such as ascorbate, glutathione, tocopherols and carotenoids (Noctor and Foyer, 1998, Alscher et al., 2002, Asada, 2006). It is believed that ascorbate plays an important role as a non-enzymatic antioxidant against oxidative stress, acting as a scavenger, removing the hydrogen peroxide generated during photosynthetic processes (Calatayud et al., 1999, Kranner et al., 2008). Transcriptomic analysis lead with the lichen algae *Trebouxia gelatinosa* indicated that only a manganese superoxide dismutase (*MnSOD*) (Carniel et al., 2016), and an APx were overexpressed in response to dehydration (Banchi et al. 2018). In addition, Kranner et al. (2002) observed that desiccation caused oxidation of almost all reduced glutathione (GSH) in *Lobaria pulmonaria*, *Peltigera polydactila* and *Pseudvernia furfuracea*, and rehydration caused the opposite effect. However,

after a long period of dehydration, *P. furfuracea* recovered rapidly the initial rate of GSH, whereas *P. polydactylon* did not re-establish the initial level of GSH. Kranner et al. (2003, 2006) concluded that an adequate adaption to desiccation in lichen holobionts is correlated with the ability to rapidly reestablish normal species-specific concentrations of antioxidants, redox potential and enzymatic activities during rehydration process. However, Kranner et al. (2005) found no relationship between ascorbate and the DT of lichen *Cladonia vulcanii* or its photobiont. Interesingly, ascorbate could not be detected in *Asterochloris erici*, suggesting that this potent antioxidant might not be relevant in Trebouxiophyceae algae (Gasulla et al., 2009). On the other hand, antioxidants and related activities appeared to be less effective in cultures of photobionts (microalgae) isolated from their respective lichens, than those of "in thallus" photobionts (Kranner et al., 2005). In sum, from the scarce and somewhat contradictory literature regarding DT and the physiological response to oxidative stress in lichens and lichen algae no clear conclusions can be drawn. This is probably due to the different experimental designs and procedures employed in those studies, most of which do not reflect the natural environmental conditions in which those organisms thrive.

Other strategies involved in DT, such as the storage of osmocompatible solutes, has been related to the maintanance of vacuolar volume and/or the stabilization of intracellular medium through vitrification (Crowe et al., 1998, Proctor et al., 2007), during the period of metabolism shutdown (Pammenter and Berjak, 1999, Royles et al., 2013). In land algae, the accumulation of compatible osmolytes, acting as osmoregulators, is mainly reflected by the presence of polyols such as glycerol and arabitol, sugars such as sucrose, trehalose and glucosylglycerol, and amino acids (Yancey, 2005, Gustavs et al., 2010). Among the osmocompatible solutes, sugars play an important role in desiccation, since their molecular composition allows the interpolation between the polar groups of phospholipids, enzymes and proteins, and functional groups of others macromolecules (Obendorf, 1997, Steadman et al., 1996), which it is believed that could guarantee the structural integrity of the membranes (Steadman et al., 1996, Centeno et al., 2016). In addition, the cyanobacteria *Nostoc commune* Vaucher accumulated trehalose, a non-reducing disaccharide, during desiccation (Fukuda et al., 2008, Tamaru et al., 2005, Sakamoto et al., 2009). This compound is accumulated by organisms that withstand environmental stresses such as drought, heat or very low temperatures (Wingler, 2002, Eastmond and Graham, 2003). Trehalose is also found in high amounts during desiccation of *Selaginella lepidophylla* (Adams et al., 1990) and seems to modulate the responses that lead to the induction of drought defense mechanisms at the beginning of the water deficit imposition in *Barbacenia purpurea* a desiccation tolerant

organism (Suguiyama et al., 2014). In the aquatic microalga *Botryococcus braunii,* sugars such as arabinose, galactose, fucose, mannose may be involved in cell osmoprotection during anabiosis, that is the suspension of the vital activities, during drying (Banerjee et al., 2002). In the aeroterrestrial Charophyte green alga *Klebsormidium crenulatum*, contiguous sequences for enzymes of the galactinol/raffinose metabolism were expressed in samples submitted to desiccation, suggesting that the raffinose series oligosaccharides (RSO) function as compatible solutes (Holzinger et al., 2014). Futhermore, it is believed that polyols, such as galactinol, myo-inositol, mannitol and sorbitol, may further contribute to avoid the deleterious effects of free radicals/ROS (Smirnoff and Cumbes, 1989, Nishizawa et al., 2008). The presence of sugar alcohols such as sorbitol and andribitol in desiccation tolerant algae has also been observed in some terrestrial species of Trebouxiophyceae algae, such as *Stichococcus* sp., *Chlorella* spp., *Apatococcus lobatus (*Chodat) JB Petersen and *Coccomyxa* sp. (Gustavs et al., 2010). More recently, Kosugi et al. (2013) observed that arabitol provided by the fungus partner increased the capacity to dissipate energy of lichenized microalgae *Trebouxia* sp..

Fait et al. (2006) observed changes in the metabolic and transcript profile during the development of *Arabidopsis* seeds, indicating a metabolism decrease during the drying phase. In seeds of *Erythrina speciosa*, a native species of the Brazilian Atlantic Forest, the decrease of the metabolism occurred even before the greatest loss of water and could be observed through a clear change in the metabolic profile (Hell et al., 2019). In the resurrection plant *B. purpurea*, it was observed that water deficiency induced protection mechanisms including photosynthesis inactivation (Suguiyama et al., 2016) and chlorophyll degradation (Suguiyama et al., 2014). Possible alternative mechanisms have been also described for lichen holobionts (Heber, 2008). In algae, for some species the shutdown of photosystem II during desiccation was also described (Veerman et al., 2007, Kranner, 2003, Holzinger et al., 2014). In mosses and lichen is suggested that the photoprotection mechanism is dependent on the desiccation rate and independent of light (Heber et al., 2007, Heber, 2008). Gasulla and co-workers (2019) described in *Asterochloris erici* an alternative mechanism of light energy dissipation during desiccation, where activation was dependent on a sufficiently slow dehydration rate.

However, the mechanisms involved in the remarkable ability of most lichens and their photobiont to survive to the water loss and their metabolic recovery upon rewetting are still unclear. In general, most evidences presented lead to the idea that DT in lichens and especially in aeroterrestrial microalgae is a constitutive strategy. However, the synthesis of a number of protective molecules, such as sugars and their derivatives (Gustavs et al., 2010, 2011), and the apparent small proteomic changes during desiccation (Gasulla et al., 2013) in

some Trebouxiophyceae algae during the dehydration period suggest that some induced responses could also play a role in their DT. Therefore, we hypothesize that the DT of lichen photobionts would be associated with the species-specific protection mechanisms of both constitutive and inducible nature. Moreover, these mechanisms could be related with the antioxidant system activation and the metabolism shutdown.

Due to the wide distribution of the algae, DT seems to be the ecophysiological key responsible for the dispersion of these organisms on a global scale. In this way, the incredible survival capacity of lichens and their green algae seems to be directly related to metabolic adjustments and rearrangements, protection and repair (Demura et al., 2014). In recent years it has increased the interest in understanding the tolerance mechanisms since it may be biotechnology useful, especially within the context of global climate. Despite this, the genetic basis, the complex series of metabolic events and the structural arrangements that support the stress tolerance of lichen green algae are largely unknown (Leprince and Buitink, 2010).

1.2 The Objectives of the PhD Thesis

Based on the evidences and hypotheses mentioned at **1.1**, the **general goal of the research project that sustains the current pH Thesis, is to study the composition and metabolic changes and the defense systems in two lichen microalgae with different ecology and putatively distinct hydric responses, *Trebouxia sp.* TR9 and *Coccomyxa simplex*, and relate those physiological traits with the DT of these species.** To achieve this aim, metabolomics analyses along with determinations of transcriptional expression of selected genes and antioxidant enzymes will be performed. Thus, it is expected that the results generated in this project will contribute to the prospecting of mechanisms and genes responsible for DT in lichens microalgae. As indicated, the present project will be carried out with two species of lichen microalgae, which presents different behavior to deal with D/R tolerance. One of these algae is *Coccomyxa simplex* (formerly, *C. solorina saccatae*), the phycobiont of *Solorina saccata*, foliaceous lichen widely distributed within relatively humid areas from the Mediterranean mountains to the arctic. It usually grows on calcareous rocks, typically in crevices and always in protected conditions (Krog and Swinscow, 1986), and is subjected to slowly developed summer dry periods. The complete genome of this microalga is not yet available; however, the nuclear genome of *Coccomyxa* C-169, a free-living algae (Guiry, 2012), phylogenetically close to lichen species of the same genus (Del Campo et al., Unpublished results), was sequenced in a considerable proportion and is available on a data DOE Joint Genome Inst. The other microalga is *Trebouxia sp.* TR9, which is one of the photobionts of the lichen *Ramalina farinacea*, epiphytic fruticose ascolichen distributed from the Mediterranean-Atlantic to the Southern boreal regions (Krog and Osthagen, 1980). In these habitats, it is subjected to desiccation during long summer days followed by rehydration as a result of dew during the night, or rain during the rainy periods of the spring and fall seasons (Casano et al., 2011). This lichen is a case in which the fungus is associated with two different species of phycobiont *Trebouxia* (referred as TR1 and TR9) coexisting in the same thallus (Del Campo et al., 2010, 2013, Casano et al. 2011). The two phycobionts respond differently to environmental conditions (Casano et al., 2011), including to the oxidative stress (Del Hoyo et al., 2011, Álvarez et al., 2012, Casano et al., 2011, Casano et al., 2015).

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2. RESEARCH PAPER I

Contrasting strategies used by lichen microalgae to cope with desiccation–rehydration stress revealed by metabolite profiling and cell wall analysis

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Contrasting strategies used by lichen microalgae to cope with desiccation–rehydration stress revealed by metabolite profiling and cell wall analysis

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Summary

Most lichens in general, and their phycobionts in particular, are desiccation tolerant, but their mechanisms of desiccation tolerance (DT) remain obscure. The physiological responses and cell wall features of two putatively contrasting lichen-forming microalgae, *Trebouxia* sp. TR9 (TR9), isolated from *Ramalina farinacea* (adapted to frequent desiccation-rehydration cycles), and *Coccomyxa solorina-saccatae* (*Csol*), obtained from *Solorina saccata* (growing in usually humid limestone crevices, subjected to seasonal dry periods) was characterized. Microalgal cultures were desiccated under 25%–30% RH and then rehydrated. Under these conditions, RWC and ψ_w decreased faster and simultaneously during dehydration in *Csol*, whereas TR9 maintained its ψ_w until 70% RWC. The metabolic profile indicated that polyols played a key role in DT of both microalgae. However, TR9 constitutively accumulated higher amounts of polyols, whereas *Csol* induced the polyol synthesis under desiccation–rehydration. *Csol* also accumulated ascorbic acid, while TR9 synthesized protective raffinose-family oligosaccharides (RFOs) and increased its content of phenolics. Additionally, TR9 exhibited thicker and qualitatively different cell wall and extracellular polymeric layer compared with *Csol*, indicating higher water retention capability. The findings were consistent with the notion that lichen microalgae would have evolved distinct strategies to cope with desiccation–rehydration stress in correspondence with the water regime of their respective habitats.

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Introduction

Lichens are symbiotic associations between at least two organisms, a heterotrophic fungus (mycobiont) and photosynthetic partners (photobionts), which can be either cyanobacteria or eukaryotic algae, and other symbionts such as bacteria (Grube and Berg, 2009; Molins *et al.*, 2013). Lichenization is a clearly successful symbiosis because lichens grow in almost all terrestrial habitats, including extreme environments such as deserts and high mountains. Lichens are poikilohydrics because their water content is mainly determined by the environmental water availability and most of them are desiccation tolerant. In a desiccated state, water accounts for only about 10% of the fresh weight of the lichen thalli (Beckett *et al.*, 2008), but most lichens are able to survive in anabiosis until water becomes available, allowing them to resume their normal metabolism (Fos *et al.*, 1999; Jensen *et al.*, 1999; Alpert, 2006). Most of the ultrastructural, molecular and physiological bases that sustain the extraordinary capacity of lichens to thrive under extreme conditions remain unknown.

Desiccation tolerance (DT) is a multifaceted phenomenon found in several organisms, including algae (Lüttge and Büdel, 2010), bryophytes (Proctor *et al.*, 2007), ferns (Farrant *et al.*, 2009; Yobi *et al.*, 2013) and a small group of angiosperms called “resurrection plants” (Gaff and Oliver, 2013). In resurrection plants, constitutive protective mechanisms slow the rate of water loss and facilitate the ordered expression of inducible components of desiccation–rehydration tolerance (Lüttge and Büdel, 2010; Dinakar and Bartels, 2013; Gechev *et al.*, 2013, Zagorchev *et al.*, 2013; Suguiyama *et al.*, 2014). Lichens do not develop either root-like structures or a hydrophobic cuticle, and their microalgae are not as profusely vacuolated as the photosynthetic cells of vascular plants, making desiccation and rehydration more rapid in these organisms than

in plants (Álvarez *et al*., 2015 and citations therein). While desiccation–rehydration can take days or weeks in resurrection plants (Moore *et al*., 2009; Suguiyama *et al*., 2014), both processes take minutes or hours in entire lichen thalli (Beckett *et al*., 2008) and isolated lichen microalgae (Gasulla *et al*., 2009). Lichens and microalgae are homoiochlorophyllous and rapid changes in water content can disrupt the normal chloroplast and mitochondrial functions and increase ROS formation (Navrot *et al*., 2007; Jardim-Messeder *et al*., 2015). Indeed, some evidence indicates that microalgae have evolved potent mechanisms to dissipate excess absorbed light (Heber *et al*., 2011; Guéra *et al*., 2015), avoiding ROS formation in chloroplasts, and would have uncoupled the respiratory electron flow from phosphorylation to dissipate energy as heat, thus preventing a redox imbalance (Beckett *et al*. 2008). These arguments led to the notion that DT in lichens and especially in terrestrial microalgae is a constitutive strategy. The high amount of diverse protective sugars and their derivatives (Gustavs *et al*., 2010, 2011), and the apparently small proteome changes during desiccation (Gasulla *et al*., 2013) in some Trebouxiophyceae algae further support this contention. However, knowledge of the molecular, biochemical and ultrastructural bases of the physiological response to desiccation–rehydration in green algae remains scarce (Holzinger and Karsten, 2013). The available evidence suggests that a wide range of DT mechanisms and likely different strategies to cope with rapid changes in water content are present within the green microalgal group.

One of obvious consequences of water loss is the progressive reduction of protoplast volume (Moore *et al*., 2008), which can perturb the subtle and complex network of connections between the plasma membrane and the cell wall (CW). Therefore, CW folding, accompanying the cell shrinkage, is particularly important to avoid damage due to mechanical stress provoked by desiccation (Moore *et al*., 2013 and citations therein). Indeed, one of the most distinct and common features of resurrection plants is the higher flexibility of their CWs in comparison with the CWs of non-desiccation tolerant plants (Moore *et al*., 2008). Mechanical stress caused by desiccation–rehydration seems to be especially dangerous in non-vacuolated cells such as terrestrial green microalgae. Therefore, it is reasonable to suppose that desiccation-tolerant microalgae should have CWs that follow water movement, as observed in structural studies of drought-induced alterations at the mycobiont–photobiont interface in several foliose lichens (Honegger *et al*., 1996), probably as consequence of a particular and species-specific biochemical composition-ultrastructural arrangement (Karsten and Holzinger, 2012; Holzinger and Karsten, 2013).

The CWs of green algae in general, and those of symbiotic microalgae in particular, have been scarcely studied (Domozych *et al*., 2012). Unusual polysaccharides such as β-galactofuranans have been characterized as CW components of the green symbiotic microalgae *Trebouxia* and *Asterochloris* (Cordeiro *et al*., 2005, 2007, 2008). In addition, a (1-6) linked O-methylated mannogalactan was isolated from *Cocccomyxa mucigena*, the symbiotic partner of the lichen *Peltigera aphthosa* (Cordeiro *et al*., 2010). Recently, we studied the CW and extracellular polymeric substances (EPS) of the two *Trebouxia* phycobionts of the lichen *Ramalina farinacea* (provisionally named *T.* sp. TR1 and TR9). In TR9 algae, the CW consists of three layers (Casano *et al*., 2011) and contains hot-water soluble β-galactofuranan(s) (Casano *et al*., 2015). A three-layered CW was reported in both lichen-forming and free living *Coccomyxa* species (Honegger and Brunner, 1981). No information on the CW polysaccharides of *Coccomyxa solorina-saccatae* (*Csol*), the algal partner of *Solorina saccata* (L.) Ach., was found in the literature.

In vascular plants, the CW hydration status, which is mainly related to acidic pectic polysaccharides and their side chains, plays a role in preventing water loss during desiccation (Leucci *et al*., 2008; Moore *et al*., 2008; Gribaa *et al*., 2013). Therefore, in addition to its contribution in maintaining flexibility, CW composition directly influences its own hydric status. No pectic polymers have been reported in the CWs of microalgae thus far (Domozych *et al*., 2012), but they were encountered in the EPS secreted by many microorganisms, including green algae, being attached to their cell surface or released into the surrounding environment (Eder and Lütz-Meindl, 2008). The presence of EPS in the pericellular space largely influences the processes of ion exchange and cell protection against extreme environmental conditions, such as desiccation (Maksimova *et al*., 2004; Knowles and Castenholz, 2008; Pereira *et al*., 2009). Knowles and Castenholz (2008) demonstrated that the addition of EPS extracted from *Nostoc* sp. CCMEE 6160 significantly enhanced the DT of the normally EPS-lacking *Chlorella* sp. CCMEE 6038. The composition of EPS seems to be of great importance for protecting against desiccation. In the case of the *Ramalina farinacea* phycobionts, TR9 produced two times more EPS than TR1 and also contains a higher content of uronic acids than TR1, indicating that the presence of acidic, charged polymeric compounds surrounding lichen-forming *Trebouxia* cells could play an important role in creating a protective microenvironment against stress conditions (Casano *et al*., 2015).

Based on the above-mentioned evidence, we hypothesized that lichen-forming microalgae have evolved different strategies to cope with desiccation–rehydration stress, imposed by the length and frequency of the dry–wet periods under the micro- and macro-environmental conditions where each lichen thrives. Therefore, in the present study we analysed the behavior of two contrasting lichen-forming microalgae, TR9 and *Csol*, isolated from *Ramalina*

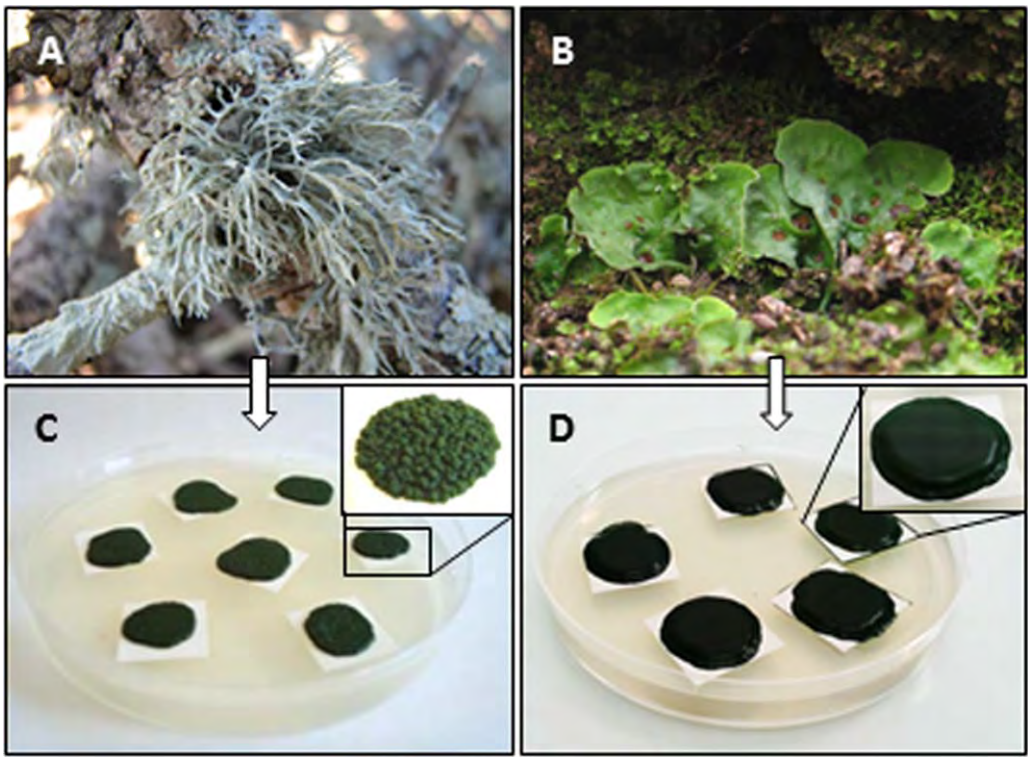

Fig. 1. General aspects of *Ramalina farinacea* and *Solorina saccata* lichens and their isolated phycobionts. *R. farinacea* (**A**) and *S. saccata* (**B**) thalli and axenic cultures of their respective microalgae TR9 (**C**) and *Csol* (**D**). The image of *S. saccata* was gently ceded by the Institució Catalana d'Història Natural.

farinacea (L.) Ach. and *Solorina saccata* (L.) Ach. (Fig. 1) respectively, during desiccation–rehydration. *Ramalina farinacea* is an epiphytic fruticose ascolichen distributed from the Mediterranean-Atlantic to the Southern boreal regions. In these habitats, it is subjected to desiccation during long summer days followed by rehydration as a result of dew during the night, or rain during the rainy periods of the spring and fall seasons. On the other hand, *Solorina saccata* is a foliaceous lichen, widely distributed within humid areas from the Mediterranean mountains to the arctic. It usually grows on calcareous rocks, typically in crevices and always in protected conditions (Krog and Swinscow, 1986), and is subjected to slowly developed summer dry periods. According to http://lichenportal.org/portal/taxa/index.php?taxon=56000, *S. saccata* associates with *Csol* as a unique photobiont. To our knowledge, no studies on the physiology of *Csol* have been performed to date. Herein, we analysed in detail the dynamics of water loss–gain in isolated cultures of TR9 and *Csol* during the course of a desiccation–rehydration cycle, in which the changes in metabolite profiling, focused mainly on organic acids, sugars and sugar alcohols, were also monitored. Additionally, the main biochemical features of the CW and EPS of *Csol* were determined and compared with those recently reported for TR9 (Casano *et al.*, 2015), with the aim to shed light on the structural and functional basis of the extraordinary capacity of lichen microalgae to survive under cyclic desiccation–rehydration.

Results and discussion

TR9 and Csol display distinct responses to desiccation and rehydration

The evolution of the hydric status of the two isolated microalgae during exposure to desiccating conditions (25%–30% RH) and then to rehydration (100% RH) was monitored using two complementary water parameters, RWC and ψ_w. The dynamics of water loss in TR9 and *Csol* were completely different (Fig. 2). According to the RWC curves (Fig. 2A and inset), TR9 dried slower and more linearly than *Csol*, reaching maximal desiccation after 8.5 h, when its water content dropped to about 17%. The rate of water loss during the entire period averaged 79 ($\pm$10.5) μg H_2O h^{-1} mg^{-1} FW. In contrast, dehydration in *Csol* microalgae followed a biphasic trend, with an initial phase characterized by a sharp RWC decrease to about 20% in 3 h, in which the water loss rate was about 320 ($\pm$41.3) μg H_2O h^{-1} mg^{-1} FW. In the second phase, water was lost more slowly, at a rate of 55 ($\pm$11.5) μg H_2O h^{-1} mg^{-1} FW. The minimal RWC of *Csol* was 8.5%, after 7.5 h of

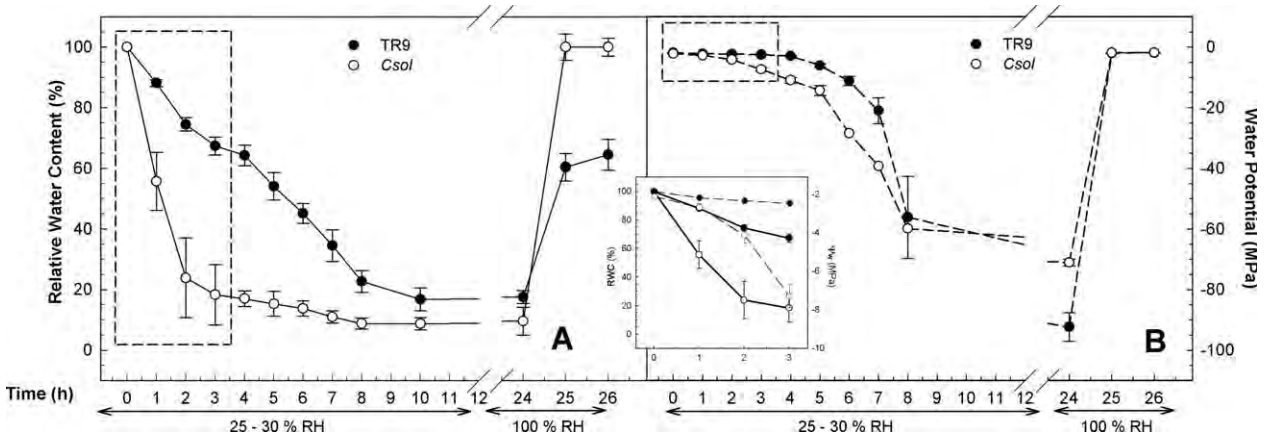

Fig. 2. Progression of hydric parameters of TR9 and *Csol* microalgae during the desiccation-rehydration process. Relative water content (**A**) and ψ_w (**B**) of isolated TR9 and *Csol* subjected to desiccating conditions for 24 h followed by 2 h of rehydration. Values are the mean ± SE of two independent experiments ($n = 3$). The inset represents a combined image of highlighted parts (*dotted lines*) of A and B.

desiccation. No further water losses were observed in either TR9 or *Csol* after 24 h of desiccation. Differences between TR9 and *Csol* were also evident during rehydration. Indeed, even though water uptake was rapid in both microalgae, 100% RWC was achieved by *Csol* in 1 h, while TR9 reached about 60% RWC in the same period (Fig. 2A) and only completed its full hydration after 24 h. Isolated lichen-forming microalgae *Asterochloris erici* submitted to an atmosphere with 20% RH became desiccated in about 1.5 h (Gasulla *et al*., 2009), suggesting a lower water retention capability of this phycobiont in relation to both *Csol* and TR9. These differences could also be explained, at least partially, by disparities in the experimental conditions of the desiccation treatments. Interestingly, a comparative analysis of two *Selaginella* spp. (spike-mosses, Lycophyta) with different sensitivity to desiccation produced RWC curves (Yobi *et al*., 2012, 2013) similar to those observed in the present study. *Selaginella lepidophylla* (desiccation-tolerant) exhibited a RWC curve very similar to that of TR9, while *Csol* seemed to resemble the desiccation-sensitive moss *S. moellendorffii*.

The analysis of ψ_w provided a complementary perspective on the progression of the hydric status of the lichen-forming microalgae throughout the desiccation-rehydration process (Fig. 2B and inset). It was surprising that ψ_w of both microalgae never reached values near 0, or above −1.8 MPa in highly hydrated phycobionts, as can be excepted (for example, see Kosugi *et al*., 2014). This could be due to the matrix effect of the nylon membrane employed for algae culture and handling, which were maintained during ψ_w measurements. Adhesive intermolecular forces between the water and the surface of membrane fibers could have interfered, decreasing ψ_w values especially in the range of −1.5 to 0 MPa. Below this range, the matrix effect of the membrane would be negligible. In sum, ψ_w values depicted in Fig. 2B cannot be considered in absolute terms (especially in the case of highly hydrated cells), but they are entirely suitable for comparing the hydric behavior of TR9 and *Csol* since all of them were determined under the same experimental conditions. In general, ψ_w decreased more rapidly in *Csol* than in TR9 during dehydration. The marked differences between the RWC and ψ_w trends of each microalga in the first hours of desiccation are noteworthy (Fig. 2B and inset). During this period, both water parameters concurrently diminished in *Csol*. Contrastingly, TR9 preserved its ψ_w even though it lost water equivalent to about 30% of its RWC after 3 h of dehydration, probably due to the fact that most of the loose water was retained by hydrophilic EPS attached their cell walls (see below). Thereafter, when water became available, both microalgae recovered their initial ψ_w values at the same time (1 h, Fig. 2B), even though the recovery of all lost water was much slower in TR9 than in *Csol*. In summary, the time courses of RWC and ψ_w clearly suggest contrasting physiological behaviors of the two microalgae under study. *Csol* did not appear to restrict water exchange with its surroundings, and thus its hydric status quickly reflects the environmental water availability, probably because it is normally high for prolonged periods in the natural habitat where *Solorina saccata* proliferates. In contrast, *Trebouxia* sp. TR9 seems to have evolved features/mechanisms, which allow to slow the rates of water loss and gain with the consequent preservation of the thermodynamic state of cell water during the initial phases of desiccation and rehydration. This physiological strategy could be of crucial importance to maintain certain key metabolic pathways [e.g., photosynthesis (Gasulla *et al*., 2009)] and to undertake metabolic adjustments to cope with the potential damages caused by rapid changes in water availability as those TR9 can encounter in its natural environment. To shed light on the bases of these two different behaviors, we comparatively analysed the possible changes in some aspects of the metabolic profile and the compositions of the CW and EPS of TR9 and *Csol*.

Metabolic differences in the desiccation and rehydration of TR9 and Csol

The metabolite profiling was focused on organic acids, sugars, and sugar alcohols during the desiccation and rehydration periods. Both microalgae responded to desiccation by decreasing their primary metabolism. During desiccation, the amounts of some intermediates of the tricarboxylic acid (TCA) cycle tended to decrease (Fig. 3), indicating that the carbon was mainly directed to metabolic pathways leading to the synthesis of protective compounds instead of being consumed for energy production in the mitochondria. Succinic acid seemed to be of key importance in the *switch off* the TCA cycle since it dramatically diminished in both TR9 and *Csol*, while other TCA intermediates followed different trends depending on the algal species. Citric and malic acids were not affected by desiccation in TR9; however, they significantly decreased in *Csol*. The maintenance of some TCA cycle intermediates in TR9 could be relevant for rapid recovery of mitochondrial energy metabolism during rehydration. The increase of lactic acid during rehydration in *Csol* indicates the existence of fermentative metabolism, which might be a result of lower mitochondrial respiration substrates, such as citric and malic acids. Interestingly, malonic acid, which inhibits succinate dehydrogenase in the TCA cycle (Zhang *et al.*, 2011), also decreased in TR9 (Fig. 3); however, this did not appear to result in higher activity of the cycle, as suggested by the decrease in substrates for the enzymes involved in the cycle.

Low cell respiration might reduce the formation of ROS in the mitochondrial electron transport chain. According to recent studies on different desiccation-tolerant species/organs, a decrease in the TCA cycle intermediates was observed in *Arabidopsis* seeds (Fait *et al.*, 2006), in

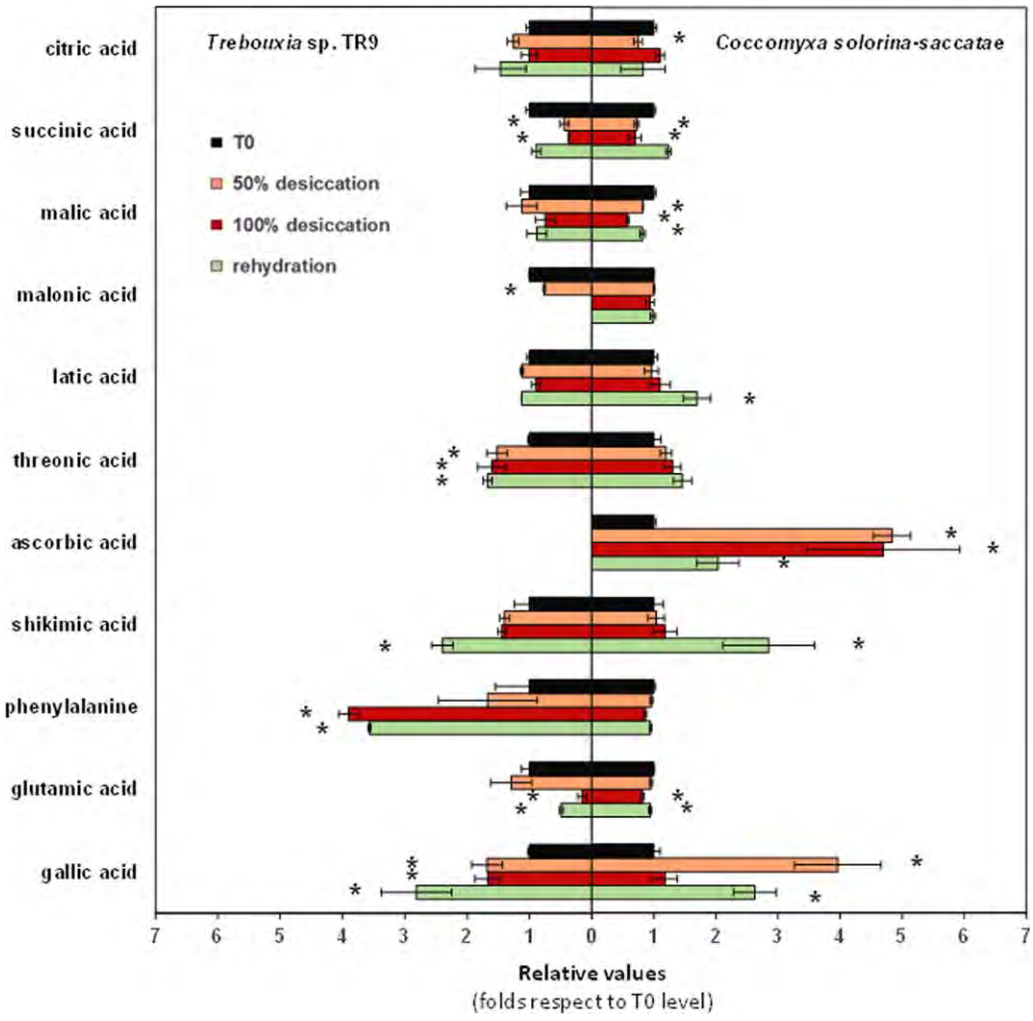

Fig. 3. Metabolite profiling of organic acids of the microalgae TR9 and *Csol* during desiccation–rehydration. *Colored bars* indicate different time points: T0 (*black*), 50% desiccation (*orange*), 100% desiccation (*red*) and rehydration (*light green*). Values are the mean ± SE of two independent experiments ($n = 5$), normalized by the T0 values of each microalgae. The asterisk indicates significant differences ($p < 0.05$) between each desiccation–rehydration stage respect the corresponding initial values (T0) following *t*-test.

Selaginella lepidophylla (Yobi *et al*., 2013) and in the leaves of *Barbacenia purpurea* (Suguiyama *et al*., 2014) during desiccation. Therefore, the *switch off* of the respiratory metabolism seems to be an evolutionary conserved mechanism within the context of a more complex strategy of DT. In fact, the *switch off* of mitochondrial metabolism alone is not sufficient to maintain redox homeostasis and avoid oxidative stress (Pammenter and Berjak, 1999, and references therein). Redox homeostasis depends on the rate of ROS generation and the activity of the antioxidant metabolites and enzymes. The metabolic profiling depicted in Fig. 3 also indicated differences in the protection strategies of the two microalgae. The constitutive amounts of phenylalanine and shikimic and gallic acids were higher in the fully hydrated cells of TR9 than in *Csol* (Supporting Information Table S1). In addition, TR9 increased its phenylalanine and shikimic acid contents during desiccation by up to four times, whereas no change was observed in *Csol* (Fig. 3). Gallic acid and its derivatives are widely present in vascular plants and constitute an important group of secondary polyphenolic metabolites with antioxidant properties (Lu *et al*., 2006). Recently, gallate was isolated from the green alga *Spirogyra* sp. (Kang *et al*., 2015). However, to our knowledge, the present study is the first to report gallic acid in lichen microalgae. Ascorbic acid, a ubiquitous antioxidant, which was present in detectable amounts only in *Csol*, increased during dehydration (Fig. 3).

Aeroterrestrial green algae, including *Coccomyxa* spp., synthesize and accumulate polyols, such as glycerol, erythritol, arabitol, ribitol, mannitol, sorbitol and volemitol, with putative protective roles against desiccation (Feige and Kremer, 1980; Gustavs *et al*., 2010, 2011). The metabolic profiling of TR9 and *Csol* also showed that polyol type and quantity differed between the two microalgae. Despite a few exceptions (including ribitol), some polyols, such as pinitol and galactosylglycerol, increased in TR9 at 50% dehydration (Fig. 4). Moreover, TR9 exhibited significantly higher total amount of soluble carbon compounds, which include mainly polyols (ca. 30-fold on a cell basis) in comparison to *Csol* (Table 1). Sugar alcohols also accounted for a higher proportion of the total soluble sugars in TR9 than in *Csol*, irrespective of their hydric status (Supporting Information Fig. S1). Interestingly, while the high level of soluble carbon compounds was constitutive in TR9, the synthesis of these compounds in *Csol* seemed to be transiently induced by water loss (Table 1). Sugar alcohols play a role as organic osmocompatible solutes, stabilizers of proteins, and serve as rapidly available respiratory substrates for metabolism maintenance under stress and repair during recovery (Holzinger and Karsten, 2013 and citations therein). Polyols are also potent antioxidant scavengers (Shen *et al*., 1999; Nishizawa *et al*., 2008). In this way, the ROS scavenging system in TR9 is highly supported by a polyol-based mechanism, which was consistent with the higher amounts of myoinositol, a precursor for the synthesis of other polyols (Supporting Information Table S1). This metabolic strategy of TR9 might be an ecological advantage because C and N in TR9 could be preferentially used for growth and biomass accumulation instead of producing large amounts of costly antioxidant enzymes. A consistently lower basal activity of antioxidant enzymes was observed in TR9 microalgae in comparison to other closely related microalgae of the same genus (del Hoyo *et al*., 2011; Álvarez *et al*., 2012) and the *Coccomyxa* genus (Álvarez, 2015). A significant decrease in glucose was observed in TR9 during dehydration and after recovery (Table 1). Decrease of hexose sugars may be associated with increased hexokinase activity, which is important to provide and incorporate phosphorylated sugars into different metabolic routes (e.g., sucrose and polyol synthesis) (Whittaker *et al*., 2001).

Sucrose and RFOs are soluble compounds implicated in the maintenance of cell membrane integrity and glass state formation during desiccation (Hoekstra *et al*., 2001). Djilianov *et al*. (2011) proposed that higher absolute values of sucrose and RFOs could increase survival following rapid desiccation of the resurrection species *Haberlea rhodopensis*. Although we found lower absolute values of sucrose in TR9 microalgae than in *Csol* during desiccation and rehydration, raffinose and stachyose were only detected in TR9 (Table 1). These findings are consistent with undetectable levels of galactinol in TR9, probably as a consequence of RFO synthesis, and with the increasing amounts of this polyol in *Csol* during desiccation and rehydration (Fig. 4). In fact, Peters *et al*. (2007) observed that the ratio between sucrose and RFOs in the resurrection plant *Xerophyta viscosa* was also important during dehydration. From this perspective, only TR9 showed a stable Suc/RFO ratio during dehydration and rehydration, as a possible additional mechanism of cell membrane protection (Table 1 and Supporting Information Fig. S1).

The composition of the cell wall and EPS are quite distinct between TR9 and Csol

The CW mechanically controls cell volume and turgor pressure (Le Gall *et al*., 2015), playing a central role in water exchange with the environment. *Csol* and TR9 both have a trilaminar CW (Casano *et al*., 2011; Honegger and Brunner, 1981). However, the wall layers are clearly distinct in *Csol* while in TR9 they have diffuse margins, suggesting quantitative and qualitative differences between the CWs of the two microalgae. Indeed, the CW yield of *Csol* differed from that of TR9 (*Csol* CW yield was 2.5 times lower; Table 2), probably as consequence of the differences in CW thickness, that is, about 413 and 203 nm for TR9 and *Csol*, respectively (Casano *et al*.,

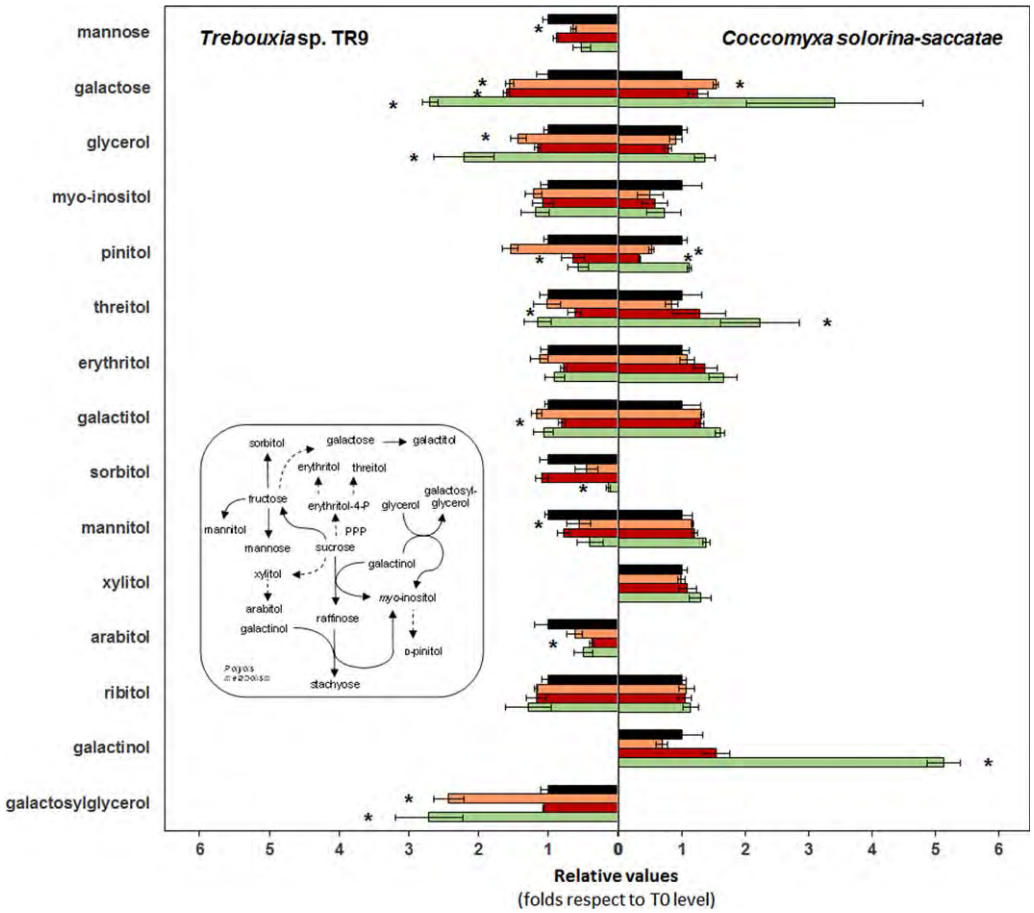

Fig. 4. Metabolite profiling of sugars and polyols of the microalgae TR9 and *Csol* during desiccation–rehydration. For details, see the legend of Fig. 3. Small box inside the chart illustrates the biosynthetic pathway of sugars and polyols.

Table 1. Soluble sugars in TR9 and *Csol* microalgae during desiccation and recovery.

Microalga	Soluble carbohydrates	T0	50% desiccation	100% desiccation	Rehydration
			($\mu g/10^6$ cell)[a]		
***Trebouxia* sp.TR9**					
	Polyols[b]	8.542 ± 0.302	9.164 ± 0.087	9.137 ± 0.830	7.890 ± 0.355
	Glucose	1.092 ± 0.012	0.204 ± 0.016 *	0.209 ± 0.018 *	0.131 ± 0.015 *
	Fructose	0.020 ± 0.009	0.026 ± 0.004	0.026 ± 0.006	0.017 ± 0.002
	Sucrose	0.023 ± 0.003	0.023 ± 0.004	0.029 ± 0.001	0.031 ± 0.002
	Raffinose	n.d.	n.d.	0.013 ± 0.018 *	0.007 ± 0.001 *
	Stachyose	0.002 ± 0.001	0.003 ± 0.001	0.004 ± 0.002	n.d.
Coccomyxa solorina-saccatae					
	Polyols	0.298 ± 0.044	0.399 ± 0.045	0.372 ± 0.032	0.387 ± 0.012
	Glucose	0.079 ± 0.018	0.057 ± 0.007	0.054 ± 0.004	0.099 ± 0.003
	Fructose	0.005 ± 0.002	0.005 ± 0.001	0.005 ± 0.001	0.011 ± 0.001
	Sucrose	0.021 ± 0.008	0.028 ± 0.008	0.052 ± 0.004 *	0.049 ± 0.003 *
	Raffinose	n.d.	n.d.	n.d.	n.d.
	Stachyose	n.d.	n.d.	n.d.	n.d.

Cell content of soluble sugars was determined using HPAEC/PAD in ethanolic extracts from samples of TR9 and *Csol* subjected to desiccation-rehydration. Values represent mean ± SE, $n = 5$. Asterisk indicates significant differences ($p < 0.05$) between each desiccation–rehydration stage respect the corresponding initial values (T0) following *t*-test. n.d., not detected.
a. TR9 and *Csol* cells were counted in pre-weighted and carefully resuspended aliquots using a hemocytometer ($n = 10$–12).
b. Polyols were estimated as soluble carbon compounds (Borges *et al.*, 2006).

Table 2. Features of the cell wall and EPS of TR9 and *Csol* microalgae.

Microalga	*Trebouxia* sp.TR9	*C. solorina-saccatae*
Cell wall		
Yield (μg cell wall/10^6 cell)	11.31	4.25
Neutral monosaccharides (mol%)		
• Arabinose	0.9	3.9
• Rhamnose	24.3	10.7
• Fucose	0.0	2.7
• Xylose	15.2	4.2
• Mannose	8.0	25.9
• Galactose	47.3	20.3
• Glucose	4.3	32.3
EPS		
Yield (μg EPS/10^6 cell)	1.85	0.65
Uronic acids (%)	10.9	7.4
Carbohydrate/protein ratio	2.2	17.0
Neutral monosaccharides (mol%)		
• Arabinose	1.2	2.1
• Rhamnose	5.0	13.9
• Fucose	0.4	1.5
• Xylose	1.3	3.3
• Mannose	2.8	14.3
• Galactose	50.1	42.4
• Glucose	28.3	15.1

2011; Álvarez, 2015). Glycosyl composition analysis also showed remarkable differences in the proportion of neutral monosaccharides between the CWs of TR9 and *Csol* (Table 2). Glucose, mannose, galactose and rhamnose were the predominant monosaccharides found in the crude CW of *Csol*, although arabinose, xylose and fucose were also present. In contrast, galactose accounted for almost 50% of the CW sugars in TR9, followed by rhamnose and xylose, as previously reported by Casano *et al*. (2015). In *Csol*, glycosyl linkage analysis revealed the predominance of a non-branched glucan with the main chain composed of 4-linked glucopyranosyl residues (Table 3), consistent with the presence of cellulose. In fact, cellulose was previously suggested as the fibrillary component of the outer layer of *Coccomyxa* sp. CWs (Honegger and Brunner, 1981). Although amylose has been commonly reported as a compound that adheres to CWs during polysaccharide extraction from *Trebouxia* photobionts (Cordeiro *et al*., 2008), its presence was not reported in the water-soluble polysaccharides obtained from *Coccomyxa mucigena* (Cordeiro *et al*., 2010). This strengthens the suggestion that the high proportion of 4-linked glucopyranosyl residues in *Csol* corresponds to a cellulose-like polymer. The second most abundant polysaccharide of the *Csol* CW was a 4-linked mannan that was poorly substituted at the *O*-2 or *O*-3 positions, probably by galactosyl or mannosyl residues (Table 3). As far as we know, this mannan was not reported in *Coccomyxa* spp. walls. However, two ($1 \rightarrow 4$) linked mannans substituted at *O*-2 with Man*p* or at *O*-6 with Gal*f* residues were isolated from the whole lichen *Roccella decipiens* (Carbonero *et al*., 2005). In addition, the detection of 6-Gal*p* and 3,6 Gal*p* units suggests that a β 1-6 mannogalactan similar to that reported for *Coccomyxa mucigena* (Cordeiro *et al*., 2010) could also be

Table 3. Glycosyl linkage analysis of the cell wall and EPS of *Coccomyxa solorina-saccatae*.

	Cell Wall	EPS
Linkage	(%)	
t*-Rha*p	3.2	3.2
t*-Fuc*p	2.7	0.5
t*-Ara*p	1.1	0.2
t*-Xyl*p	2.8	2.8
2-Rha*p*	3.6	5.4
t*-Man*p	3.7	8.0
4-Rha*p*	1.5	0.3
t*-Glc*p	0.8	1.9
t*-Gal*f	4.0	0.9
t*-Gal*p	3.9	6.7
2-Fuc*p*	–	1.1
4-Ara*p* *or* 5-Ara*f*	2.1	1.7
3-Ara*p*	0.7	0.4
2-Xyl*p* +4-Xyl*p*	1.4	–
2,3-Rha*p*	2.4	–
3,4-Rha*p*	–	0.1
4-Xyl*p*	–	0.8
2,3-Rha*p*	–	5.6
2,4-Rha*p*	–	0.3
3-Glc*p*	0.1	0.3
2-Man*p*+3-Man*p*	3.1	2.8
2-Glc*p*	1.3	4.1
3-Gal*f*	–	6.1
4-Man*p*	14.1	0.8
6-Man*p*	0.6	1.1
2-Gal*p*	–	0.5
4-Gal*p*	3.7	8.2
4-Glc*p*	28.5	7.1
6-Glc*p*	–	0.2
2,3-Man*p*	1.9	0.7
3,4-Man*p*	0.6	–
6-Gal*p*	6.2	9.2
3,4-Gal*p*	–	2.7
3,4-Glc*p*	0.6	0.6
2,3-Glc*p*	–	0.3
2,4-Man*p*	1.1	0.4
2,4-Glc*p*	0.2	0.6
4,6-Man*p*	–	0.2
2,3,4-Gal*p*	–	0.7
3,6-Man*p*	0.3	0.9
2,6-Man*p*	–	0.2
4,6-Glc*p*	0.6	1.0
3,6-Gal*p*	2.4	7.8
4,6-Gal*p*	–	0.8
2,6-Gal*p*	–	0.4
3,4,6-Gal*p*	–	2.0
3,4,6-Glc*p*	–	0.1
3,4,6-Man*p*	–	0.3

–, Not detected.

present in *Csol* CWs. This galactan would be branched at every 2.5 units by either *t*-Man*p* or *t*-Gal*p* residues. Therefore, the CW composition of *Csol* was quite distinct from that reported for the *Trebouxia* TR9 strain, in which the predominant polysaccharide was a 3-linked galactofuranan (Casano *et al*., 2015) instead of glucan, mannan, and galactopyranan reported for *Csol*. Moreover, TR9 also contains highly branched rhamnose-rich polysaccharides (ca. 24% of total polysaccharides) (Casano *et al*., 2015), whereas in the *Csol* CW, these polymers were much less substituted and accounted for about 10% of the sugar residues (Tables 2 and 3).

In vascular plants, the presence of acidic pectic polysaccharides in the CW is a key element in the control of cell hydration status (Le Gall *et al*., 2015). Under our experimental conditions, uronic acids were not detected as components of the CW polysaccharides of either microalgae. Accordingly, no pectic polymers have been reported in the microalgal CWs, but they have been found as part of the EPS of many microorganisms, including green algae (Domozych *et al*., 2012). EPS are formed by a complex mixture of polymeric substances with hygroscopic properties (Knowles and Castnholz, 2008). In the current study, uronic acids were found in the EPS of both microalgae although they were 1.5-fold higher in TR9, which also had three times more EPS than *Csol* (Table 2). These results suggest that the ability to retain a water layer around the cell is higher in TR9, which could contribute to a decreased rate of water loss during desiccation.

The neutral sugars of EPS expressed by the cell surface were 1.2 times higher in TR9 than in *Csol* (Supporting Information Table S2). Galactose was the predominant monosaccharide found in the EPS of both microalgae (Table 2). Glucose, mannose, and rhamnose each accounted for about 15% of the sugars in the *Csol* EPS, while glucose alone accounted for about 30% of the sugars in the TR9 EPS. Glycosyl linkage analyses revealed a complex picture of polysaccharides in the *Csol* EPS (Table 3). The detection of 6-Gal*p* along with 4,6; 2,6 and 3,4,6-galactopyranosyl residues suggested the presence of a highly branched galactan as the predominant polysaccharide. The high proportion of *t*-Man*p* indicates that this monosaccharide might account for most of the galactan substitutions. Therefore, our results indicate that a mannogalactan, similar to that found in the CW of *C. mucigena*, is the main component of the *Csol* EPS. In addition, a polymer formed by 2-Rha*p* residues branched at *O*−3 position appeared to represent a considerable proportion of the EPS. A linear 3-linked galactofuranan, as indicated by the proportion of 3-Gal*f* and the absence of 3,6-Gal*f*, and a cellulose like-polymer, as deduced by the occurrence of 4-linked glucopyranosyl residues, were also found as minor EPS polysaccharides. The presence of 4–6 Glc*p* also suggested that "hemicelluloses" could be a minor component of the *Csol* EPS. In comparison with the TR9 EPS, in which galactans were the predominant polysaccharides (Casano *et al*., 2015), the *Csol* EPS were much more heterogeneous and constituted a complex mixture of polysaccharides.

One of the most striking differences between TR9 and *Csol* concerns the proportion of Gal*f* in their polysaccharides (Casano *et al*., 2015 and Table 3, respectively). In the CW and EPS of TR9, Gal*f* accounted for about 30%, whereas in *Csol*, this sugar accounted for about 5% of the monosaccharides. Although the Gal*f* form is thermodynamically less favorable than its pyranose form, it has been found in many non-mammalian organisms such as bacteria, fungi, and green algae and is considered crucial for the elaboration of their protective surface coat (CW and secreted EPS) (Tefsen *et al*., 2011). The absence of Gal*f* in the external coat often results in decreased fitness and sensitivity to osmotic stress. Moreover, in *Aspergillus* spp., a Gal*f* deficiency was associated with a thinner CW (Tefsen *et al*., 2011 and citations therein). Accordingly, the remarkably higher proportion of Gal*f* in TR9 could contribute to its thicker external coat, improving its water retention capacity compared with *Csol*.

In addition to carbohydrates, proteins were also detected as part of the EPS from both microalgae. The protein fraction was proportionally more abundant in TR9 than in *Csol* (Table 2 and Supporting Information Table S2). The exoproteomes of the two phycobionts also showed distinct bidimensional electrophoretic patterns (Fig. 5). At least 75 and 64 spots were distinguished in TR9 and *Csol*, respectively, using silver staining (Fig. 5A and C). The TR9 exoproteome was highly acidic, with most of its polypeptides showing IPs between 3.0 and 4.5, and molecular masses higher than 50 kDa (Fig. 5A). In contrast, the *Csol* exopolypeptides showed IPs ranging mostly from 3.8 to 4.7, and relatively lower molecular masses compared with TR9 (Fig. 5C). It is well known that EPS protect a variety of microorganisms from rapid water loss (Knowles and Castnholz, 2008; Pereira *et al*., 2009), and the presence of proteins with enzymatic activity or water-retaining properties may contribute to this protective role. Indeed, some of the TR9 and *Csol* spots were preliminary identified as glycoside hydrolase(s) (data not shown) and glycoproteins (Fig. 5). Alcian Blue dying indicated the presence of seven acidic/glycosylated exopolypeptides in TR9 (Fig. 5B) and six in *Csol* (Fig. 5D). Periodic Acid/Schiff Reagent provided similar results. Interestingly, the glycoproteins detected in the TR9 exoproteome were within the most abundant spots (e.g., spots 2, 6 and 7 in Fig. 5A and B), whereas in *Csol*, except for spot 3, all of the glycoproteins were minor spots (Fig. 5C and D). In this context, the higher amount of exoproteins, including glycoproteins, in TR9 could also play a role in its slower water exchange with the environment.

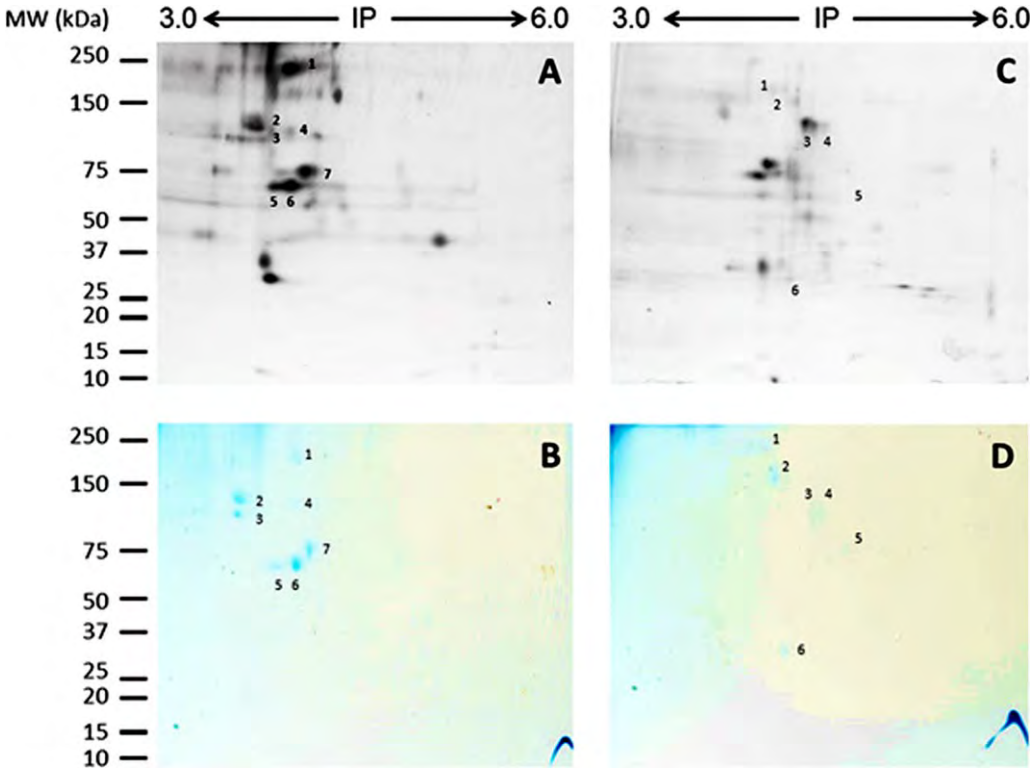

Fig. 5. Extracellular proteome of the microalgae TR9 and *Csol*. Total (**A, C**) and glycosylated (**B, D**) polypeptides present in TR9 (A, B) and *Csol* (B, D) EPS were separated using 2D-PAGE and revealed with appropriated staining.

Concluding remarks

Trebouxia sp. TR9 and *C. solorina-saccatae* are two microalgae isolated from lichens that thrive in distinct ecological contexts. In its natural habitat, TR9 faces relatively rapid daily cycles of desiccation-rehydration, whereas *Csol* is subjected to seasonal drying–wetting periods in which water exchanges occur at comparatively slower rates. The dynamics of the hydric parameters observed in our experiments are consistent with the notion that the DT strategy of each microalga is fitted for the water regime of the respective lichen habitat. These two distinct strategies to cope with the cellular stress associated with desiccation-rehydration seem to be supported, at least in part, by differences in the basal levels of key metabolites or inducible metabolic adjustments, and in the features of the CW and the hydroactive EPS. Polyol-based protective mechanisms are present in both microalgae. However, TR9 maintained constitutively higher amounts that were unchanged during desiccation, contrasting with the lower but inducible levels of these metabolites observed in *Csol*. Both TR9 and *Csol* exhibited reduced TCA activity during desiccation, possibly as a common mechanism to prevent ROS increase; however, each microalga apparently diverted its metabolic flux to distinct fates. TR9 induced the synthesis of protective RFOs and antioxidant phenolics while *Csol* invested in the accumulation of ascorbic acid. Moreover, the differences observed in the CWs and EPS between TR9 and *Csol* indicate that their rates of water exchange could also be determined by the features of their "external coats." All of the quantitative and qualitative characteristics of the CW and EPS of TR9 point toward a higher capability of water retention in comparison to *Csol*. In summary, although both microalgae are desiccation tolerant, TR9 appears to be predisposed to tolerate desiccation mainly in a constitutive manner together with some inducible components, whereas inducible processes in *Csol* appear to play a more crucial role. These findings are consistent with the ecological contexts in which these microalgae naturally occur.

Experimental procedures

Microalgae isolation and culture

The *Trebouxia* sp. TR9 microalga was isolated from the lichen *R. farinacea* collected at S[a] El Toro (Castellón, Spain; 39°54′16″N, 0°48′22″W) (Fig. 1A and C) as previously described (Casano *et al.*, 2011). Axenically cultured *Coccomyxa solorina-saccatae* (strain 216-12) (Fig. 1D) was obtained from Sammlung von Algenkulturen at Gottingen University (Germany). According to this algal bank, *Csol* was isolated from the lichen *Solorina saccata* (Fig. 1B) found in

Großer St. Bernhard, Switzerland (45°57′4″N, 7°12′32″W). Both microalgae were cultured under axenic conditions on small Polyvinylidene fluoride membrane (0.45 μm pore size, Durapore®, Millipore, The United States) squares (4 cm^2) in semisolid Bold 3N medium (Bold and Packer, 1962) in a growth chamber at 15°C, under a 14-h/10-h light/dark cycle (light conditions: 25 μmol PAR m^{-2} s^{-1}).

Desiccation–rehydration treatment

After 21 days, the cultures on the nylon squares (ca. 180–200 mg FW) were removed from the culture medium and subjected to desiccation in a closed container (18.5 l internal volume) with 10–15 g of silica gel (25%–30% RH) for up to 24 h. The other environmental conditions were a temperature of 20°C, and a 14-h/10-h light/dark cycle (25 μmol PAR m^{-2} s^{-1}). Thereafter, cultures were transferred onto watered filter paper placed in sealed Petri dishes, which rapidly created a water vapor-saturated environment, for up to 2 h under 20°C and 25 μmol PAR m^{-2} s^{-1}. At indicated times (Fig. 2), samples were collected from the desiccation or rehydration treatment, weighed, and immediately used for the determination of water parameters or frozen in liquid N_2 and maintained at −80°C until use.

Determination of water parameters

The hydric status of the cultures was monitored using RWC and ψ_w determinations. The RWC was determined according to the formula: $RWC = [(FW - DW)/(TW - DW)] \times 100$, where FW is the actual cell mass at a given stage of desiccation–rehydration, DW is the dry cell mass, and TW is the fully hydrated cell mass. It should be noted that TW is not synonymous of "water-saturated weight" because it corresponded to the culture mass immediately determined after extracting it from its Petri dish, without any previous incubation on water-saturated filter paper, to avoid TW overestimations due to intercellular-capillary water accumulation. The ψ_w of the microalgal cultures was estimated using a Dewpoint Potentiometer Decagon WP4 (Decagon Devices, The United States) following the manufacturer's instructions. In this method sample ψ_w equilibrates with that of the air in a sealed chamber, where a photoelectric cell detects when condensation first appears. The time requested to reach steady measures varied with the sample hydric status, but no exceeding 10 min. Two calibrations were performed, the first one carried out in the equipment continuous mode, according the manufacturer's instructions, and the second one using three replicates of each microalga equilibrated in a −5 MPa polyethyleneglycol 6000 solution (according to Michel and Kaufman, 1973).

Metabolic profiling and soluble carbohydrate analysis

The metabolic profile of the two microalgae was analysed in samples collected at the beginning of desiccation, at 50% and 100% maximal desiccation, and at rehydration (1 h). Microalgal samples were collected in liquid nitrogen, lyophilized and 20 mg were extracted in 500 μl of methanol:chloroform:water (12:5:1) using ribitol (0.2 mg ml^{-1}) as an internal standard, exactly as described by Suguiyama *et al.* (2014). The abundance of ribitol (exogenous) was also evaluated in the absence of biological samples. The endogenous ribitol (i.e., total minus exogenous ribitol) of both microalgae did not significantly change within the experiments (Fig. 4), which allowed the use of this molecule as internal standard. The metabolic profile was performed with derivatized samples using a gas chromatography–mass spectrometry (GC-MS) system (Agilent GC 6890 and MSD 5973N series, Agilent, The United States) (Suguiyama *et al.*, 2014). GC was performed on a 30-m HP5 column with a film thickness of 0.25 μm (Supelco, Bellfonte, The United States), using the following temperature program: 5 min of isothermal heating at 70°C, followed by a 5°C min^{-1} oven temperature ramp to 280°C, and a final 1 min of heating at 280°C. For soluble carbohydrate analysis, the extracts were deionized through anion exchange columns and a volume containing 200 μg of sugar was analysed using high-performance anion exchange chromatography coupled with a pulsed amperometric detector (HPAEC/PAD, Dionex ICS-3000, The United States) as described by Carvalho *et al.* (2013). Sugars were identified by co-chromatography using authentic standards (Sigma-Aldrich Co., The United States).

Extraction of cell walls and EPS from Trebouxia sp. TR9 and Coccomyxa solorina-saccatae

Cell walls of TR9 and *Csol* were extracted from 21-days cultures (ca. 2.5 g FW) similar to the method described by Casano *et al.* (2015) except that the latter microalga was broken up through ten 1-min cycles of ultrasonication (600 Watt). After cell disruption, the CWs were collected by centrifugation (10 000*g*, 10 min) and washed with 50 mM NaCl and ultrapure water. Cell debris was removed by successive washes with organic solvents. The EPS from both microalgae were obtained by gently stirring cells with ultrapure water or water containing protease inhibitors (for protein analyses) as described previously (Casano *et al.*, 2015).

Determination of carbohydrate and protein contents

Total carbohydrates were determined using the phenol-sulfuric acid method (DuBois *et al.*, 1956) with glucose as the standard. Uronic acids were assayed with *m*-hydroxydiphenyl (Fisher Co.) in the presence of sulfamate (Filisetti-Cozzi and Carpita, 1991), using galacturonic acid (Sigma Co.) as the standard. The total proteins in the EPS were measured according to Bradford (1976), using bovine serum albumin (Sigma-Aldrich Co., The United States) as the standard.

Sugar composition and sugar linkage analyses

Glycosyl composition analyses of CW and EPS were performed using the GC/MS alditol acetate procedure. Briefly, approximately 300–400 μg of the samples were used for the analyses. Inositol (20 μg) was added to each sample as the internal standard. The samples and standard sugars were hydrolyzed in 2 M trifluoroacetic acid for 8 h in a sealed tube at 100°C, reduced with $NaBD_4$, and acetylated by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for the detection of amino sugars). The resulting alditol acetates were analysed on an Agilent 7890A GC interfaced with a 5975C

MSD, in electron impact ionization mode. Separation was performed on a 30-m Supelco SP2331 bonded phase fused silica capillary column. Sugar linkage analysis was performed using partially O-methylated alditol acetates and GC-MS as described previously (Casano *et al*., 2015).

Analysis of extracellular proteins

The extracellular proteome of both microalgae was analysed using 2D electrophoresis. EPS samples (equivalent to 10 μg of total proteins, according to Bradford's method) were resuspended in 125 μl of IEF buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) Triton X-100, 2% IPG buffer 3-6 (GE Healthcare, The United States), 130 mM DTT] and loaded onto pH 3–6, 7-cm IPG strips because more than 90% of the EPS proteins had IP values lower than 6.0 (data not shown). IEF was carried out at 20°C with a current limit of 50 μA/strip at approximately 26 kVh in a PROTEAN®^{TM} IEF Cell (Bio-Rad, The United States). Thereafter, the IPG strips were equilibrated in two steps of 15 min each in 150 mM Tris–HCl, pH 6.8, 6 M urea, 20% (w/v) glycerol, 2% (w/v) SDS, including 2.0% (w/v) DTT in the first step and 4.5% w/v iodoacetamide and 0.002% (w/v) bromophenol blue in the second step. The SDS-PAGE was performed in 4%–15% (linear gradient) acrylamide gels. The gels were stained with either Silver Stain Plus^{TM} (Bio-Rad) for detecting total proteins or Alcian Blue (Jay *et al*., 1989) and Periodic Acid/Schiff Reagent (Zacharius *et al*., 1969) for visualizing glycoproteins.

Statistics

The biochemical analyses of the CWs and EPS were repeated twice, starting with new cultures each time. The evolution of water parameters and of the metabolic profile during desiccation-rehydration was determined in two to three independent experiments, starting with new cultures each time, with three or six replicates per treatment for each point of the analyses. Data were analysed using ANOVA with *a posteriori* comparison of the means using Tukey's test.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Comparison of the relative initial (T0) content of metabolites between TR9 and *Csol*.

Table S2. Distribution of the EPS on the cell surface of TR9 and *Csol* microalgae.

Fig. S1. Carbohydrate analysis of the microalgae TR9 and *Csol* during desiccation-rehydration. The relative contribution of the total major soluble carbohydrates of each compound is depicted as a percentage.

3. RESEARCH PAPER II

Tolerance to Cyclic Desiccation in Lichen Microalgae is Related to Habitat Preference and Involves Specific Priming of the Antioxidant System

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Tolerance to Cyclic Desiccation in Lichen Microalgae is Related to Habitat Preference and Involves Specific Priming of the Antioxidant System

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Oxidative stress is a crucial challenge for lichens exposed to cyclic desiccation and rehydration (D/R). However, strategies to overcome this potential stress are still being unraveled. Therefore, the physiological performance and antioxidant mechanisms of two lichen microalgae, *Trebouxia* sp. (TR9) and *Coccomyxa simplex* (Csol), were analyzed. TR9 was isolated from *Ramalina farinacea*, a Mediterranean fruticose epiphytic lichen adapted to xeric habitats, while Csol is the phycobiont of *Solorina saccata*, a foliaceous lichen that grows on humid rock crevices. The tolerance to desiccation of both species was tested by subjecting them to different drying conditions and to four consecutive daily cycles of D/R. Our results show that a relative humidity close to that of their habitats was crucial to maintain the photosynthetic rates. Concerning antioxidant enzymes, in general, manganese superoxide dismutases (MnSODs) were induced after desiccation and decreased after rehydration. In TR9, catalase (CAT)-A increased, and its activity was maintained after four cycles of D/R. Ascorbate peroxidase activity was detected only in Csol, while glutathione reductase increased only in TR9. Transcript levels of antioxidant enzymes indicate that most isoforms of MnSOD and FeSOD were induced by desiccation and repressed after rehydration. *CAT2* gene expression was also upregulated and maintained at higher levels even after four cycles of D/R in accordance with enzymatic activities. To our knowledge, this is the first study to include the complete set of the main antioxidant enzymes in desiccation-tolerant microalgae. The results highlight the species-specific induction of the antioxidant system during cyclic D/R, suggesting a priming of oxidative defence metabolism.

Keywords: Antioxidant • Coccomyxa • Desiccation tolerance • Lichen microalga • Priming • Trebouxia.

Introduction

The water status of lichens varies passively with surrounding environmental conditions as they are subject to continuous and relatively rapid cycles of desiccation and rehydration (D/R). During desiccation, most lichens enter into a latent state, named anhydrobiosis, until the water becomes available again and then they recover their normal metabolism. This behavior is lethal for most living beings; however, most lichens orchestrate a repertoire of physiological, biochemical and molecular mechanisms to prevent cellular damage during desiccation. In this context, some of the mechanisms include the accumulation of sugars, and some derivatives enable maintenance of cell membrane integrity and glass state formation (Centeno et al. 2016). In addition, the constitutive synthesis of late-embryogenesis-abundant proteins helps to prevent protein denaturation (Gasulla et al. 2009, Carniel et al. 2016), and the presence of polar oligogalactolipids preserves the thylakoid structure (Gasulla et al. 2016).

One of the most important challenges that lichens in general, and their microalgae in particular, have to cope with is the oxidative stress associated with changes in water content. Aerobic metabolism, such as photosynthesis and cell respiration, unavoidably and constantly generates reactive oxygen species (ROS) in chloroplasts, mitochondria, peroxisomes, etc. (Gill and Tuteja 2010, Inupakutika et al. 2016). Under optimal conditions, ROS do not cause cellular damage since they can be kept at life-compatible levels by antioxidant mechanisms (Inupakutika et al. 2016 and references therein). However, redox homeostasis can be perturbed by different stress factors such as D/R. During the initial part of their rapid dehydration and the first minutes upon rehydration, a burst of intracellular ROS occurs in lichen microalgae (Weissman et al. 2005a, Catalá et al. 2010, Álvarez et al. 2015). This occurs in part because lichen algae do not significantly modify their content of photosynthetic pigments during D/R. Therefore, the chloroplast can increase its ROS formation rate since CO_2 fixation is rapidly impaired, whereas light continues to be absorbed by chlorophyll and electrons transported to O_2 at both PSI and PSII (Mubarakshina Borisova et al. 2012, Roach and Krieger-Liszkay 2014, Smirnoff and Arnaud 2019), forming primarily singlet oxygen, superoxide anion radicals ($O_2^{\bullet -}$) and H_2O_2, and secondarily, hydroxyl radical ($OH^{\bullet}$), the most reactive and toxic ROS. In addition, $OH^{\bullet}$ can also be generated at a neutral pH by the Fenton reaction between H_2O_2 and $O_2^{\bullet -}$ catalyzed by transition metals such as Fe (Fe^{2+}, Fe^{3+}) (Inupakutika et al. 2016).

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The increased and unbalanced formation of ROS is thought to be one of the most important sources of damage to proteins, lipids and nucleic acids, which ultimately results in cellular injury and death (Gill and Tuteja 2010). Lichens employ several mechanisms to avoid ROS formation, such as the activation of the xanthophyll cycle (Fernández-Marín et al. 2010) and alternative mechanisms that dissipate the excess of energy during desiccation (Gasulla et al. 2009, Komura et al. 2010, Heber et al. 2011). Complementarily, scavenging of formed ROS can be carried out by enzymatic or nonenzymatic antioxidants. Among the nonenzymatic constituents, reduced glutathione (GSH) (Kranner et al. 2005), polyols, phenolic compounds and ascorbic acid (Centeno et al. 2016) are within the most important water-soluble antioxidants in lichens. The principal enzymatic constituents include superoxide dismutase (SOD), ascorbate peroxidase (APx), catalase (CAT), glutathione reductase (GR), monodehydroascorbate reductase and dehydroascorbate reductase. There is a profuse and compelling body of evidence that supports a positive correlation between drought resistance and the activity of the antioxidant system in vascular plants (e.g. Kranner et al. 2002, Farrant et al. 2003, Dinakar and Bartels 2013, Georgieva et al. 2017).

It could be expected that lichens as holobionts and their microalgae partners, in particular, show higher levels of desiccation tolerance (DT) and have higher antioxidant enzymatic activity than less-tolerant species. However, from the few comparative studies carried out with lichens and/or isolated photobionts, no clear relationship between DT and levels of antioxidant enzymes can be formed. Mayaba and Beckett (2001) observed that the activities of SOD, CAT and APx were similar under wetting and drying conditions in *Peltigera polydactyla*, *Ramalina celastri* and *Teloschistes capensis*, which thrive in moist, xeric and extremely xeric habitats, respectively. Kranner (2002) did not observe a correlation between GR activity and the different degrees of DT of three lichens, *Lobaria pulmonaria*, *P. polydactyla* and *Pseudevernia furfuracea*. Weissman et al. (2005b) even reported that after rehydration, *Ramalina lacera* loses almost all CAT activity, and SOD activity decreases by 50–70%. On the other hand, in cultured isolated photobionts, antioxidants and antioxidant-related activities seem to be less effective than those of lichenized photobionts, and a clear activation of the studied antioxidant enzymes in response to desiccation has not been observed (Kranner et al. 2005, Gasulla et al. 2009). Carniel et al. (2016) also observed, through a transcriptomic analysis, that only one antioxidant gene, a manganese superoxide dismutase (*MnSOD*), was overexpressed in the lichen alga *Trebouxia gelatinosa* in response to dehydration. More recently, the same group has confirmed this result and reported an increase in transcript levels corresponding to an APx during dehydration (Banchi et al. 2018). In summary, within the limits of these scarce and partial data, it seems clear that high concentrations of antioxidants and/or antioxidant enzyme activities do not indicate 'per se' that a species could be tolerant to desiccation-induced oxidative stress. Alternatively, and in line with the proposed by Kranner et al. (2002) and Kranner et al. (2003), the ability to maintain, during desiccation, or rapidly re-establish, during rehydration, some critical levels of antioxidants and enzyme activities seems to be characteristic of well-adapted species. Accordingly, recent results with the resurrection plant *Haberlea rhodopensis* demonstrated that the polyphenolic antioxidant and antioxidant enzymes involved in the ascorbate-glutathione cycle increased during desiccation, peaking at an air-dry state (Georgieva et al. 2017). It is proposed that the induction of antioxidant metabolism during dehydration can protect the plant from oxidative stress during drying and afterwards during the early stages of rehydration.

In the present study, we employed two microalgae, *Trebouxia* sp. TR9 (TR9) and *Coccomyxa simplex* (Csol), isolated from lichens with different hydric requirements. TR9 is a phycobiont of the lichen *Ramalina farinacea*, a Mediterranean epiphytic fruticose ascolichen that can withstand long desiccation periods and frequent daily D/R cycles. On the other hand, Csol was isolated from *Solorina saccata*, a foliaceous lichen that is widely distributed within relatively more humid areas from the Mediterranean mountains to the Arctic where it grows on calcareous rocks, typically in crevices and always under sheltered conditions (Krog and Swinscow 1986). In a previous study, performed with these algae species exposed to one cycle of desiccation at 25% relative humidity (RH) followed by rehydration at saturating humidity, metabolite profiling and cell wall analysis suggested that TR9 could be predisposed to tolerate desiccation mainly by constitutive mechanisms together with some inducible components (Centeno et al. 2016). In contrast, in Csol, inducible responses seem to play a more crucial role, suggesting that each microalga has evolved different strategies of DT according to the water regimen of their habitats. Although the ecology of *R. farinacea* and *S. saccata* and the physiological studies (Centeno et al. 2016) indicated that TR9 and Csol differ in their DT degree, it has never been empirically demonstrated. In addition, a common feature of studies concerning the effects of desiccation on isolated phycobionts is that algae are grown on semi-solid cultures for weeks and then submitted to a single cycle of D/R (e.g. Carniel et al. 2016, Centeno et al. 2016, Banchi et al. 2018). However, in their natural habitats, they are exposed to seasonal and/or almost continuous daily cycles of D/R. Thus, the initial steps in the present study were to test the tolerance of both phycobionts to different drying conditions. Thereafter, we followed an approach that tended to mimic the daily changes in the water regimen that the selected microalgae could experience in their natural habitat. TR9 and Csol underwent consecutive daily cycles of D/R under 'species-specific' conditions of dehydration for each alga (22–25% RH for TR9 and 55% RH for Csol) to further determine their degree of DT (estimated as recovery of photochemical efficiency upon rehydration). Additionally, we carried out a comparative analysis of the activity of the main antioxidant enzymes and their gene expression during four cycles of D/R to shed light on the role of the antioxidant system in the acquisition of DT in lichen algae.

Results and Discussion

Assessing the desiccation tolerance of TR9 and CsoI, two lichen microalgae with different habitat preferences, through their photosynthetic response to different environmental conditions

To assess the possible differences in the degree of DT between two ecologically distinct lichen microalgae, isolated TR9 and CsoI were dried and maintained up to 3 months in atmospheres with a RH of 22–25%, 37% (these RHs are within the range of RH during Mediterranean summer days) and 56% (this RH is within the range of that during summer days in *S. saccata* habitats). The capacity to recover the photosynthetic activity (estimated as the maximum quantum efficiency yield of PSII, F_v/F_m) upon rehydration was monitored up to 3 months (**Fig. 1A–C**). Isolated TR9 microalgae showed higher initial F_v/F_m rates (0.75) compared with CsoI microalgae (0.65). In addition, TR9 microalgae were capable of recovering the photosynthetic activity after desiccation for 1, 2 and 3 months, even when different air-dried conditions were applied (25%, 37% and 56% RH), showing a slight decrease over time (**Fig. 1A–C**). In contrast, CsoI microalgae showed a drastic drop in F_v/F_m ratio from the first month of exposure at both 25% and 37% RH (**Fig. 1A, B**), suggesting that the photosynthetic machinery of this species becomes irreversibly damaged during desiccation under such conditions. However, when exposed to 56% RH, CsoI microalgae demonstrated a better recovery of photosynthetic levels (**Fig. 1C**). These results support the idea that TR9 and CsoI microalgae have a higher physiological recovery when exposed to conditions similar to those of their natural habitats. TR9 is a phycobiont of *R. farinacea*, a lichen adapted to xeric habitats and usually subjected to daily/seasonal cycles of D/R, while CsoI thrives within *S. saccata*, a lichen that grows in relatively more humid areas (Krog and Swinscow 1986, Centeno et al. 2016). These results agree with those of Gray et al. (2007), who investigated the photorecovery of phylogenetically close desert and aquatic algae and found that dehydration-tolerant algae recovered photosynthesis upon rehydration from a desiccated state faster than their aquatic relatives.

In view of the abovementioned results and those from a previous study in which, when desiccated under 25% RH, CsoI lost water much faster than TR9 (Centeno et al. 2016), a new set of experiments was performed aiming to evaluate the hydric status and the photosynthetic recovery capacity after up to four cycles of D/R within a more natural context. Therefore, desiccation was performed under species-specific RH (25% to TR9 and 56% to CsoI) and two different drying velocities (slow and rapid). The results showed that there were no differences between the RWC (RWC) curves of both species; 50% of the total desiccation (50D) occurred after approximately 3–4 h at slow desiccation and between 1 and 2 h at rapid desiccation (**Fig. 1D, E**). These results contrast with previous results (Centeno et al. 2016) and emphasize the notion that the hydric behavior of each lichen algae seems to be highly dependent not only on the RH during desiccation in absolute terms but also on the temporal evolution of this parameter during drying.

Indeed, when submitted to rapid desiccation conditions along four D/R cycles, CsoI microalgae did not completely recover its initial F_v/F_m level, which seemed to decrease after subsequent cycles, reaching 0.379 after 16 h rehydration in the last cycle (**Fig. 1G**). In contrast, TR9 microalgae could maintain their photosynthetic activity even after four cycles of D/R (~0.700). In addition, TR9 appeared to recover the photosynthetic activity faster, with a complete recovery after 1 h of rehydration, while in CsoI, the recovery appeared to be progressive and partial (**Fig. 1G**). Additional experiments in which the F_v/F_m were also measured during four D/R cycles employing other RH conditions (RH 90–50%, 50%, 30% and 10%) yielded similar results (Supplementary Fig. S3). These data support the idea that TR9 microalgae fully recover their photosynthetic capacity independently of the humidity condition applied, contrasting with the more sensitive behavior of CsoI.

Moreover, when subjected to daily slow desiccation cycles in air-dried conditions similar to those of their natural habitat (**Fig. 1F**), both species were able to fully recover their photosynthetic rates after the four D/R cycles. In poikilohydric organisms, survival to desiccation is considered to rely on the presence of constitutive mechanisms and the induction of mechanisms of repair only after R (Oliver et al. 1998, Farrant and Moore 2011). Our results suggest that drying velocity may play a crucial role in CsoI microalgae function (**Fig. 1F, G**), which could be related to the induction of a protective system during the desiccation step.

Both microalgae presented low F_v/F_m when completely desiccated (100D) (**Fig. 1F, G**) under slow and rapid drying conditions. It is known that dehydration suppresses photosynthesis in both desiccation-sensitive and desiccation-tolerant algae (Kranner et al. 2003, Gray et al. 2007, Holzinger et al. 2014). However, as reported by Challabathula et al. (2018) 'although the photosynthetic rates decrease in both desiccation-tolerant and sensitive plants during drought, the remarkable difference lies in the complete recovery of photosynthesis after rehydration in desiccation-tolerant plants'.

On the molecular and cellular level, the desiccation process is associated with increased ROS formation as by-products of metabolism, particularly in the electron transport chains of respiration and photosynthesis (Kranner et al. 2008). In the same way, during rehydration, a rapid and important increase in ROS can also occur (Weissman et al. 2005a, Catalá et al. 2010, Álvarez et al. 2015). Therefore, within the wide range of DT mechanisms to cope with D/R, one of the most important is based on a coordinated regulation of the antioxidant system to preserve redox homeostasis (Alscher et al. 2002, Gechev et al. 2013).

The following step in our research was aimed at providing insights into the role of the antioxidant system as a species-specific strategy of TR9 and CsoI to cope with D/R. We carried out a comparative analysis of the activity of the main

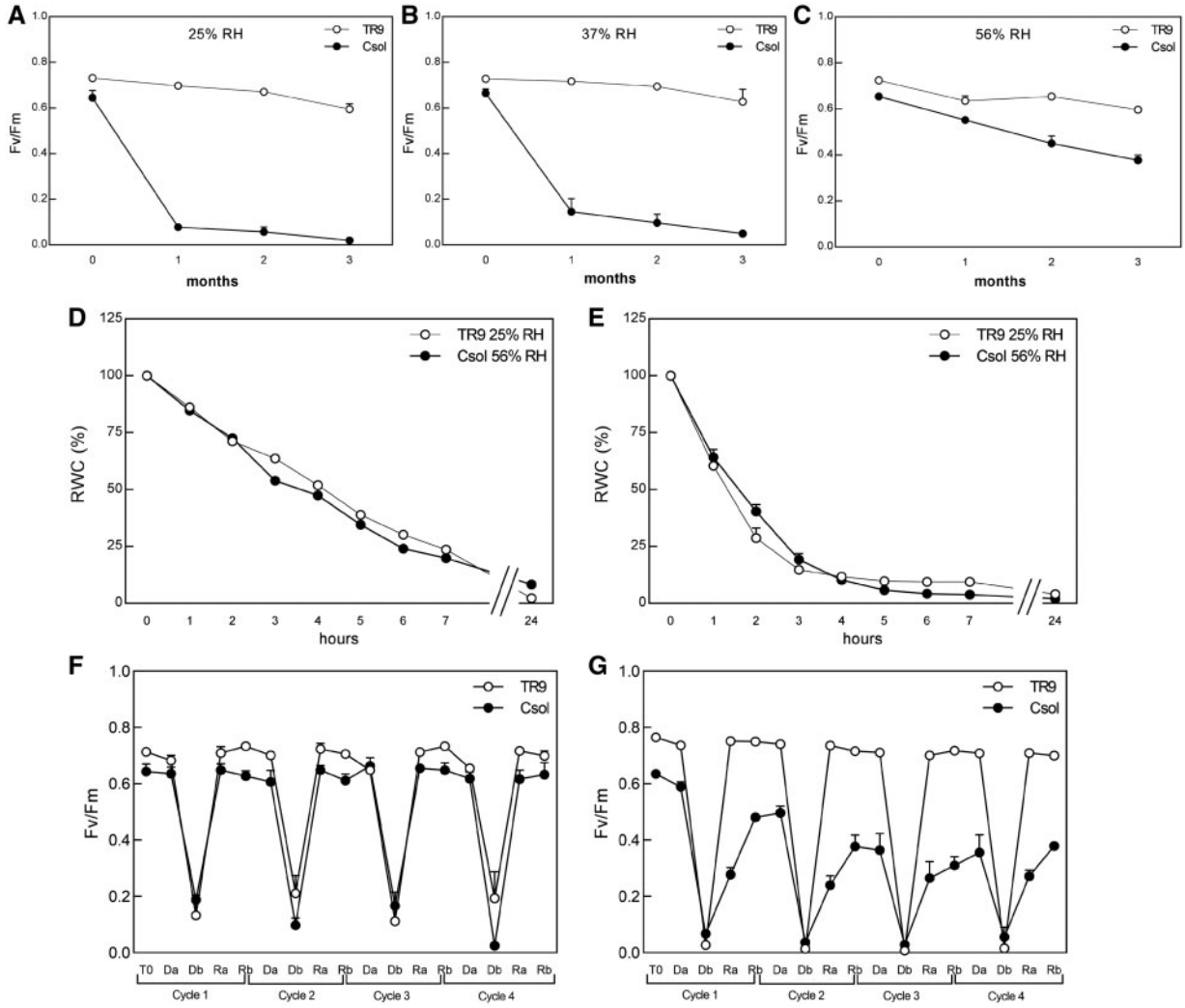

Fig. 1 Effects of the desiccation condition on the RWC and maximal photochemical yield of PSII (F_v/F_m) in TR9 and Csol microalgae. (A–C) F_v/F_m recovery after rehydration from desiccation under 25%, 37% and 56% RH for up to 3 months. RWC evolution at slow (D) and rapid desiccation (E) conditions. F_v/F_m changes in microalgae submitted to slow (F) and rapid (G) desiccation. Error bars show the standard error. T0, T0′ and T0″ indicate initial control, and controls of cycles 2 and 4, respectively; Da and Db indicate desiccation treatments with 50% and 100% of the minimal RWC value, respectively; Ra and Rb indicate treatments after 1 and 16 h of rehydration, respectively.

antioxidant enzymes and their gene expression during four cycles of D/R in these lichen algae.

Antioxidant enzyme response induced by desiccation-rehydration cycles

In resurrection plants, an increase in the antioxidant activities of APx, GR, SOD and CAT has been reported during dehydration. This activity can remain high at lower water contents (Farrant, 2000, Kranner et al. 2002, Farrant et al. 2003, Dinakar and Bartels 2013, Georgieva et al. 2017).

In lichens and their photobionts, antioxidant mechanisms include protective enzymes, such as SOD, CAT, peroxidases, GR and APx, in combination with nonenzymatic substances such as glutathione, α-tocopherol and ascorbic acid (Kranner et al. 2005, Kranner and Birtić 2005, Weissman et al. 2005a, Weissman et al. 2005b). SOD acts as the first line of defence by converting $O_2\bullet^-$ into H_2O_2, preventing the formation of highly toxic compounds. Then, CAT and APx transform H_2O_2 into water. On the other hand, GR, monodehydroascorbate reductase and dehydroascorbate reductase are involved in the regeneration of reduced glutatione and ascorbic acid (Inupakutika et al. 2016).

In the present study, the antioxidant enzyme activities were measured by spectrophotometric assay and zymogram analysis. In general, results indicate a diverse antioxidant response during the D/R cycle treatments.

SODs are a group of metallo-enzymes containing Fe, Mn or CuZn in their prosthetic group and are present in mitochondria, chloroplasts, cytosol, peroxisomes and apoplast (Alscher et al. 2002). There is no evidence of the presence of CuZnSOD isoforms, at least in most Chlorophyta algae (Inupakutika et al. 2016). The zymogram technique allowed us to separate the SOD isoforms according to their electrophoretic mobility into three groups of MnSODs, named MnSOD-A, MnSOD-B and MnSOD-C, and one FeSOD isoform (**Figs. 2A, 3A**). The activity

of each band/band group was estimated by image analysis (**Figs. 2B–E, 3B–E**). No clear trend was found regarding the SOD activity of TR9 photobionts (**Fig. 2A–E**), despite the increase in MnSOD-B and MnSOD-C isoforms, during cycles 2 and 4 (**Fig. 2C, D**). However, it is noteworthy that the increased values of T0′ and T0″ activities of both groups of MnSOD isoforms suggest an acclimation induced by successive cycles of D/R. On the other hand, changes in SOD activity were more striking in Csol photobionts. The activity of all isoforms, including FeSOD, clearly increased under desiccation and/or rehydration conditions but did not significantly change the initial activity levels of cycles 2 and 4 (T0′ and T0″, respectively, **Fig. 3B–E**). The different response at the level of SOD activity carried out by TR9 and Csol seems to reveal contrasting strategies from the first defence line of the antioxidant system on. Gasulla et al. (2009) found that SOD activity declined in *Asterochloris erici* during the desiccation period and during the first 24 h of recovery. A recovery in the antioxidant activity was observed only after 48 h at RH 67%.

CAT is involved in the rapid detoxification of H_2O_2 (Kranner et al. 2008), especially under conditions of high rates of H_2O_2 production (e.g. high SOD activity), due the low affinity for H_2O_2 of CAT (Smirnoff and Arnaud 2019). Their activity in lichens has been shown to vary greatly among species (Silberstein et al. 1996, Mayaba and Becket 2001, Weissman et al. 2005b). The activity of this enzyme was assayed by zymogram and semi-quantified as described for SOD. Zymograms revealed two bands of CAT isoforms in TR9 (**Fig. 2F**): CAT-A, which progressively increased during the second D/R cycle compared with the initial T0 and appeared to maintain its enzymatic activity during the subsequent D/R cycles (**Fig. 2G**). On the other hand, no significant differences were detected in the activity of the second isoform CAT-B (**Fig. 2H**). In contrast, no significant changes were observed in CAT activity (spectrometrically measured) in TR9 photobionts under Pb stress, which increases the production of ROS (Álvarez et al. 2012). Only one CAT isoform was found in Csol, which progressively increased through the four cycles of D/R (**Fig. 3F, G**).

APx is relatively specific to H_2O_2 since it does not metabolize other peroxides at high rates and participates in the 'ascorbate-glutathione' pathway in which H_2O_2 reduction is ultimately linked to NAD(P)H oxidation via ascorbate and glutathione pools (Noctor et al. 2012). According to previous results, no detectable levels of ascorbate were found in *Trebouxia* lichen photobionts (Kranner et al. 2005, Gasulla et al. 2009), including TR9 (Centeno et al. 2016). In this species, GR activity was characterized by a relatively low initial T0 level (0.051 ± 0.012 μmol NADPH·min^{-1}·mg^{-1}), which was significantly increased after D/R cycles 2 and 4 (**Fig. 2I**). A similar behavior was observed in the same microalgae exposed to other stressful conditions that

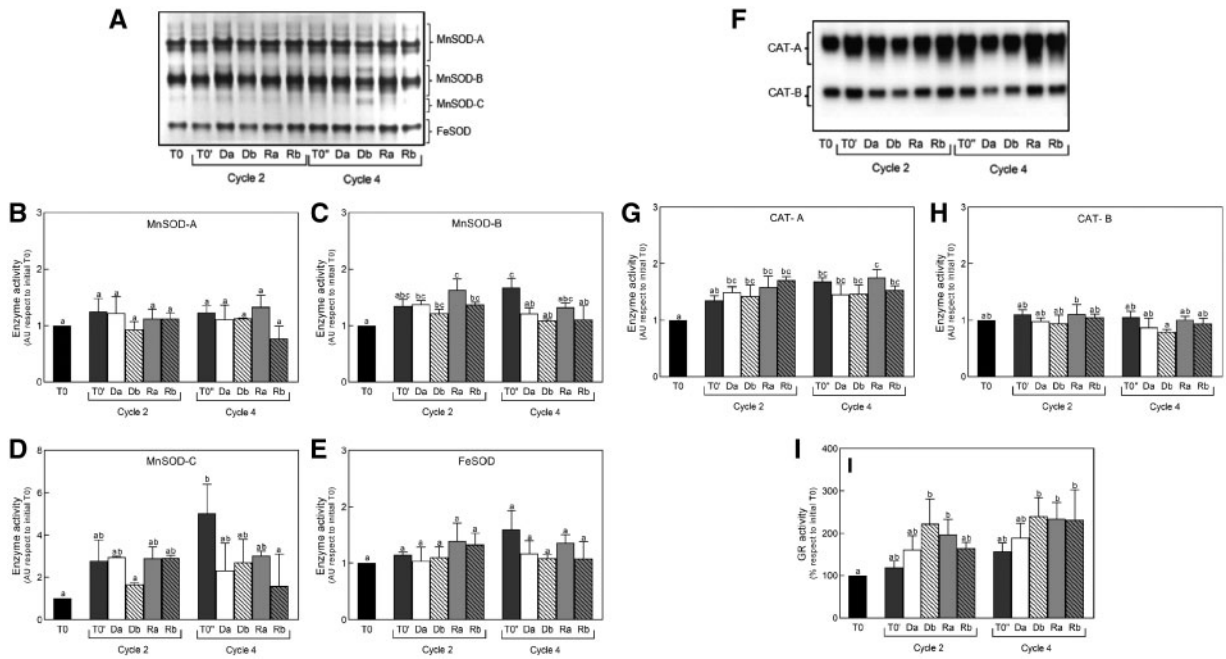

Fig. 2 Effects of cyclic desiccation-rehydration on SOD, CAT and GR activities in TR9 microalgae. (A) Image showing a representative SOD zymogram. SOD isoforms were grouped as indicated according their electrophoretic mobility and metal cofactor. (B–E) Enzyme activity of groups of SOD isoforms (MnSOD-A, MnSOD-B, MnSOD-C, FeSOD) calculated by the mean image analysis of three to six zymograms from independent biological samples. (F) Image depicting a representative CAT zymogram. (G, H) CAT activity was estimated from the image analysis of three to six zymograms from independent biological samples. Values in (B–E) and (G, H) are expressed in arbitrary units (AU). (I) Total GR activity determined by spectrophotometric assay. Values are expressed as percentages with respect to the initial T0 values (μmol NADPH·min^{-1}·mg^{-1} = 0.051 ± 0.012). Error bars show the standard error. Black bars indicate initial control treatment (T0); dark gray bars indicate controls of cycles 2 and 4 (T0′ and T0″, respectively); white solid and striped bars indicate desiccation treatments with 50% (Da) and 100% (Db) of the minimal RWC value, respectively; light gray solid and striped bars indicate treatments after 1 h (Ra) and 16 h (Rb) of rehydration, respectively.

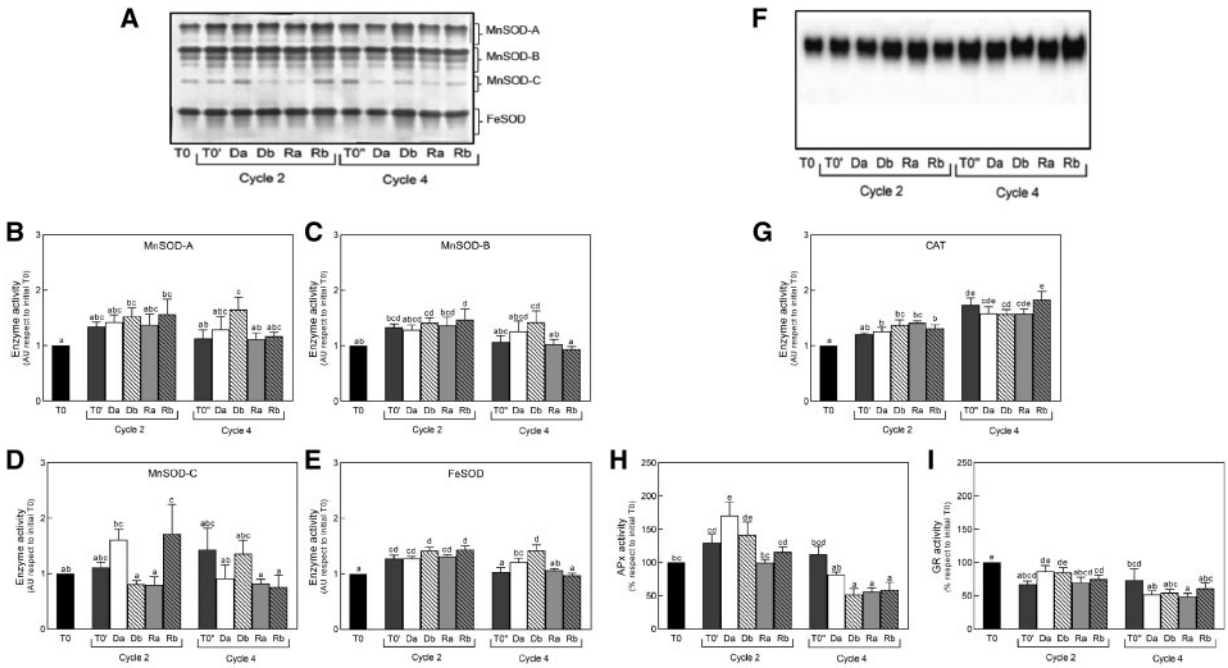

Fig. 3 Effects of cyclic desiccation-rehydration on SOD, CAT, Apx and GR activities in Csol microalgae. (A) Image showing a representative SOD zymogram in which the SOD isoforms were grouped as indicated in legend to **Fig. 2**. (B–E) Enzyme activity of groups of the SOD isoforms (MnSOD-A, MnSOD-B, MnSOD-C, FeSOD) calculated by the mean image analysis of three to six zymograms from independent biological samples. (F) Image depicting a representative CAT zymogram. (G) CAT activity was estimated from image analysis of three to six zymograms from independent biological samples. Values are expressed in AU. (H) Total Apx and (I) GR activity were determined by spectrophotometric assay. Values are expressed in percentages with respect to initial T0 values (μmol ascorbate·min^{-1}·mg^{-1} = 0.790 ± 0.207 and μmol NADPH·min^{-1}·mg^{-1} = 0.262 ± 0.098, respectively). Values represent the mean of three biological replicates of two independent experiments. Error bars show the standard error. Details of color and pattern of bars are given in the legend to **Fig. 2**.

promote ROS formation, such as Pb and xenobiotic treatments, in which GR activity was highly induced from low basal levels (del Hoyo et al. 2011, Álvarez et al. 2012).

On the other hand, Csol presented a marked increase in APx activity during desiccation of cycle 2, which then returned to the initial T0 (0.790 ± 0.207 μmol ascorbate·min^{-1}·mg^{-1}) levels during rehydration of the same cycle (**Fig. 3H**). A progressive decrease in APx activity was observed during cycle 4. Notably, Csol GR activity was characterized by a high initial level (0.262 ± 0.098 μmol NADPH·min^{-1}·mg^{-1}) that was approximately five times higher than that of TR9. In Csol, GR activity (**Fig. 3I**) showed a quite stable behavior during cycle 2, and a relative decrease in cycle 4 similar to that of APx activity. These results lead to the idea that, in the Csol photobiont, APx, GR and the 'ascorbate-glutathione' pathway could be relevant during the first cycles of D/R treatments, while SOD and CAT could play a more important role in later steps of cyclic D/R conditions.

The lack of ascorbate in TR9 photobionts, as shown previously (Centeno et al. 2016), and the induction of GR activity under cyclic D/R (this study) could indicate that this microalga uses, in addition to CAT, an alternative route(s) to the ascorbate-glutathione pathway for keeping the H_2O_2 level within a range compatible with life preservation. GSH could be linked to H_2O_2 and/or organic peroxide reduction by at least two ascorbate-independent routes involving certain types of peroxiredoxin (PRX) and/or glutathione S-transferase (Noctor et al. 2012). In addition, phenolic substrate can be oxidized by H_2O_2 using type III peroxidase in the vacuole (Smirnoff and Arnaud 2019). Moreover, our previous studies based on metabolomics analysis showed that TR9 photobionts have more polyols and phenolic compounds than Csol photobionts, which could also help act as ROS scavengers, avoiding oxidative damage (Centeno et al. 2016).

Despite the advances in the last few years, it has not yet been possible to establish a clear relationship between stress tolerance level and antioxidant mechanisms. To improve our knowledge of the role of antioxidant enzymes in the DT of TR9 and Csol photobionts, a detailed analysis of the transcript levels of all SOD, CAT, APx and GR encoding genes was carried out.

Changes in antioxidant transcript levels induced by desiccation-rehydration cycles

This is the first study to characterize changes in the almost/ nar complete set of the main antioxidant transcripts of lichen microalgae within the context of several daily cycles of D/R. TBLAST analysis revealed the existence of 10 genes encoding antioxidant enzymes in the TR9 microalgae, of which two corresponded to *CAT*, five corresponded to *SOD*, two corresponded to *GR* and one corresponded to gamma-glutamylcysteine ligase (*GCL*) enzyme (**Fig. 4**). In the case of Csol, the transcripts of the antioxidant genes matched as follows: four

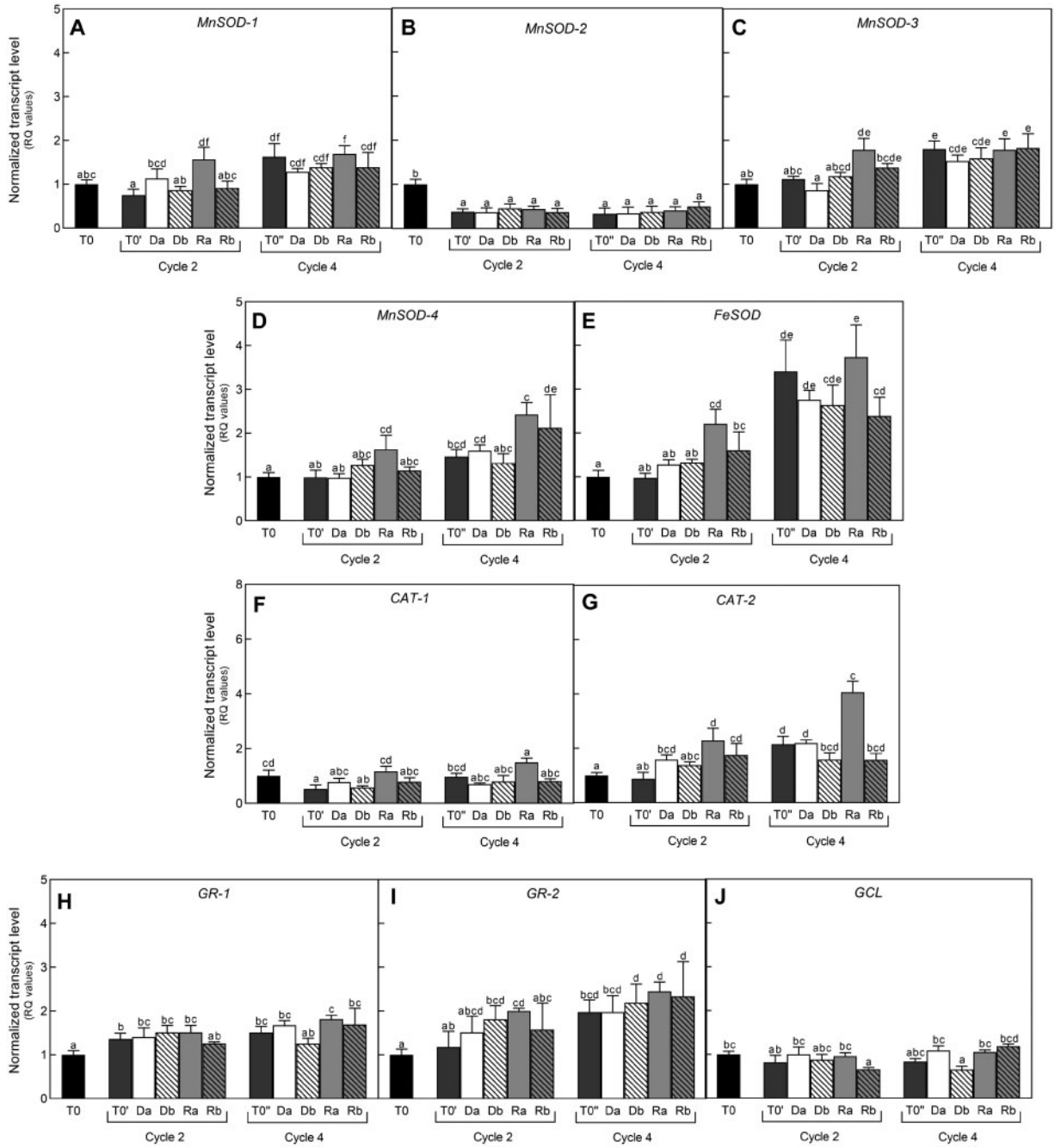

Fig. 4 Changes in the transcript levels of antioxidant encoding genes of TR9 microalgae exposed to cyclic desiccation-rehydration. mRNA levels of genes encoding SOD, *MnSOD-1*, *MnSOD-2*, *MnSOD-3*, *MnSOD-4*, *FeSOD* (A–E, respectively), CAT, *CAT-1 and CAT2* (F, G, respectively), GR, *GR-1*, *GR-2* (H, I, respectively) and *GCL* (J) genes. Values were calculated with $2^{-\Delta\Delta Ct}$ relative quantification for five biological replicates. Error bars show the standard error. Details of color and pattern of bars are given in the legend to **Fig. 2**.

to *CAT*; six to *SOD*; three to *GR*; one to *GCL*; and two to *APx* nonredundant transcripts. From these Csol genes, three *CAT*, one *SOD*, one *GR* and one *APx* were not included in the quantitative expression analysis because their transcripts were barely detectable, indicating that these genes are nonfunctional, at least under the conditions employed in our study. The expressed Csol antioxidant genes are shown in **Fig. 5**.

FeSODs are located in the chloroplast; MnSODs are located in the mitochondrion and peroxisome; and CuZn SODs are located in the chloroplast, cytosol, and possibly in the extracellular space (Alscher et al. 2002). Most eukaryotic algae contain either FeSOD or MnSOD or both (Wang et al. 2011). The results of the TR9 transcript level demonstrated different transcriptional responses during D/R cycles depending on the SOD isozyme-encoding gene (**Fig. 4**). While transcript levels of *MnSOD-2* decreased and those of *MnSOD-1* and *MnSOD-3* slightly increased (**Fig. 4A–C**, respectively), the amount of *MnSOD-4* and *FeSOD* transcripts increased during D/R cycles

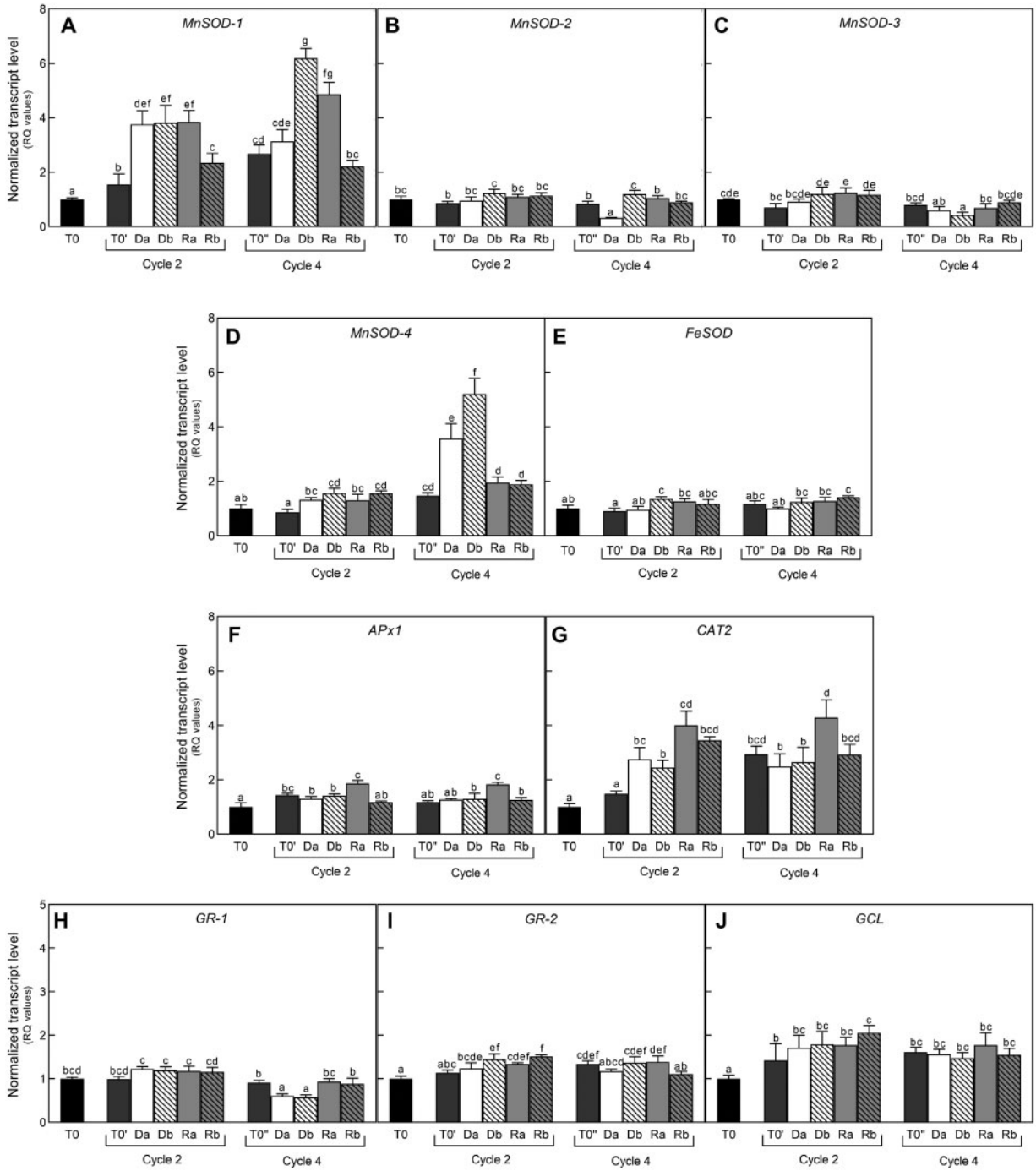

Fig. 5 Changes in the transcript levels of antioxidant encoding genes of CsoI microalgae exposed to cyclic desiccation-rehydration. mRNA levels of genes encoding SOD: *MnSOD-1*, *MnSOD-2*, *MnSOD-3*, *MnSOD-4*, *FeSOD* (A–E, respectively); APx: *APx1* (F); CAT: *CAT* (G); GR: *GR-1*, *GR-2* (H, I, respectively) and *GCL* (J). Values were calculated with $2^{-\Delta\Delta Ct}$ relative quantification for five biological replicates. Details of color and pattern of bars are given in the legend to **Fig. 2**.

(**Fig. 4D and E**, respectively). In CsoI, the *MnSOD-1* and *MnSOD-4* transcripts also increased compared with the initial control during the successive cycles of D/R (**Fig. 5A, D**) and demonstrated a transcriptional increase during desiccation treatments (50D and 100D) at cycle 4. In this species, the expression of the other two *MnSOD* and *FeSOD* genes did not change (**Fig. 5B, C, E**). Studies have shown that SOD transcripts are induced in plant species under different stresses, such as heat, cold, drought, osmotic, salt and oxidative stresses (Wang et al. 2011, Feng et al. 2016, Zhou et al. 2017). In tomato, among nine SOD genes, four (SlSOD2, SlSOD5, SlSOD6 and SlSOD8) were upregulated under drought treatment (Feng et al. 2016). In *T. gelatinosa*, an over-expression of one putatively mitochondrial *MnSOD* gene and enzyme upregulation upon dehydration

was observed, probably as a response to *MnSOD* mRNAs built up in preparation to rehydration (Carniel et al. 2016).

Ascorbate and thiol peroxidases have numerous isoforms and are found in all subcellular compartments (Smirnoff and Arnaud 2019). Some studies have reported an increase in stress resistance as a result of the over-expression of peroxidases, such as APx (Dietz 2016, Maruta et al. 2016). In *Klebsormidium crenulatum*, an alpine aeroterrestrial alga, desiccation induced the transcriptional upregulation of genes, including those involved in the ascorbate-glutathione pathway (Holzinger et al. 2014). As mentioned earlier, *TR9* and *Csol* seem use different metabolic strategies for keeping the H_2O_2 within homeostatic levels. The absence of detectable levels of APx-encoding genes supports the absence of ascorbate in *Trebouxia* phycobionts (Kranner et al. 2005, Gasulla et al. 2009) and with previous results concerning the metabolic profile of *TR9* microalgae (Centeno et al. 2016). On the other hand, ascorbate seems to play an important role in Csol microalgae (Centeno et al. 2016). Ascorbate peroxidase-encoding gene (*APx1*) transcript levels analysis revealed an increase during the first hour of rehydration (**Fig. 5F**) and this gene is probably related to the activation and/or preservation of the antioxidant system to deal with high ROS formation inside cell compartments. The desiccation-induced increase in APx activity observed during the second D/R cycle (**Fig. 3H**) could be partially explained by the transcriptional induction of the *APx1* gene. Besides, the overall trend of APx activity in the second D/R cycle closely resembled the variation for ascorbic acid previously observed during one cycle of D/R in the same microalga (Centeno et al. 2016). This is consistent with strong evidences demonstrating that the stability of APx is highly dependent on the concentration of ascorbic acid (Shigeoka et al. 2002 and references therein).

Corroborating the zymogram analysis, CAT genes were expressed in TR9 and Csol. Both *CAT* isoforms, *CAT1* and *CAT2*, found in TR9 and *CAT2* from Csol presented a similar profile (**Figs. 4F, G, 5G**). The transcript level increased significantly during D/R cycles compared with the initial T0 and rehydration after 1 h of rehydration (1R) (**Figs. 4G, 5G**). CAT is most likely restricted to peroxisomes (Smirnoff and Arnaud 2019). Peroxisomal CAT mutants have been studied in *Arabidopsis* and tobacco and show a key role in H_2O_2 removal (Queval et al. 2007). Gechev et al. (2013) studied the CAT-encoding genes in the resurrection plant *H. rhodopensis*, and their transcript levels were high under desiccated and rehydrated conditions.

Previous studies have shown that over-expression of GR leads to an increase in cellular GSH levels in response to biotic and abiotic stresses (Foyer et al. 2001, Kouřil et al. 2003). The synthesis of GSH is a two-step process catalyzed by GCL and glutathione synthetase. GCL is the rate-limiting enzyme, as it is subject to feedback inhibition by the overproduction of GSH (Foyer et al. 2001). *GR* and *GCL* transcripts were quantified in both species (**Figs. 4, 5**H–J, respectively). In TR9 photobionts, the transcript levels of *GR1* and *GCL*, which are key enzymes for GSH synthesis, remained approximately constant throughout the experiment. However, expression of the gene encoding the *GR2* isoform progressively increased during D/R cycles (**Fig. 4I**), resembling the GR activity profile observed under the same conditions (**Fig. 2I**). These results strongly suggest that the *GR2* gene could be involved in the antioxidant response of TR9 to cyclic D/R. On the other hand, in Csol, the *GR1* (**Fig. 5H**) transcript level decreased at D/R cycle 4, while transcript levels of *GR2* (**Fig. 5I**) and *GCL* (**Fig. 5J**) slightly increased during D/R cycles compared with their initial T0 values. Under oxidative stress conditions, post-translational activation of GCL could contribute to the rapid increase in GSH synthesis in higher plants (Rausch et al. 2007). In *T. gelatinosa*, the upregulation of three microsomal glutathione *S-transferases* occurred during dehydration, suggesting that they are a part of the mechanism to keep the intracellular redox homeostasis (Carniel et al. 2016). Yobi *et al.* (2012) found that the levels of γ-glutamyl amino acids were significantly higher in *Selaginella lepidophylla* (desiccation-tolerant) than in *Selaginella moellendorffii* (desiccation-sensitive) in response to dehydration, which indicates that γ-glutamyl amino acids or glutathione are involved in the acquisition of DT. Therefore, the maintenance of *GCL* levels in TR9 and their increase in Csol could indicate different strategies to maintain glutathione homeostasis during D/R cycles.

A holistic view of transcriptions levels suggests the existence of an acclimation process, from a permanent hydrated state to cyclic desiccation periods. Some genes, such as TR9 *MnSOD-1*, *MnSOD-3*, *MnSOD-4*, *FeSOD*, *GR-2* and Csol *MnSOD-1*, *CAT*, *GR-2* and *GCL*, were likely induced by the increase in ROS formation provoked by cyclic D/R conditions. It should be noted that the induction of such a number of antioxidant genes, along with their enzymatic activities, has not been observed in lichen algae until the present study. This study has been possible by two essential facts: (i) we searched for and studied all the genes encoding the main antioxidant enzymes, and (ii) our study was not limited to one cycle of D/R but to several cycles, allowing us to observe acclimation processes that often occur under natural conditions. It is highly plausible that the failure to find inducible antioxidant mechanisms associated with DT in lichen thalli (obviously collected from its natural habitat) could be because those specimens were already acclimated. On the other hand, the lack of antioxidant response of isolated lichen algae to desiccation (Kranner et al. 2002; Gasulla et al. 2009) could be because cultures were only submitted to a single D/R cycle, which probably was not enough to acclimate. Our results with nonacclimated microalgae clearly indicate that, at least in part, their DT rely on their capacity to induce certain key genes along several D/R cycles.

The physiological process by which plants are able to activate defence responses faster, better or both is called *priming*. This may be initiated in response to an environmental cue and may also persist as a residual effect following an initial exposure to the stress (Filippou et al. 2013). In our results, the induction of antioxidant genes pointed to above could be included as *primed* responses within a specific biological context, such as lichen algae, which are endowed with relatively strong constitutive mechanisms of defence, suggesting a strategy of metabolic economy to cope with D/R stress. In addition, the fine and fast control of ROS levels seems to be crucial if most lichen algae are often subjected to daily variation in water availability. If part

of the enzymatic antioxidant system acts as a prompt and regulated response (according to the current ROS formation rate), it will confer a more efficient strategy against oxidative damage associated with desiccation stress.

Materials and Methods

Microalgae isolation and culture

The *Trebouxia* sp. TR9 was isolated from the lichen *R. farinacea* according to Casano et al. (2011), and *C. simplex* (formerly known as *C. solorinae-saccatae* strain 216-12) was obtained from Sammlung von Algenkulturen at Göttingen University (Germany). Both microalgae were axenically cultured on small nylon square membranes in semi-solid Bold 3N medium (Bold and Packer 1962) in a growth chamber maintained at a continuous temperature of 15°C and with light/dark cycles of 14 h (25 μmol PAR·m^{-2}·s^{-1})/10 h. All experiments were carried out with 21-day-old cultures (~180–200 mg FW).

Desiccation-rehydration treatments

In the first experiments, TR9 and CsoI cultures on the nylon squares were removed from the culture medium and subjected to slow desiccation in a closed container with saturated solutions of either potassium acetate (22–25% RH), $MgCl_2$ (35–37% RH) or $Mg(NO_3)_2$ (53–58% RH) within the growth chamber for up to 90 d. At the indicated times (**Fig. 1**), five cultures of each species were transferred onto water vapor-saturated sealed Petri dishes (Centeno et al. 2016) for up to 24 h under growth chamber conditions. The maximal photochemical PSII efficiency by chlorophyll *a* fluorescence was measured shortly before the end of the dark period in rehydrated samples.

The following experiments were performed in a phytotron (KK 115 Top+, Pol-Eko-Apparatus, Poland) in which, in addition to temperature and light conditions, some air hydric parameters such as RH and the speed of air movement can be controlled. Temperature was set at 15°C and the photoperiod in 14 h of light (25 μmol PAR·m^{-2}·s^{-1}) and 10 h of darkness. Desiccation treatment started with the light phase and rehydration began with 4–6 h under light followed by 10 h of darkness. For rapid desiccation experiments, cultures of each microalgae on their nylon supports were subjected to daily cycles of 8–10 h desiccation at 25–30% RH for TR9 or 55–60% for CsoI, moderate air movement (20% of maximum capacity), followed by 14–16 h rehydration in water vapor-saturated sealed Petri dishes. The slow desiccation experiments were performed under the same conditions except that the lowest air speed was employed. At the indicated intervals, 5–8 samples of TR9 and CsoI were collected and weighed to follow their RWC (**Fig. 1**) as described by Centeno et al. (2016). In other experiments, cultures of each microalgae were subjected up to four cycles of either rapid or slow desiccation. At 0%, 50% and 100% desiccation and after 1 and 14–16 h rehydration, five to eight samples were acclimated to darkness (when necessary), and their remnant photosynthetic capacity was assayed by fluorometry as described below. For the transcript level determination and the assay of antioxidant enzymes, TR9 and CsoI cultures were submitted to up to four daily cycles of slow D/R. At 0%, 50% and 100% desiccation and after 1 and 14–16 h R, five samples of each microalgae were collected. Every sample was fractionated into three aliquots (~40–100 mg each) and immediately frozen in liquid N_2 and maintained at −80°C until use.

Measurements of chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was measured in dark-acclimated algae using a modulated pulse fluorometer PEA (Plant Efficiency Analyser, Hansatech, UK). The initial, remnant or recovered photosynthetic capacity was assessed through the fluorescence parameter F_v/F_m (variable fluorescence/maximum fluorescence), which estimates the maximum quantum yield of PSII (Baker and Oxborough 2004). The minimum fluorescence (F_o) was obtained after dark adaption for 15 min and the maximum fluorescence (F_m) was determined with a 2.2 s saturating pulse (3,500 μmol photons·m^{-2}·s^{-1}) of red LED's light (peak wavelength 650 nm). Maximal variable fluorescence (F_v) was calculated as F_m–F_o.

Protein extraction and antioxidant enzyme activities

Frozen aliquots of TR9 and CsoI were homogenized using a steel microbead homogenizer (Retsch, MM400, Germany) in the presence of 50 mM Tris–Cl (pH 7.5), 2 mM EDTA, 1 mM ascorbic acid, 10% (v/v) glycerol and 1% (w/v) insoluble polyvinyl pyrrolidone. The homogenates were centrifuged at 20,000×*g* for 20 min at 4°C, and the supernatants were used for antioxidant enzyme assays. The total protein concentration in the extracts was determined spectrophotometrically with Coomassie Brilliant Blue according to Bradford (1976), using bovine serum albumin as a standard. The activities of the antioxidant enzymes APx and GR were carried out by spectrophotometric assays as described by Amako et al. (1994) and Schaedle and Bassham (1977), respectively.

In addition, SOD and CAT isoforms were assayed through specific zymograms in which 10 μg of total proteins were usually electrophoresed under native conditions, and the activity band(s) of each enzyme was revealed by specific staining (Woodbury et al. 1971, Álvarez et al. 2012). MnSOD isoforms were distinguished from FeSOD isoforms as described (Álvarez et al. 2012). SOD isoforms were grouped into four groups (**Figs. 2A, 3A**). The intensity (activity) of each SOD isoform group and CAT isoform(s) was estimated using ImageJ software and posteriorly corrected for possible protein loading biases (**Figs. 2B–H, 3B–G**). For this correction, the same extracts were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (Supplementary Fig. S1). At least two well-defined bands in all lanes were quantified using ImageJ.

Transcript levels of genes codifying antioxidant enzymes

RNA was extracted from half-algal culture (50–100 mg FW) following the protocol of Jones et al. (1985). The quality of isolated RNAs was checked on denaturing agarose gels. RNA was quantified using a NanoDrop ND-1000™ spectrophotometer (Daemyung, Korea). Coextracted DNA contaminants were degraded using the DNAse I RNA-free kit (Invitrogen, California, USA), and the complementary DNA (cDNA) was synthesized with the RevertAid First Strand cDNA Synthesis kit (Thermo-Fisher Scientific, Massachusetts, USA) using the supplied oligo-dT primer following the manufacturer's guidelines.

Sequences encoding enzymes involved in the antioxidant cell system were searched for in the translated TR9 transcriptome database (housed in our computers in the University of Alcalá) employing *Arabidopsis thaliana* cDNA sequences and the TBLASTX tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The cDNA sequences of antioxidant genes in the close relative *Coccomyxa subellipsoidea* C-169 (NCBI: txid 574566) were used to design a set of primers to amplify the respective homologous genes in CsoI employing cDNA as a template (Supplementary Table S1). Amplification products were sequenced with an ABI 3130 Genetic analyzer using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, California, USA).

Specific primers were designed for each antioxidant gene of TR9 (Supplementary Table S2) and CsoI (Supplementary Table S3) for transcript quantification by real-time PCR (qPCR). PCR amplification was carried out in a 10 μl total volume containing 1 μl of 10-fold diluted cDNA, 0.5 μl each primer and 5 μl 2X Fast SYBR-Green Master Mix (Applied Biosystems, CA, USA) using a 7500 Fast Real-Time PCR system (Applied Biosystems) under standard conditions of the apparatus. Five biological replicates and two technical replicates were used in this study. Two negative controls were included for each primer pair, in which cDNA was replaced by water or total RNA. The absence of nonspecific PCR products and primer dimers were verified by dissociation curves and by agarose gel electrophoresis. The amplification efficiency of each set of primers was checked to be higher than 95% following the standard curve method described by da Costa et al. (2015). Transcript quantifications were normalized to three reference genes (Supplementary Fig. S2): *Ap47* (Clathrin adaptor complexes subunit), *Act* (Actin) and *PP2A* (Serine/threonine protein phosphatase) following the strategy proposed by Vandesompele et al. (2002). Reference gene selection was based on studies conducted in other plant species subjected to abiotic stresses, including salinity and drought stress (da Costa et al. 2015 and references therein).

Statistical analysis

The changes in RWC and antioxidant enzyme activities during D/R cycles were repeated twice, starting with new cultures each time. All analyses were conducted with three or five replicates per treatment for each point of the analyses. Data were analyzed using the multiple sample comparison (Statgraphics Centurion XVII, 2016, StatPoint Technologies 2016) Fisher's least significant difference (LSD) test ($P < 0.05$).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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4. RESEARCH PAPER III

Polyols-related gene expression is affected by cyclic desiccation in lichen microalgae

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Highlights

- Expression of *myo*-inositol and raffinose-related genes is modulated by cyclic D/R
- NO concentration is induced by cyclic D/R, with a burst during rewetting
- Polyols play an important role on the osmoregulation
- NO signaling assists on the regulation of the osmoprotectants
- A primed metabolic state is suggested to occur

Abstract

The accumulation of compatible solutes, as sugar and polyols, in response to stress is a widely metabolic adaptation. However, the signaling process involved in this regulation remains unclear. Lichens and their microalgae have to cope with a variety of stresses associated with sudden changes in water content during daily cycles of desiccation-rehydration (D/R). In this way, we hypothesized: (1) the differences in desiccation tolerance among lichen-forming microalgae could be associated with species-specific differences in their polyol metabolism and (2) the nitric oxide (NO), as a signaling molecule, could assist the regulation of the expression of polyol-related genes in these organisms. Therefore, we carried out a comparative analysis of the transcriptional expression of key genes involved in the synthesis of polyols and metabolite levels, along with the cellular concentration of NO in isolated lichen microalgae during consecutive daily cycles of D/R and correlated the results with the polyol profile and NO measurements. Two microalgae were employed, *Trebouxia sp*. TR9 (TR9) and *Coccomyxa simplex* (Csol), obtained from lichens with different hydric requirements. Our results revealed that, in both microalgae, the expression of *myo*-inositol and raffinose-related genes is modulated by cyclic D/R. Although, genes seem more prone to be transcriptionally upregulated by cyclic D/R in Csol than in TR9, indicating a more inducible defensive response in Csol in contrast with a constitutive defense system in TR9. Correlation analysis provided evidences that NO may act as a signaling molecule, through the modulation of polyol biosynthetic pathway and may be related to a primed memory state, by assisting in the rapidly and more precise metabolic response to subsequent D/R cycles.

Key words: *Coccomyxa*, desiccation tolerance, transcriptional expression, nitric oxide, lichen microalga, *Trebouxia*.

1. Introduction

Lichens are desiccation-tolerant symbiotic associations exposed to seasonal and/or almost continuous daily cycles of desiccation-rehydration (D/R) in their natural habitats. Following the air-drying, they can survive in a state of suspended animation and when water became available again, they resume their normal metabolism (Kranner et al., 2008). During the daily cycles of D/R, lichens and their microalgae have to cope with a variety of stresses associated with sudden changes in water content, one of them is the osmotic stress and other, the oxidative stress. Polyols seem to play important roles to cope with both stresses and represent the major type of osmoprotectant sugar in green microalgae (Roser et al., 1992; Centeno et al., 2016). Chemically, polyols are alcohols containing multiple hydroxyl groups; they are reduction products of aldoses or ketoses (Longo et al., 2018). Several studies have also shown the importance of polyols in abiotic stress, suggesting a role as osmoprotectants, cell-membrane stabilizers and/or reactive oxygen species

(ROS) scavengers, thereby preventing oxidative damage of membranes and enzymes (Nishizawa et al., 2008; Keunen et al., 2013; Palma et al., 2013).

Accumulation of compatible solutes in response to stress is a metabolic adaptation found in a number of stress-tolerant organisms, ranging from algae to higher plants (Yancey et al., 1982; Suprasanna et al., 2016). Compatible solutes belong to different classes of chemically diverse molecules, which include sugars and polyols. They are electrically neutral molecules, do not inhibit enzyme activity and increase the cell's ability to preserve osmotic balance during stress condition, thus maintaining cellular structure and function (Rathinasabapathi, 2000). In microalgae, sugars and polyols are commonly the most important osmoprotectants in terms of cellular osmotic responses to abiotic stress (Brown and Hellebust, 1978, Shetty et al., 2019). Myo-inositol is the central compound involved in polyol biosynthesis, derived from glucose-6-phosphate, it serves as a precursor to a number of polyol metabolites (Loewus and Murthy, 2000), for instance, mannitol and sorbitol. These two compounds have been reported to play an important role in osmoregulation, e.g. of the marine algae *Platymonas suecica* (Helebust, 1976), *Stichococcus bacillaris* (Brown and Hellebust, 1978), and *Picochlorum* (Foflonker, 2016), in response to salinity stress. Moreover, studies on myo-inositol metabolism in *Mesembryanthemum crystallinum* a halophyte iceplant, showed that myo-inositol is converted to the osmoprotectants D-ononitol and D-pinitol in a two-step pathway, which is regulated by stress (Vernon and Bohnert, 1992; Rammesmayer et al., 1995; Nelson et al., 1999).

On the other hand, aerobic metabolism, such as photosynthesis and cell respiration, constantly generates ROS in chloroplasts, mitochondria, peroxisomes, etc. (Gill and Tuteja 2010; Inupakutika et al., 2016). Under optimal conditions, when redox homeostasis is not perturbed, ROS do not cause cellular damage since they can be kept at life-compatible levels by antioxidant mechanisms (Inupakutika et al., 2016 and references therein). However, during the initial part of their rapid dehydration and the first minutes upon rehydration, a burst of intracellular ROS occurs in lichen microalgae (Catalá et al., 2010; Álvarez et al., 2015). The increased and unbalanced ROS formation is thought to be one of the most important sources of damage to proteins, lipids and nucleic acids, which ultimately results in cellular injury and death (Gill and Tuteja, 2010). Lichens employ several mechanisms to avoid ROS formation, such as the activation of the xanthophyll cycle (Fernández-Marın et al., 2010) and alternative mechanisms that dissipate the excess of energy during desiccation (Heber, 2008 and cites therein; Gasulla et al., 2009, 2019a).

Complementarily, ROS scavenging can be carried out by antioxidant enzymes (Inupakutika et al., 2016; Hell et al., 2019) and nonenzymatic antioxidants. Among these later compounds, it can be found reduced glutathione (GSH) (Kranner et al., 2005; Hell et al., 2019), phenolics and ascorbic acid (Centeno et al., 2016; Hell et al., 2019). In addition, polyols are within the most important water-soluble antioxidants in lichens (Aubert et al., 2007; Centeno et al., 2016).

Nitric oxide (NO) has been recognized as one signaling molecule involved in the regulation of diverse biochemical and physiological processes (Weissman, et al., 2005). NO functions involve signal transduction, cell death, interaction with ROS production and degradation (Beligni and Lamattina 2002; Palmieri et al., 2008; Wang et al., 2013). In the green algae *Ostreococcus tauri* a functional nitric oxide synthase (NOS) was also characterized (Foresi et al., 2010). NO was reported to be involved in stress responses (Mallick et al., 2002; Chen et al., 2003), reducing the damage produced by photo-oxidative stress in *Chlorella pyrenoidosa* (Chen et al., 2003), and playing an important role in the regulation of lipid peroxidation and photo-oxidative stress during rehydration of the lichen *Ramalina farinacea* and its isolated phycobiont(s) (*Trebouxia sp*.), and the lichen microalga *Asterochloris erici* (Catalá et al., 2010). As a free radical, in biological system NO is found as oxidized stable forms, mainly as S-nitrosothiols (RSNOs). Inside the cells NO might react with thiol-containing biomolecules yielding RSNOs or S-nitrosoproteins, which act as natural reservoir of NO in cells, preserving NO bioavailability (Stamler et al., 1992). Indeed, RSNOs and NO can control protein activity in post-transcriptional modifications via S-nitrosylation pathway (Salgado et al., 2013). However, the participation of NO in the signaling process of cell drying and rewetting, and its possible influence on the polyols involved in the response to cyclic D/R in aeroterrestrial microalgae remains to be elucidated. We hypothesized that, (1) the differences in desiccation tolerance among lichen-forming microalgae could be associated with specie-specific differences in their polyol metabolism and (2) the NO, as a signaling molecule, could assist the regulation of the expression of polyol-related genes in these organisms.

Therefore, in this study we studied the polyol metabolism, estimated by determinations of the transcript abundance of genes encoding some of key enzymes and metabolite levels, along with the cellular concentration of NO (in terms of RSNOs) in isolated lichen microalgae during desiccation-rehydration cycles. For those purposes two microalgae were employed, *Trebouxia* sp. TR9 (TR9) and *Coccomyxa simplex* (Csol), obtained from lichens with different hydric

requirements. TR9 is a phycobiont of the lichen *Ramalina farinacea*, a Mediterranean epiphytic fruticose ascolichen that can withstand long desiccation periods and frequent daily D/R cycles. On the other hand, Csol was isolated from *Solorina saccata*, a foliaceous lichen that is widely distributed within relatively more humid areas from the Mediterranean mountains to the Arctic where it grows on calcareous rocks, typically in crevices and always under sheltered conditions (Krog and Swinscow, 1986). Our previous study concerning the metabolic profile of TR9 and Csol submitted to one cycle of D/R, indicated that polyols played a key role in DT of both microalgae (Centeno et al., 2016). However, TR9 constitutively accumulated higher amounts of polyols, whereas Csol induced the polyol synthesis under D/R. In addition, recent results (Hell et al., 2019) highlighted the species-specific induction of the antioxidant system, at transcriptional level, in the same algae during cyclic D/R, suggesting a priming of oxidative defense metabolism and probably other components of the algal response to desiccation. In the current study, the microalgae TR9 and Csol underwent four consecutive daily cycles of D/R under 'species-specific' conditions of dehydration for each alga (22–25% RH for TR9 and 55% RH for Csol) to mimic their natural habitats. We carried out a comparative analysis of the transcriptional expression of key genes involved in the synthesis of polyols during consecutive daily cycles of D/R and correlated the results with the polyol profile and NO measurements.

2. Materials and Methods

2.1. Microalgae isolation and culture

The *Trebouxia* sp. TR9 was isolated from the lichen *R. farinacea* according to Casano et al. (2011), and *C. simplex* (formerly known as *C. solorinae-saccatae* strain 216-12) was obtained from Sammlung von Algenkulturen at Göttingen University (Germany). Both microalgae were axenically cultured on 2 x 2 cm nylon membranes in semi-solid Bold 3N medium (Bold and Packer 1962) in a growth chamber maintained at a continuous temperature of 15°C and with light/dark cycles of 14 h (25 µmol PAR·m^{-2}·s^{-1})/10 h. All experiments were carried out with 21-day-old cultures and ~180–200 mg of fresh weight (FW).

2.2. Desiccation-rehydration treatments

TR9 and Csol cultures on the nylon squares were removed from the culture medium and subjected to desiccation in a phytotron (KK 115 Top+, Pol-Eko-Apparatus, Poland) in which, in addition to temperature and light conditions, some air hydric parameters such as RH and the speed of air movement can be controlled. Temperature, irradiance and photoperiod were set at the same values as for culture. Desiccation treatment started with the light phase and rehydration began with 6 h under light followed by 10 h of darkness. Cultures of each microalgae on their nylon supports were subjected to daily cycles of 10 h desiccation at 25–30% RH for TR9 or 55–60% for Csol, moderate air movement (20% of maximum capacity), followed by 16 h rehydration in water vapor-saturated sealed Petri dishes. The evolution of the relative water content (RWC) of TR9 and Csol was monitored through the experiment as described by Hell et al. (2019). For the determination of transcript and polyol levels five samples of each microalgae were collected, according to the following treatments: Control (T0), 50 % of the minimal RWC (Da treatment), 100 % of the minimal RWC (Db treatment), and after 1 h (Ra treatment) and 16 h of rehydration (Rb treatment). Every sample was fractionated into three aliquots (~40–100 mg each) and immediately frozen in liquid N_2 and maintained at −80°C until use.

2.3. Sugar alcohol analysis

Ten mg of microalgal samples were lyophilized, extracted in 500µL of methanol:chloroform:water (12:5:1) as described by Suguiyama et al. (2014). The extracts were deionized through anion exchange columns and a volume containing 300 µg of sugar mL^{-1} were filtered on Millipore membranes Millex-HV Durapore (0.45 µm pore size, 13 mm diameter) and analyzed by high performance anion exchange chromatography coupled with a pulsed amperometric detector (HPAEC/PAD, Dionex ICS3000, The United States), with Carbo-Pac MA1 column. The elution was an isocratic method with 500 mM of sodium hydroxide, with a flow of 0.4 mL min^{-1} and run time of 54 min was analyzed using as described by Carvalho et al. (2013). Sugars were identified using authentic standards (Sigma-Aldrich Co., The United States).

2.4. Transcript levels of genes codifying enzymes involved in the sugar alcohol metabolism

RNA was extracted from 50–100 mg FW samples following the protocol of Jones et al. (1985). The quality of isolated RNAs was checked on denaturing agarose gels and each RNA was quantified using a NanoDrop ND-1000™ spectrophotometer (Daemyung, Korea). DNA

contaminants were degraded using the DNAse I RNA-free kit (Invitrogen, California, USA), and the complementary DNA (cDNA) was synthesized with the RevertAid First Strand cDNA Synthesis kit (Thermo-Fisher Scientific, Massachusetts, USA) using the supplied oligo-dT primer following the manufacturer's guidelines.

The identification of transcripts coding for enzymes involved in the sugar alcohol metabolism from TR9 and Csol was performed by searching among customized databases compiling transcript sequences from both algal species, which are housed in our computers in the University of Alcalá. The sequences of enzymes involved in the sugar alcohol metabolism from the close relative *Coccomyxa subellipsoidea* C-169 (NCBI: txid 574566) were used as a query in tblastn searches with Geneious Prime 2019.9.3 (Kearse et al., 2012). As a result, 6 transcripts were selected as the products of the expression of genes coding for enzymes involved in the sugar alcohol metabolism from TR9 and Csol. Such sequences were used to obtain pairs of primers for transcript quantification by real-time PCR (qPCR) (Supplementary Tables S1 and S2). Both strands of the resulting PCR products were sequenced with an ABI 3130 Genetic analyzer using the ABI BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, California, USA) to confirm their correctness. qPCR amplification was carried out using a 7500 Fast Real-Time PCR system (Applied Biosystems) as described (Hell et al., 2019). Five biological replicates were used in this study. The absence of nonspecific PCR products and primer dimers were verified by dissociation curves and by agarose gel electrophoresis. The amplification efficiency of each set of primers was checked to be higher than 95% following the standard curve method described by da Costa et al. (2015). Transcript quantifications were normalized to three reference genes (Supplementary Figures S1 and S2) *Ap47* (Clathrin adaptor complexes subunit), *Act* (Actin) and *PP2A* (Serine/threonine protein phosphatase) as described (Hell et al., 2019).

2.5. Estimation of S-nitrosothiol (RSNO) content

Endogenous NO was measured in terms of RSNO levels. Five mg of lyophilized samples were extracted in Mili-Q water and the RSNO content was measured with the InNO-T, nitric oxide measuring system with a specific NO sensor (amiNO-700) (Innovative Instruments, Inc Tampa, FL, USA). Aliquots of sample aqueous suspension were added to the sampling compartment, which contained 10 mL of aqueous solution of copper chloride (0.1 mol L^{-1}). This condition allowed for the detection of free NO released from the S-nitrosothiol present in the sample

homogenate. Data were compared to a standard curve obtained with S-nitrosoglutathione (GSNO) and normalized against samples weight. Calibration curves were obtained with aqueous solutions of freshly prepared GSNO (Silveira et al., 2016, 2019). The results were expressed in µmol of NO per 10^9 cell. TR9 and Csol cells were counted in pre-weighted and carefully resuspended aliquots using a hemocytometer (n=10–12)

2.6. Statistical analysis

All analyses were conducted with three or five biologically independent replicates per treatment for each point of the analyses. Data were analyzed using the multiple sample comparison (Statgraphics Centurion XVII, 2016, StatPoint Technologies 2016) Fisher's least significant difference (LSD) test ($P < 0.05$). HeatMaps were prepared using correlation coefficients for metabolites and transcripts levels calculated by applying Spearman algorithm using Past software (Hammer et al., 2001).

3. Results

The proportions of sugars and sugar alcohols, determined by HPAEC/PAD, in Csol and TR9 (Figure 1 and 2, respectively) indicated a different composition of the osmocompatible metabolites pool in each microalga, which in turn seemed to be modulated by D/R exposition. In Csol microalgae, mannitol was the major sugar alcohol, representing 63.56% of the total sugar and sugar alcohols at T0 (initial control), and its level was maintained during the subsequent D/R cycles (Figure 1). An increase of mannose, maltose and sorbitol was also detected (Figure 1) after 16h of rehydration (Rb treatment) after two D/R cycles. Even more, when cycles 2 and 4 are compared, it was observed a higher proportion of glucose and arabitol and lower levels of iso-erythritol and glycerol, during cycle 2 and the opposite was observed at cycle 4 (Figure 1). In TR9 microalgae, arabitol was the major sugar alcohol at T0 (initial control), representing 61.74% of the total sugars and sugar alcohols, and its level significantly decreased after Da treatment (50% of the minimal RWC values) after two D/R cycles. On the other hand, sorbitol significantly increased during the same stage, and its level was maintained in high proportion after two and four D/R cycles, except after 16h of rehydration (Rb treatment) at cycle 4. At this point mannitol accounted for 94,38% of the total sugars and sugar alcohols.

With the purpose of verifying the influence of NO as a signaling molecule in cyclic D/R, its concentration was analyzed during D/R cycles (Table 1). The analysis indicated higher amounts of NO in TR9 compared to Csol microalgae (Table 1). TR9 showed approx. 7 µmol of NO per 10^9 cell, while Csol presented 0.105 µmol of NO per 10^9 cell at the T0. A significant decrease of NO was observed in both microalgae as the desiccation increased with the lowest values at Db (100% of the minimal RWC value). In TR9, NO quantities were 10 and five times lower than those observed for the T0 and T0' controls, respectively, and they were below the detection limit in Csol (Table 1). In both species, NO quantities increased again upon 1h and 16h rehydration (Ra and Rb treatments respectively), achieving values similar to T0. It is noteworthy that during the rehydration of cycle 4, the NO levels significantly increased, representing approximately twice and three/six times the quantity measured in the initial controls of TR9 and Csol, respectively (Table 1).

A heatmap metabolite-NO analysis indicated a negative correlation between NO and some metabolites, measured by HPAEC/PAD, in Csol microalgae (NO-xylitol r = -0.418; NO-arabitol r = -0.404; Figure 3). A similar behavior was observed in TR9 microalgae, a negative correlation between NO and some metabolites was also observed, notably NO-maltose (r = - 0.629) and NO-xylitol (r = - 0.332). In contrast, NO-mannitol presented a positive correlation (r = 0.373) (Figure 3). The main metabolite-metabolite correlations in Csol were maltose-*myo*-inositol (r = 0.870), xylitol-arabitol (r = 0.945), xylitol-glucose (r = 0.834), arabitol-glucose (r = 0.864), sorbitol-maltose (r = 0.861), sorbitol-mannose (r = 0.967), *myo*-inositol-mannitol (r = -0.5492), mannitol-iso-erythritol (r = -0.385), maltose-mannitol (r = -0.379), iso-erythritol-xylitol (r = -0.202), iso-erythritol-arabitol (r = -0.083) and iso-erythritol-glucose (r = -0.216). For TR9, the main metabolite-metabolite correlations were: glycerol-*myo*-inositol (r = 0.736), sorbitol-*myo*-inositol (r = 0.855), sorbitol-mannose (r = 0.707), glucose-xylitol (r = -0.579), mannitol-arabitol (r = -0.345) and mannitol-glucose (r = -0.0825).

Blast searches against customized databases compiling transcript sequences from both algal species, revealed the existence of six genes encoding key enzymes involved in the metabolism of myo-inositol and raffinose (Figure 4): one gene for myo-inositol-1-phosphate synthase (MiPS), two genes for myo-inositol monophosphatase (MiPP1 and MiPP2), one gene for myo-inositol oxidase (MiOX) and two genes for raffinose synthase (RafS1 and RafS2).

Analyses of transcript levels by qPCR, demonstrated differential expression of genes in response to cyclic D/R in Csol and TR9 microalgae (Figures 5 and 6, respectively). Changes in transcript levels were more profuse in Csol than in TR9 microalgae. In Csol (Figure 5), MiPS transcripts significantly increased during cycle 2 and 4, compared to the initial control. However, MiPP1, MiPP2 and MiOX genes showed different transcriptional responses: MiPP1 transcripts remained unchanged during the D/R cycles whereas the amount of MiPP2 transcripts increased during drying of both D/R cycles. MiOX transcripts decreased during desiccation, especially at cycle 4, and increased upon fully rewetting. Regarding the raffinose synthase genes, RafS1 and RafS2 transcript levels increased (more markedly in the case of Raf2) along both D/R cycles in an oscillating manner.

In the case of TR9 (Figure 6), MiPS transcripts decreased during cycle 2, except after 1h of rehydration (Ra). In these conditions, the amount of MiPS transcripts significantly increased respect to T0' but not to initial level (T0). Even more, MiPS significantly decreased after 16h of rehydration (Rb) on both cycles. For MiPP1 and MiPP2, in general, the transcript levels slightly increased during cycle 2 and 4, but a dramatic increase was observed in MiPP2 at 16h of rehydration (Rb) of cycle 4. The level of MiOX transcripts showed only slight variations, decreasing at the beginning of cycle 2 and 4 (T0' and T0", respectively). In respect to RafS transcript levels, no significant differences along the D/R cycles were observed in RafS1, whereas they markedly increased during rehydration of cycle 4 in the case of RAfS2.

In order to evaluate 1- the contribution of NO as a signaling molecule on gene expression regulation and 2- the relation between the transcript level and sugar and sugar alcohol metabolites during desiccation/rehydration process, a correlation analysis was performed (Figure 7).

In Csol, the Spearman analysis indicated a negative correlation between NO and the transcript level of almost all studied genes: MiPS (r = -0.591), MiPP1 (r = -0.645), MiPP2 (r = -0.182), RafS1 (r = -03554) and RafS2 (r = -0.264). MiOX was the only gene which presented a positive r value (r = 0.327) (Figure 7). The correlation analysis between transcript levels and metabolites indicate only one positive case, between RafS2 and mannitol (r = 0.682), and eight negative correlations: MiPP-glycerol (r = -0.591), MiPP2-sorbitol (r = -0.596), MiPP2-maltose (r = -0.579), MiPP2-mannose (r = -0.502), RafS1-glycerol (r = -0.509), RafS1-maltose (r = -0.505), RafS2-*myo*-inositol (r = - 0.514) and RafS2-maltose (r = -0.569).

In the case of TR9, the Spearman analysis indicated a positive correlation between NO and the transcript levels of two genes: MiPP1 (r = 0.336) and MiOX (r = 0.2181). The negative NO-transcripts correlations were: MiPS (r = -0.018), MiPP2 (r = -0.464), RafS1 (r = -0.309) and RafS2 (r = -0.236). The correlation between transcript level-metabolite indicates a positive correlation between: MiPP2-iso-erythritol (r = 0.736), RafS2-myo-inositol (r = 0.673), RafS2-glycerol (r = 0.536) and RafS2-sorbitol (r = 0.609). A negative correlation was found between MiPS-xylitol (r = -0.688), MiPS-mannitol (r = -0.090) and MiPS-mannose (r = -0.074).

4. Discussion

In this study, we present findings on the physiological impact of cyclic desiccation/rehydration in two microalgae, Csol and TR9, with different hydric behavior focusing on the metabolism of some key sugars and sugar alcohols. We provide evidences of the regulation of transcript levels of main sugar alcohols-related genes and the possible role of NO as a modulator of gene expression, which lead both microalgae species to the osmoprotectants accumulation.

4.1. Changes in sugar and sugar alcohol proportions

Different studies have shown the importance of polyols in abiotic stress, acting as osmoprotectants, cell-membrane stabilizers or ROS scavengers (Nishizawa et al., 2008; Keunen et al., 2013; Palma et al., 2013).

In our previous work, analyzing one cycle of D/R, the metabolic profile revealed that sugar alcohol played a key role in DT of both, Csol and TR9 microalgae. Csol induced the polyol synthesis under D/R whereas TR9 microalgae constitutively accumulated higher amounts of polyols and synthesized protective raffinose-family oligosaccharides (RFOs) (Centeno, 2016). The results presented herein are regarding to Csol and TR9 microalgae submitted to two and four D/R cycles. The amounts and proportions of sugar and sugar alcohols observed in the present study are consistent with those from previous research (Centeno et al., 2016). Csol HPAEC/PAD analysis indicated that mannitol is the most abundant compound in fully hydrated cells (T0) and their levels are maintained during D/R cycles (Figure 1). Mannitol is involved in important physiological functions such as the control of cell turgor, increasing its intracellular concentration at low water activity (Yancey et al., 1982; Patel and Williamson, 2006), and as non-enzymic antioxidant by quenching ROS (Patel and Williamson, 2006). Mannitol and sorbitol had been reported as

important osmoregulators (Foflonker, 2016) in the marine algae *Platymonas suecica* (Helebust, 1976), *Stichococcus chloranthus* and *Stichococcus bacillaris* (Brown and Hellebust, 1978). On the other hand, Shetty and co-workers (2019), highlighted that sorbitol and mannitol have not been detected in appreciable quantities in other algal species indicating that this might be a species-specific mechanism. In our analysis, high proportions of mannitol and sorbitol has been found in both Csol and TR9 (Figure 1 and 2).

The increase of mannose, maltose and sorbitol proportion after fully rehydration at cycle 2 in Csol could also indicate the role of these compounds as compatibles solutes or carbon sources. Curiously, sorbitol was not detected in Csol at the first D/R cycle on previous metabolic profile analyses (Centeno et al., 2016), leading to the idea that sorbitol increases in a response to cyclic desiccation, induced by water uptake (Figure 1).

In the case of TR9 microalgae, arabitol represented the major sugar of fully hydrated cell (T0, initial control, Figure 2). Interestingly, this compound was reported in isolated *Trebouxia* sp. from *Ramalina yasudae*, as essential in the role of dissipating excess light energy into heat, protecting the photobiont from photoinhibition (Kosugi et al., 2013). As discussed by Centeno et al. (2016), the abundant initial level of arabitol in TR9 (Figure 2) may represent the constitutive strategy used by this microalga to cope with sudden or sporadic changes in water availability. However, the proportion of this compound significant decay at the same stage that sorbitol increased (Figure 2 and Table S3), suggesting a shift in the sugar alcohol metabolism induced by cyclic desiccation. Even more, mannitol increase was observed after 16h of rehydration of cycle 4 (Rb, Figure 2), suggesting an antioxidant role by quenching ROS during water uptake. In two *Trebouxia* photobionts from Antarctic lichens submitted to desiccation stress, considerable levels of ribitol, glycerol, myo-inositol and sorbitol were also detected (Sadowsky et al., 2016).

In Csol, an opposite response was found between the proportion of glucose and arabitol during cycle 2 and iso-erythritol and glycerol during cycle 4 (Figure 1), suggesting a shifting on compound preference. Glycerol has been reported as a common but effective compatible solute produced by most salt sensitive algal species under high saline stress (Shetty et al., 2019). The increase of glycerol concentration under stress salinity was recorded for *Chlamydomonas pulsatilla* (Hellebust et al., 1985), *C. reinhardtii* (León and Gaván, 1994), *C. mexicana* (Salama et al., 2013), *Chlamydomonas* sp. JSC4 (Ho et al., 2017) and more recently was also described in isolated photobiont from the lichen *Wahlenbergiella striatula* (Gasulla et al., 2019b). Erythritol

has been proposed as an osmotic protector (Williamson et al., 2002) and its presence was reported as a stress specific metabolite related to dehydration in *Oropetium thomaeum*, a desiccation tolerant grass (Zhang et al., 2018). Erythritol level was also higher in the desiccation tolerant grass *Sporobolus stapfianus,* submitted to dehydration stress (Oliver et al., 2011). Interestingly, a comparative metabolic analysis between *S. lepidophylla* and its desiccation susceptible relative *Selaginella moellendorffii,* demonstrated that polyols such as sorbitol and xylitol, were 100 times more abundant in *S. lepidophylla* compared to *S. moellendorffii* (Yobi et al., 2012 reviewed by Pampurova and Dijck et al., 2014). The levels of arabitol, erythritol and glycerol were also increased in *S. lepidophylla* (Yobi et al., 2012).

4.2. NOx concentration and its effect on sugar and sugar alcohol metabolism

Nitric oxide (NO) is an intra and intercellular signaling molecule involved in the regulation of diverse biochemical and physiological processes (Palmieri et al., 2008; Nabi et al., 2019), including the abiotic stress response (Chen et al., 2003). The results herein showed higher constitutive amount of NO in TR9 microalgae, compared to Csol (Table 1). Catalá et al. (2010), studying *Trebouxia* sp. photobiont and the lichen thalli *R. farinacea,* found that the mycobiont-derived NO has an important role in the regulation of oxidative stress and in the photooxidative protection of photobionts, especially in the early stages of lichen rehydration.

In both TR9 and Csol algae, the significant decrease of NO detected at maximal dehydration (Db, 100% of the minimal RWC value) could be explained as a result of the reduced metabolism or anabiosis. When rehydration was applied, a burst of NO was detected (Table1). This contrasts with the results of Weissman and co-workers (2005), that reported an in intracellular oxidative stress during rehydration and a rapid increase of intracellular NO production in the fungal hyphae of the lichen *R. lacera* during rehydration, but not in the algae. In this respect, our results indicate that the NO increase during rehydration occurs probably associated with the formation of other highly reactive compounds such as ROS, and suggest that this is a specie-specific mechanism. Even more, the prominent increase of NO observed at the Ra and Rb treatments at cycle 4, agree with recent results, which showed a species-specific induction of the antioxidant genes in response cyclic D/R in Csol and TR9 microalgae (Hell et al., 2019). In Csol, the negative correlation between NO-xylitol and NO-arabitol (Figure 3) and the absence of correlation between NO-iso-erytritol, could indicate a NO negative feedback, translated as the

specie preference to synthetize iso-erythritol from sucrose, instead xylitol and arabitol (Figure 4) to overcome a desiccation-rehydration stress situation, as observed during cycle 4 (Figure 1). The same negative feedback could be observed in TR9 microalgae, between NO-maltose and NO-xylitol (Figure 3) and the positive NO-mannitol correlation. These may indicate a metabolism enforcement to deviate the metabolic route to synthetize mannitol, from sucrose or glucose, rather than xylitol or maltose. Interestingly, the positive correlation found between NO-mannitol in TR9 microalgae may also be related with the mannitol increase observed after treatment 16h of rehydration at cycle 4 (Rb, Figure 2), indicating a possible role of NO as a signaling to a specific polyol increase.

In Csol, the negative correlation between iso-erythritol-xylitol (r = - 0.202), iso-erythritol-arabitol (r = - 0.083) and iso-erythritol-glucose (r = - 0.216) observed through the metabolite-metabolite correlation analysis (Figure 3) reinforces the idea that polyol pathways are oriented to iso-erythritol synthesis in this microalga. The positive correlation among osmoprotectants and important hubs, such as myo-inositol and glucose, demonstrates that in Csol the changes are highly controlled by the exposition to cyclic D/R. The strongest positive correlations between certain metabolites, as observed for xylitol-arabitol (r = 0.945) and sorbitol-mannitol (r = 0.967), corroborate the idea of directed and specific responses concerning groups or pathways of osmoprotectants in this species. On the other hand, in TR9, the correlation between mannitol-arabitol (r = -0.354) and mannitol-glucose (r = -0.0825), may indicate the metabolism redirection from arabitol and glucose at the initial fully hydrated state (T0) to mannitol after four D/R cycles (Figure 3). However, these correlations are weaker, especially considering the positive correlations of important osmoprotectants (sorbitol-mannitol; r = 0.707) or osmoprotectants and the main hubs (sorbitol-myo-inositol, r = 0.855), indicating smaller influence of the exposition to D/R or, in other words, a more constitutive character of the sugar-alcohol defense strategy in this alga.

4.3. Sugar alcohol-related transcript level

Inositols and their derivatives are functionally important classes of compounds (Saxena et al., 2014), and the *myo*-form is the most abundant in biological systems where it is considered an essential metabolite (Banerjee et al., 2007). The pathway from glucose-6-phosphate to *myo*-inositol-1-phosphate and *myo*-inositol is an important hub for the synthesis of various metabolites, including sugar and sugar alcohol compounds (Figure 4). The inositol biosynthetic pathway is

highly conserved across biological kingdoms where the rate limiting enzyme *myo*-inositol-1-phosphate synthase (MiPS) catalyzes the conversion of glucose-6-phosphate to *myo*-inositol-1-phosphate and subsequently *myo*-inositol-1-phosphate is converted to free *myo*-inositol by the enzyme *myo*-inositol mono phosphatase (MiPP) (Saxena et al., 2014). The role of inositol is related to many cellular processes including membrane formation, cell wall biogenesis, signal transduction and stress response (Downes et al., 2005). Plants maintain at least one copy of MIPS gene in a core catalytic domain, although their expression and function are not constant throughout the plant kingdom. Different MIPS isoforms, which are involved in the response to various stresses, show variable expression profiles depending on different developmental stages, as recently reviewed by Hazra et al. (2019). In green microalgae, only one MIPS isoform is known, as identified to *Chlamydomonas reinhardtii, Volvox carteri* (Hazra et al., 2019) and also in our study with Csol and TR9 (Figures 5 and 6).

Myo-inositol is also related to the Raffinose Family Oligosaccharides (RFO) metabolism, acting as a key branching point, which can lead to the galactinol/RFO pathway (ElSayed et al., 2014). Even more, *myo*-inositol can be also a product of raffinose synthesis (Peterbauer and Ritcher, 2001), since raffinose synthase (RAFS), catalyzes the reversible galactosylation of sucrose from galactinol, yielding raffinose and *myo*-inositol (Peterbauer and Ritcher, 2001). As far as we know, this is the first study to characterize the transcript level of genes related to the metabolism of myo-inositol and raffinose, in aeroterrestrial algae during cyclic D/R.

Interestingly, our results revealed a species-specific transcriptional response to cyclic D/R of the MiPS-coding gene (Figures 5 and 6). In Csol, MiPS transcripts significantly increased during cyclic D/R whereas in TR9 the MiPS transcript level were maintained in the same level as initial control, and a significant decrease was observed only during rehydration. These results reinforce the idea of a contrasting behavior between both species: TR9 seems to be constitutively prepared to cope with cyclic D/R and Csol seems to have a metabolic response induced by stress. Previous results showed that TR9 has constitutively higher amounts of polyols (8.542 ± 0.302) than Csol (0.298 ± 0.044) (Centeno et al., 2016). Even more, MiPP1 and MiPP2 transcripts, which encode the enzyme involved in the conversion of *myo*-inositol-1-phosphate into free *myo*-inositol, also indicate a relevant participation of *myo*-inositol as well as a different behavior of each microalga. In the case of TR9, the remarkable increase of MiPP2 during rehydration phase of cycle 4 could be related with the role of ROS scavenger of *myo*-inositol (Figure 6). Contrastingly, in

Csol, MiPP1 transcript levels remained unchanged whereas MiPP2 transcripts increased during drying of both D/R cycles (Figure 5). In plant species with more than one copy of MiPS, it is reported that the expression profile of different MIPS isoforms are variable and specific to different developmental stages and confronted to various stresses (Mitsuhashi et al., 2008; Khurana et al., 2012). It may be possible that the same occurs with MiPP-coding genes, and MiPP2 may be more relevant in the *myo*-inositol synthesis than MiPP1, in the response of Csol to desiccation stress. The MIOX enzyme converts *myo*-inositol to glucuronic acid (Valluru and Van de End et al., 2011). Regarding the transcript level of the MiOX-coding gene, no clear pattern was observed for TR9 (Figure 6), while they significant increased after rewetting in Csol (Figure 5). Lorence et al., (2004) presented a possible biosynthetic route to ascorbate in plants, using *myo*-inositol as the initial substrate and myo-inositol oxygenase as a possible entry point. Recent studies with *myo*-inositol oxygenase gene family in tomato also revealed its involvement in ascorbic acid accumulation (Munir et al., 2020). Interestingly, in TR9, previous metabolic profile analysis showed that AsA was below the detection limit, while Csol demonstrate an increase of AsA during D/R (Centeno et al., 2016). In agreement with these results, Hell et al. (2019) reported no detectable levels of ascorbate peroxidase transcripts in TR9 while, in contrast, this enzyme seemed to play an important role in the antioxidant protection of Csol. Therefore, the enhanced transcript level of MiOX, in Csol (Figure 5) may be related not only to glucuronic acid but also with AsA biosynthesis, however, a deeper investigation is still required.

In addition, some authors have proposed that the increasing concentration of *myo*-inositol can also stimulate the accumulation of RFOs in seeds (Karner et al., 2004) and vegetative tissues (Valluru and van den Ende, 2011). Changes in the expression of raffinose synthase genes in vegetative tissues under abiotic stresses have been demonstrated in a broad range of species, for instance, rice (Saito and Yoshida, 2011), *Arabidopsis* (Kant et al., 2008), poplar (Ko et al., 2011), cucumber (Sui et al., 2012), maize (Li et al., 2020)). Recently, the *ZmRAFS (Zea mays)* overexpression in *Arabidopsis* enhanced drought stress tolerance by increasing RFOs and *myo*-inositol levels via ZmRAFS mediated galactinol hydrolysis (Li et al., 2020). In a transcriptomic study of the aeroterrestrial green algae *K. crenulatum,* several enzymes involved in the biosynthesis of the raffinose family of oligosaccharides were also up-regulated in desiccated samples (Holzinger et al., 2014).

With the exception of a slightly increase of RafS2 during rewetting of cycle 4, no differences in the transcripts related with raffinose synthesis were observed in TR9 (Figure 6). This contrast with the strong increase in RafS1 and RafS2 transcript levels observed in Csol, especially in RafS2. These results also suggest, for both species, that one RafS isoform is more responsive to D/R stress (Figure 5 and 6). An overview of the RafS transcript levels of Csol and TR9 corroborates, once again, the hypothesis of a priming process (Hell et al., 2019), which allows the microalgae to activate their defense responses faster, during the subsequent D/R cycles. In addition, Csol genes involved in the sugar alcohol metabolism seem more prone to be transcriptionally upregulated by cyclic D/R than TR9, in agreement with the idea of a mainly constitutive defense system in TR9 and a more inducible defensive response in Csol.

4.4. NO and sugar alcohols-related transcripts

The first approach of the correlation analysis indicated the contribution of NO as a signaling molecule on gene expression regulation (Figure 7). Interestingly, the negative correlation found among NO and the transcript levels of the genes under study, indicates a decrease of *myo*-inositol synthesis related to the NO increment. The majority of encoding genes directly linked to *myo*-inositol synthesis (MiPS and MiPP) showed a negative correlation with NO, in both species (Figure 7). Moreover, RafS1 and RafS2, which generates *myo*-inositol as a product from the reaction of raffinose synthesis, also presented a negative correlation with NO. These results suggest that under desiccation and rehydration stress, the burst of NO acts as a signaling, with the purpose to redirect the polyol pathway to the synthesis of e.g. mannitol, iso-erythritol and sorbitol, instead to generating *myo*-inositol (Figure 4). In addition, in both species, NO-MiOX presented a positive correlation (Figure 7), indicating that *myo*-inositol, whose synthesis is impaired, is directed to glucuronic acid or, in the case of Csol, also to AsA synthesis (Lorence et al., 2004).

In general, the negative correlation between the transcript level of MiPS, MiPP, RafS and the studied metabolites, may indicate the main compounds formed as a result to the change in the metabolic pathway. In the case of Csol, this alteration may lead, especially, to glycerol, sorbitol, maltose, mannose synthesis. In TR9 the results indicate preferably the synthesis of xylitol, mannitol and mannose.

Conclusions

Our results show dynamic changes in the expression patterns of genes involved in *myo*-inositol and raffinose synthesis in response to cyclic D/R in two lichen microalgae and their relationship with NO production. NO may act as a signaling molecule, facilitating osmoregulation and antioxidant protection, through the modulation of polyol biosynthetic pathway. NO may be related to a primed memory state of lichen microalgae, by assisting them in the rapidly and more precise metabolic response to subsequent D/R cycles through influencing on the transcriptional expression of sugar alcohols-related genes. Thereby, this work also contributes to enhance the knowledge about the NO signaling role, although other functions from NO are not discarded as its ROS scavenger capacity.

Author contributions statement

AFH, LMC, DCC contributed to the conception and design of the study, the analysis and interpretation of data. AFH obtained most of the experimental data. MTP and ABS performed NO analysis. MHG, FG and EMC contributed to acquisition of data. AFH wrote the first draft of the manuscript. All authors contributed in revising the article critically for important intellectual content and for final approval of the version.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Table 1. Nitric oxide concentration on Csol and TR9 microalgae submitted to dessication-rehydration treatments. (T0) indicates initial control treatment; (T0' and T0") indicates the controls of cycles 2 and 4, respectively; (Da) and (Db) indicates 50% and 100% of the minimal RWC value, respectively; (Ra) and (Rb) indicates 1h and 16h of rehydration, respectively. Data represent the mean values of three replications ± standard error. Different letters indicate significant differences between treatments, according to Fisher's least significant difference (LSD) test ($P < 0.05$). [a]TR9 and Csol cells were counted in pre-weighted and carefully resuspended aliquots using a hemocytometer (n=10–12). b.d.l. below detection limit.

		NO (µmol per 10^9 cel)[a]	
		Coccomyxa simplex	*Trebouxia TR9*
Control	T0	0.105 ± 0.010 de	7.072 ± 0.690 cde
Cycle 2	T0'	0.311 ± 0.090 bc	3.456 ± 0.783 de
	Da	0.324 ± 0.042 bc	6.064 ± 0.758 cde
	Db	b.d.l.	0.643 ± 0.174 e
	Ra	0.339 ± 0.059 bcd	7.405 ± 1.072 cd
	Rb	0.115 ± 0.013 cde	9.904 ± 0.725 bc
Cycle 4	T0"	0.014 ± 0.002 e	11.613 ± 0.928 abc
	Da	0.044 ± 0.007 de	6.911 ± 0.625 cde
	Db	0.375 ± 0.052 b	6.028 ± 0.378 cde
	Ra	0.671 ± 0.136 a	15.736 ± 3.192 ab
	Rb	0.331 ± 0.060 b	16.844 ± 6.158 a

Figure 1. Changes in the proportions of sugars and sugar alcohols in Csol microalgae exposed to cyclic desiccation-rehydration. The amount of the indicated metabolites was determined by HPAEC/PAD. (T0) indicates initial control treatment; (T0' and T0") indicates the controls of cycles 2 and 4, respectively; (Da) and (Db) indicates 50% and 100% of the minimal RWC value, respectively; (Ra) and (Rb) indicates 1h and 16h of rehydration, respectively. Values represent mean ± SE, n=3. Compounds without values were below the detection limit. Fisher's least significant difference (LSD) test ($P < 0.05$) was applied, statistical analysis are shown at the Supplementary table S3.

Figure 2. Changes in the proportions of sugars and sugar alcohols in TR9 microalgae exposed to cyclic desiccation-rehydration. The amount of the indicated metabolites was determined by HPAEC/PAD. (T0) indicates initial control treatment; (T0' and T0") indicates the controls of cycles 2 and 4, respectively; (Da) and (Db) indicates 50% and 100% of the minimal RWC value, respectively; (Ra) and (Rb) indicates 1h and 16h of rehydration, respectively. Values represent mean ± SE, n=3. Compounds without values were below the detection limit. Fisher's least significant difference (LSD) test ($P < 0.05$) was applied, statistical analysis are shown at the Supplementary table S3.

Figure 3. Heatmap of metabolite-metabolite and metabolite-NO correlations along desiccation-rehydration treatments. Each square represents the correlation between the metabolite heading the column with the metabolite heading the row. Correlation coefficients and significances (p value) were calculated by applying Spearman algorithm using Past software. Each square indicates a given r value resulting from a Spearman correlation analysis in a false color scale.

Figure 4. Biosynthetic pathway of sugar alcohols. The transcripts analyzed are represented in blue. MiPS: Myo-inositol-1-phosphate synthase; MiPP1 and MiPP2: Myo-inositol-phosphate-phosphatase 1 and 2, respectively; MiOX: Myo-inositol-oxygenase; RafS1 and RafS2 - Raffinose synthase 1 and 2, respectively. Sugars and sugar alcohols metabolite determined using HPAEC/PAD, are represented in green.

Figure 5. Changes in the transcript levels of sugar alcohol and raffinose encoding genes of Csol microalgae exposed to cyclic desiccation-rehydration. mRNA levels of genes encoding *MiPS*, *MiPP1*, *MiPP2*, *MiOX*, *RafS1* and *RafS2* genes. Values were calculated with $2^{-\Delta\Delta Ct}$ relative quantification for five biological replicates. Error bars show the standard error. Black bars indicate initial control treatment (T0); dark gray bars indicate controls of cycles 2 and 4 (T0' and T0", respectively); white solid and striped bars indicate desiccation treatments with 50% (Da) and 100% (Db) of the minimal RWC value, respectively; light gray solid and striped bars indicate treatments after 1 h (Ra) and 16 h (Rb) of rehydration, respectively

Figure 6. Changes in the transcript levels of sugar alcohol and raffinose encoding genes of TR9 microalgae exposed to cyclic desiccation-rehydration. mRNA levels of genes encoding *MiPS, MiPP1, MiPP2, MiOX*, *RafS1* and *RafS2* genes. Values were calculated with $2^{-\Delta\Delta Ct}$ relative quantification for five biological replicates. Error bars show the standard error. Black bars indicate initial control treatment (T0); dark gray bars indicate controls of cycles 2 and 4 (T0' and T0", respectively); white solid and striped bars indicate desiccation treatments with 50% (Da) and 100% (Db) of the minimal RWC value, respectively; light gray solid and striped bars indicate treatments after 1 h (Ra) and 16 h (Rb) of rehydration, respectively

Figure 7. Heatmap of metabolite-transcript level correlations along desiccation-rehydration treatments. Each square represents the correlation between the metabolite heading the column with the metabolite heading the row. Correlation coefficients and significances (p value) were calculated by applying Spearman algorithm using Past software. Each square indicates a given r value resulting from a Spearman correlation analysis in a false color scale.

Figure 1

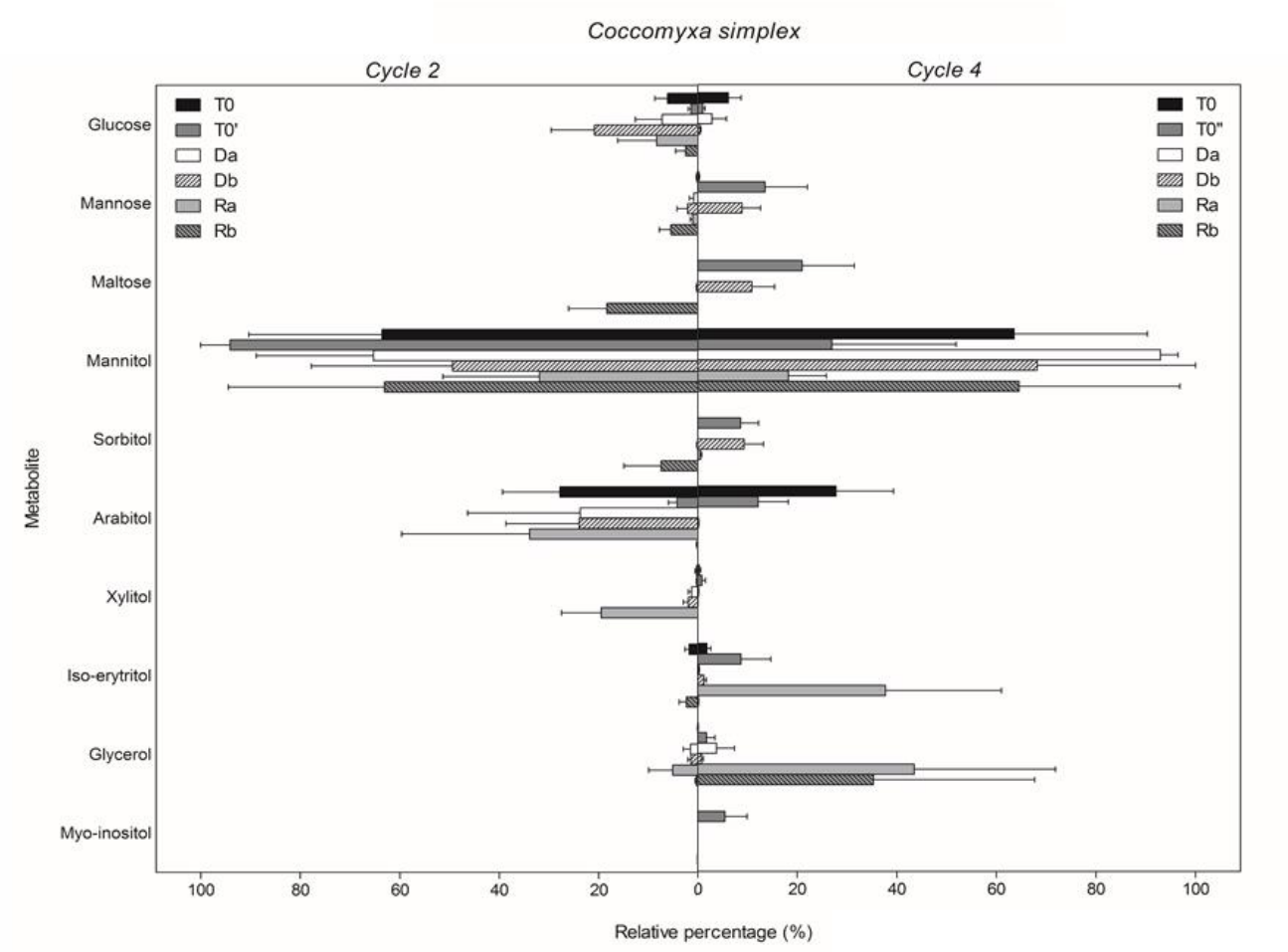

Coccomyxa simplex
Cycle 2
Cycle 4
T0
T0'
Da
Db
Ra
Rb
T0
T0"
Da
Db
Ra
Rb
Glucose
Mannose
Maltose
Mannitol
Sorbitol
Arabitol
Xylitol
Iso-erytritol
Glycerol
Myo-inositol
Metabolite
100
80
60
40
20
0
20
40
60
80
100
Relative percentage (%)

Figure 2

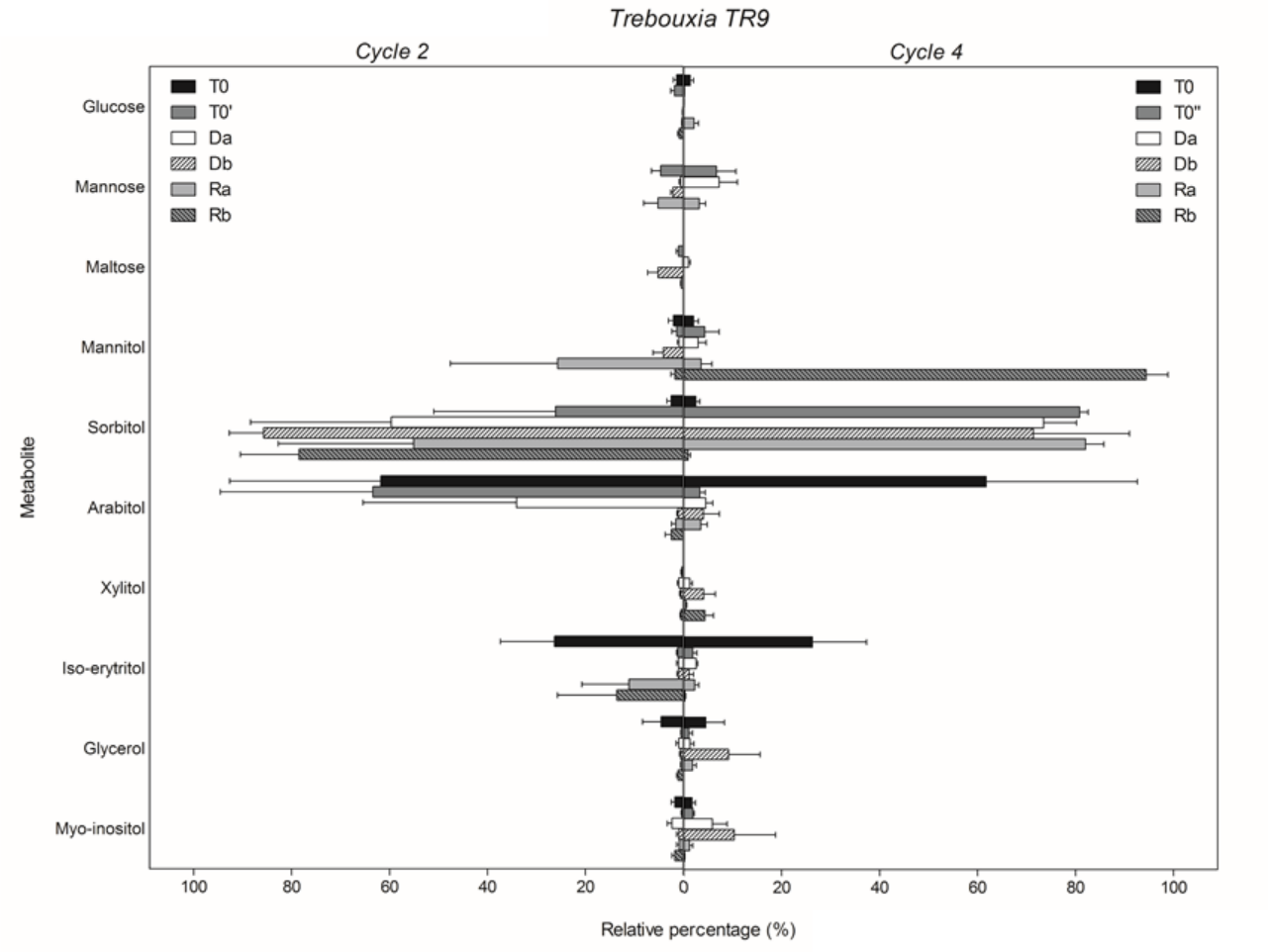
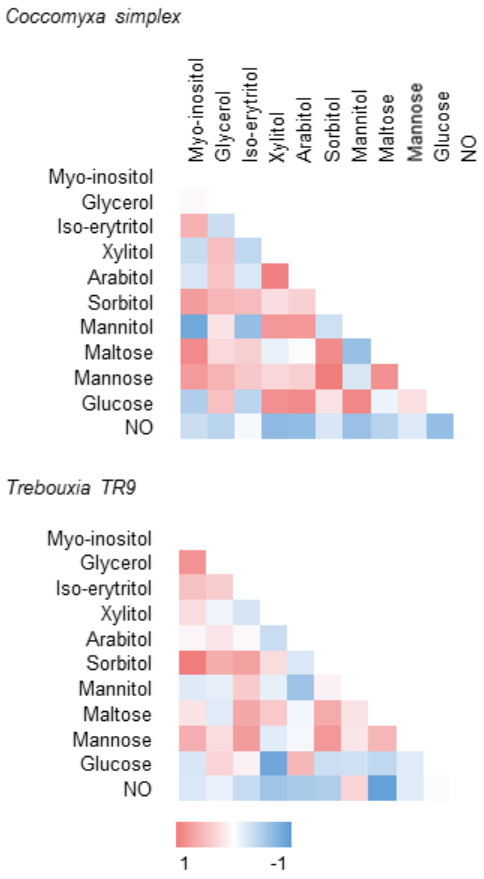

Trebouxia TR9
Cycle 2
Cycle 4
T0
T0'
T0"
Da
Db
Ra
Rb
Glucose
Mannose
Maltose
Mannitol
Sorbitol
Arabitol
Xylitol
Iso-erytritol
Glycerol
Myo-inositol
Metabolite
100
80
60
40
20
0
20
40
60
80
100
Relative percentage (%)

Figure 3


Coccomyxa simplex
Myo-inositol
Glycerol
Iso-erytritol
Xylitol
Arabitol
Sorbitol
Mannitol
Maltose
Mannose
Glucose
NO
Trebouxia TR9
Myo-inositol
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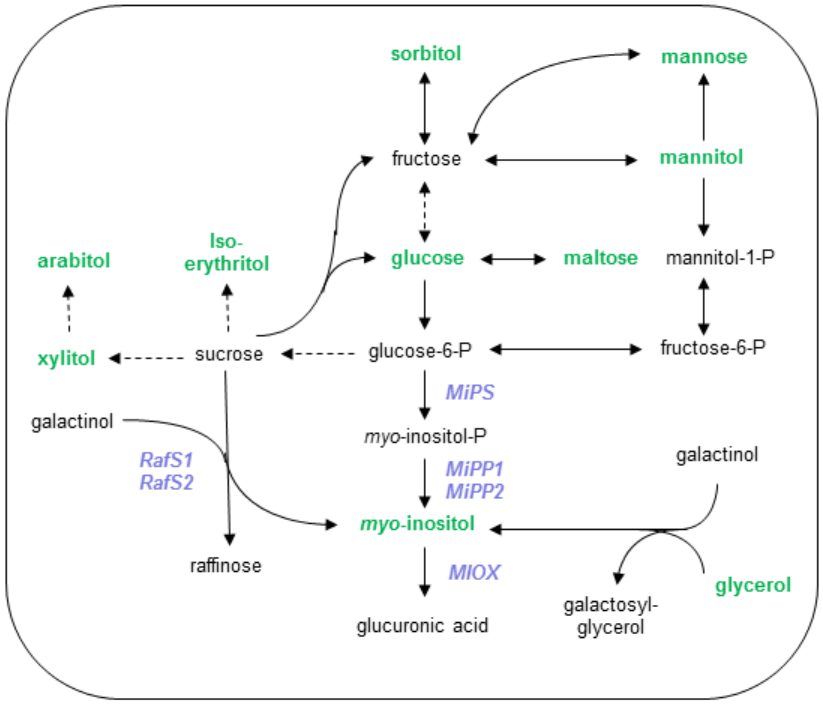
Maltose
Mannose
Glucose
NO
1
-1

Figure 4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xylitol
sucrose
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fructose-6-P
MiPS
galactinol
myo-inositol-P
RafS1
RafS2
MiPP1
MiPP2
galactinol
myo-inositol
raffinose
MIOX
glycerol
galactosyl-
glycerol
glucuronic acid

Figure 5

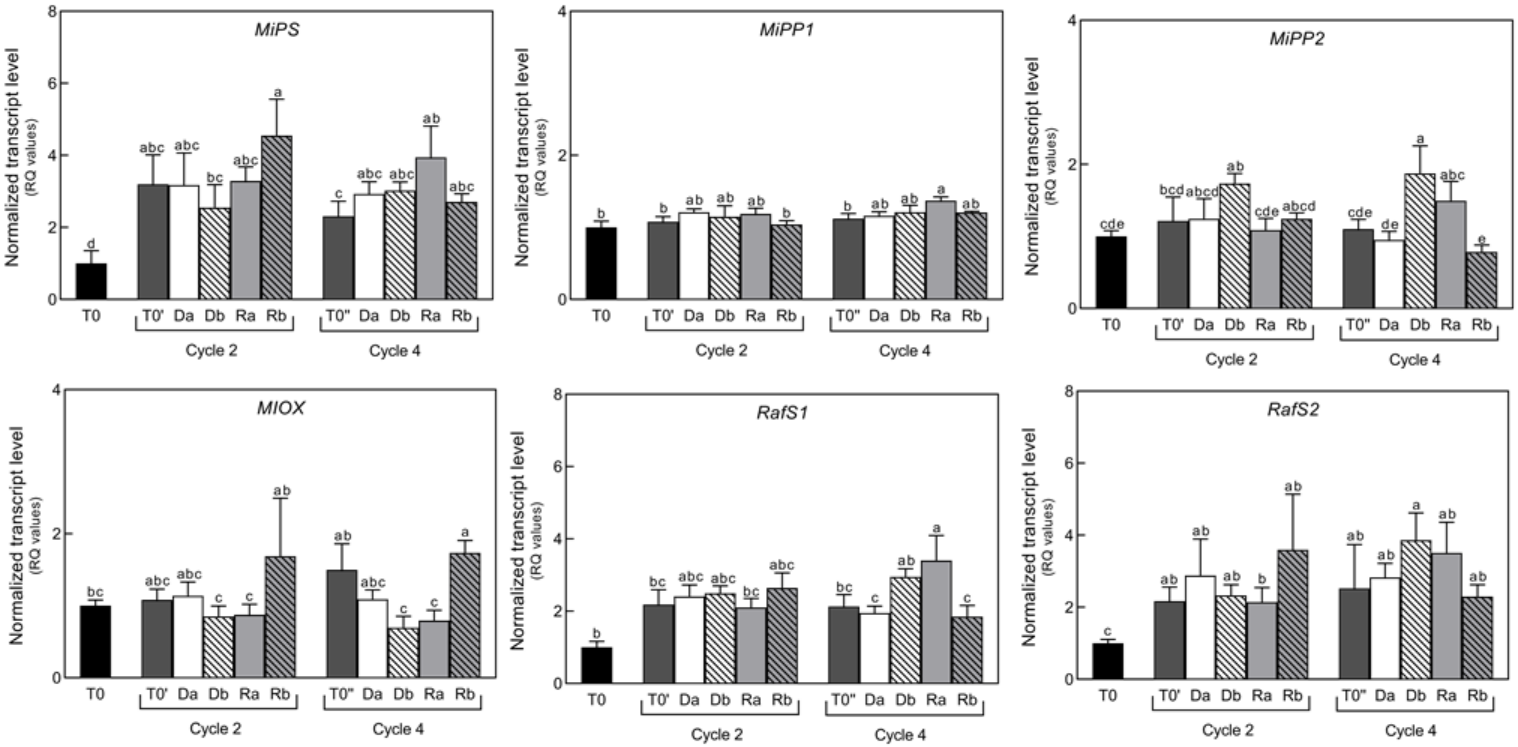
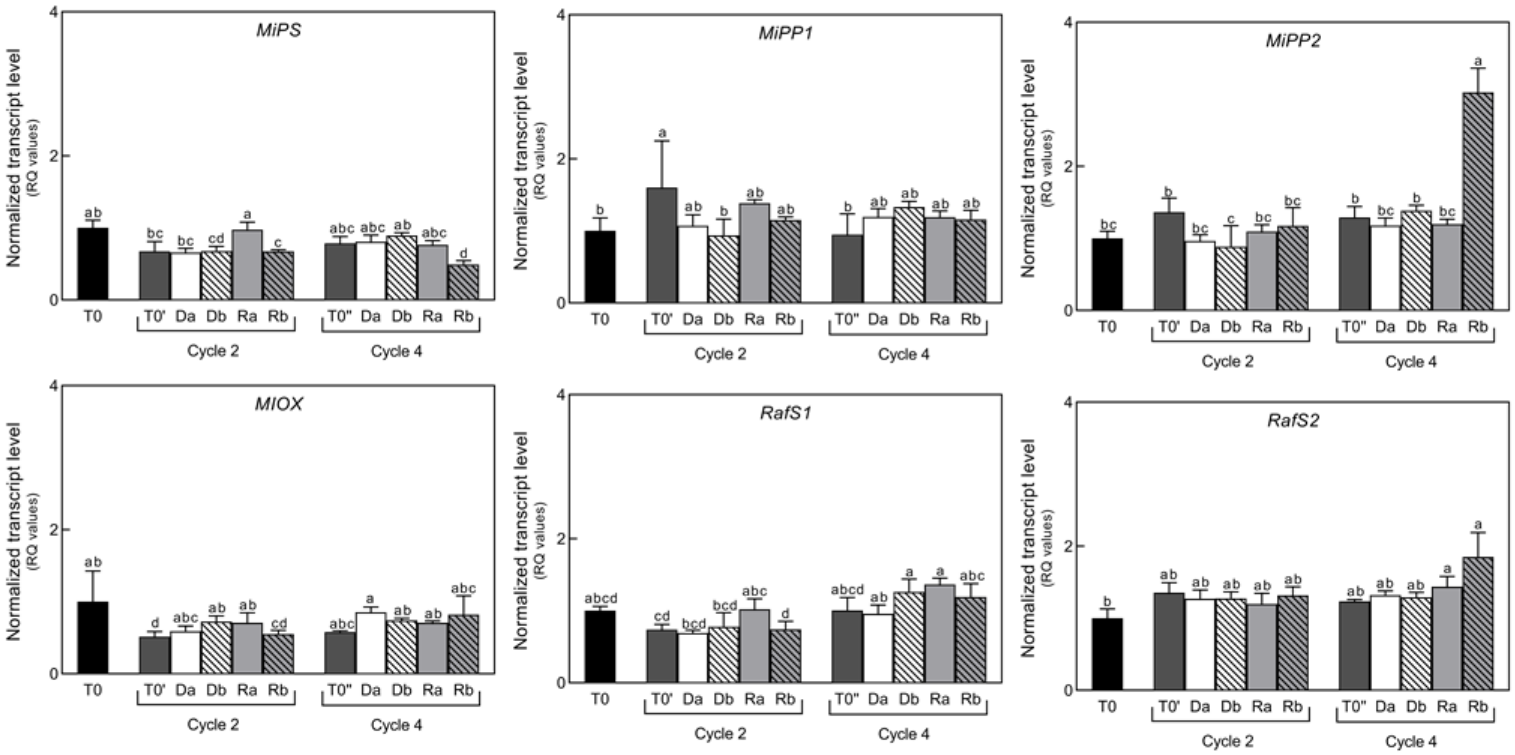

MiPS
MiPP1
MiPP2
MIOX
RafS1
RafS2
Normalized transcript level
(RQ values)
T0
T0' Da Db Ra Rb
T0" Da Db Ra Rb
Cycle 2
Cycle 4

Figure 6


MiPS
MiPP1
MiPP2
MIOX
RafS1
RafS2
Normalized transcript level
(RQ values)
T0
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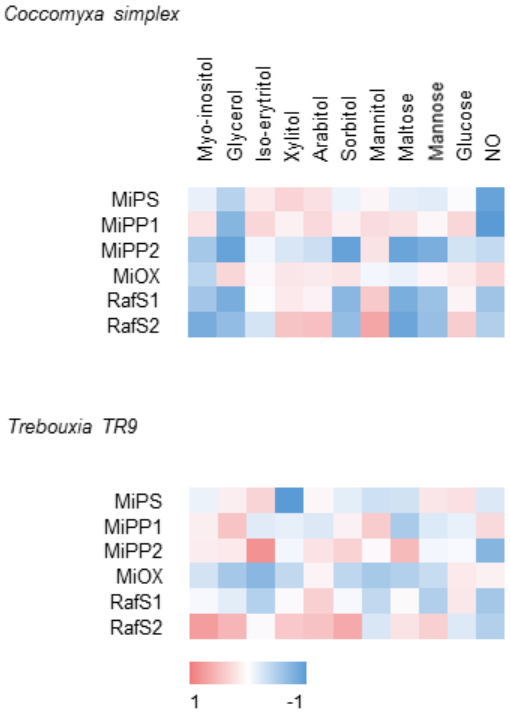
T0" Da Db Ra Rb
Cycle 2
Cycle 4

Figure 7


Coccomyxa simplex
Myo-inositol
Glycerol
Iso-erytritol
Xylitol
Arabitol
Sorbitol
Mannitol
Maltose
Mannose
Glucose
NO
MiPS
MiPP1
MiPP2
MiOX
RafS1
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RafS2
1
-1

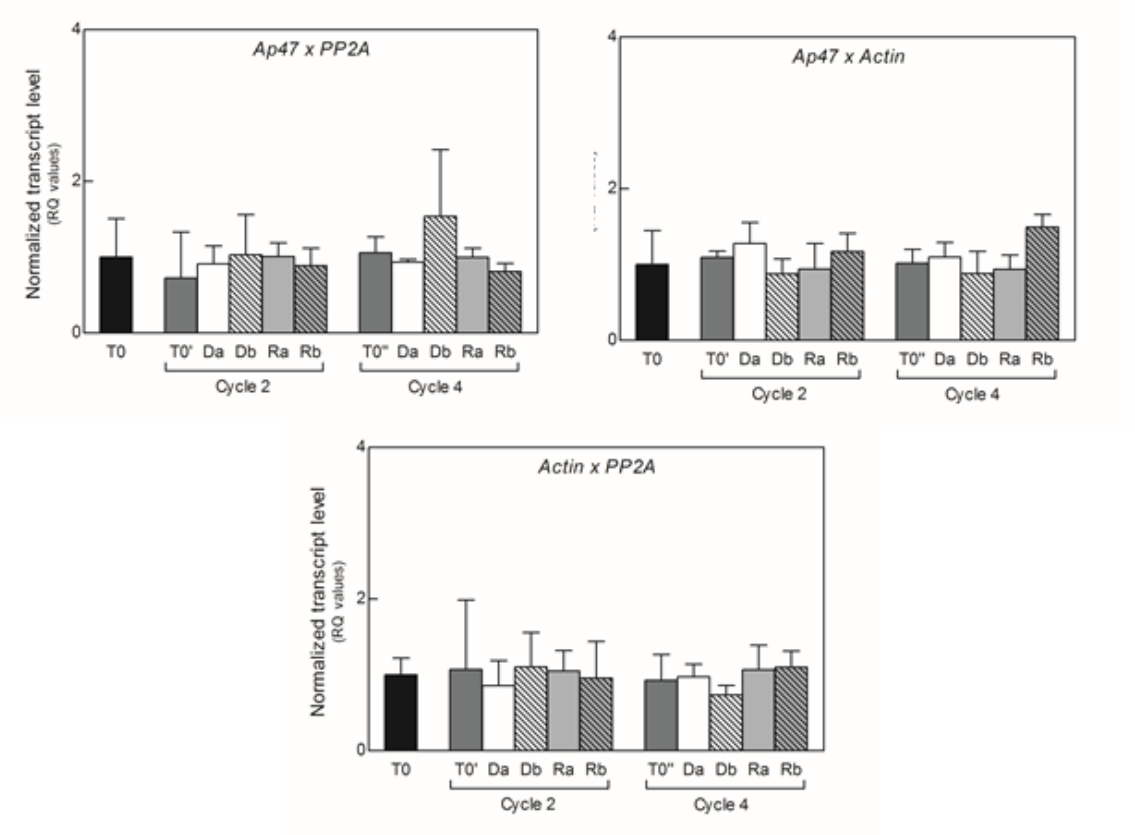

Figure S1: Comparison between housekeeping (Actin, Ap47 and PP2A), in Csol microalgae, during cyclic desiccation. Values were calculated with $2^{-\Delta\Delta Ct}$ relative quantification for five biological replicates. Error bars show the standard error. Black bars indicate initial control treatment (T0); dark gray bars indicate controls of cycles 2 and 4 (T0' and T0", respectively); white solid and striped bars indicate desiccation treatments with 50% (Da) and 100% (Db) of the minimal RWC value, respectively; light gray solid and striped bars indicate treatments after 1 h (Ra) and 16 h (Rb) of rehydration, respectively

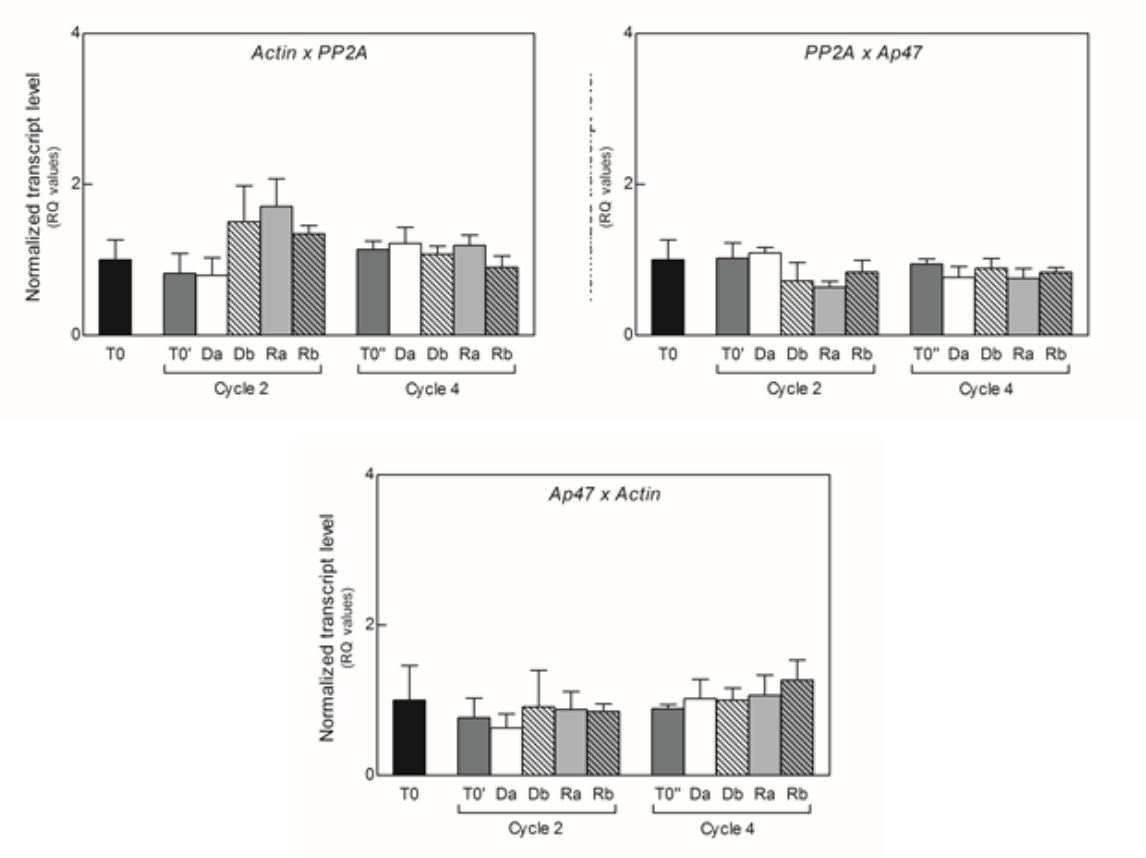

Figure S2: Comparison between housekeeping (Actin, Ap47 and PP2A), in TR9 microalgae, during cyclic desiccation. Values were calculated with $2^{-\Delta\Delta Ct}$ relative quantification for five biological replicates. Error bars show the standard error. Black bars indicate initial control treatment (T0); dark gray bars indicate controls of cycles 2 and 4 (T0' and T0", respectively); white solid and striped bars indicate desiccation treatments with 50% (Da) and 100% (Db) of the minimal RWC value, respectively; light gray solid and striped bars indicate treatments after 1 h (Ra) and 16 h (Rb) of rehydration, respectively

Table S1- Oligonucleotide sequences of sugar alcohol and raffinose genes, and reference genes of *Coccomyxa simplex* employed for transcript quantification by a real-time PCR. F, forward; R, reverse. pb, pair of bases.

Transcript ID	Accession number	Primer sequences (5' – 3')	Amplicon size (pb)
CsolAct	MK422473	F: CCTACGTGGCCATCGACTAT R: CTGCCAATCGTGATCACCTG	106
CsolAp47	MK422476	F: TCAACGACAAGGTGCTGTTC R: CGCACACACTGATGGAACTT	91
CsolPP2A	MK422485	F: AGCAGTTCAACCACACCAAC R: CAGGAGTAGCCCTCCATGAC	73
CsolMiPS	MT683896	F: ACATCTCCAGCCTCAACCTC R: TGCAGCTCAAAGTCCAACAC	64
CsolMiPP1	MT683894	F: AACCAACTTTGCACATGGCT R: GCAGTGTACGTCTTGGTCAC	132
CsolMiPP2	MT683895	F: GTTCTGGGAGTGCTAGACCA R: CGAACAGGCTCACCATTCAG	89
CsolMiOX	MT683893	F: AGACCGTGGACTACGTCAGA R: GACAGGTCTGCGTCTGTTTC	151
CsolRafS1	MT683897	F: ATCAACTGCATGTGCCACTC R: CGGCCAGAAATCATCTGACG	84
CsolRafS2	MT683898	F: GACTTCTATCCGCGCAATCC R: CTGGATGCTTGCTCTGGAAC	121

Table S2- Oligonucleotide sequences of sugar alcohol and raffinose genes, and reference genes of *Trebouxia* sp. TR9 employed for transcript quantification by a real-time PCR. F, forward; R, reverse. pb, pair of bases.

Transcript ID	Accession number	Primer sequences (5' – 3')	Amplicon size (pb)
TR9Act	MH068774	F: GAGCGAGGTTACAGTTTCAC R: GGTTGAACTTGAAGCAGCAG	210
TR9Ap47	MH068775	F: CTTGTCATTGAGGCCAAGCTT R: TGGTGGGAGCCTTGAAGATG	71
TR9PP2A	MH068779	F: TCTCGGATCTGCTAGAGCTGTTC R:CTGAGTGATAGCCTCTGTCTACATAGTCT	96
TR9MiPS	MT683902	F: ATCAAGCCCACTCACTCCAA R: CCATCCTTGGTCATCCAGGT	155
TR9MiPP1	MT683900	F: TCCTCAAGGTTCTCCAGCAG R: GTACTCAGAGTCAGCGTTGC	86
TR9MiPP2	MT683901	F: TCACAGCCATAAGGGAAGCA R: GTCAGGTCATTGGTGAAGCC	85
TR9MiOX	MT683899	F: CGCCTGTACAGTAGCACAAC R: GGAGGAAGGGCTGTCTTCTT	125
TR9RafS1	MT683903	F: CTCCATCAAGCAGCACTTCC R: TCTCGCGGGTAGAAGTCATC	123
TR9RafS2	MT683904	F: GAGGTCCAGGTTGCCTGTAT R: TCACACACAGCACTTCTCCT	116

Table S3. Changes in the proportions of sugars and sugar alcohols in Csol and TR9 microalgae exposed to cyclic desiccation-rehydration. (T0) indicates initial control treatment; (T0' and T0") indicates the controls of cycles 2 and 4, respectively; (Da) and (Db) indicates 50% and 100% of the minimal RWC value, respectively; (Ra) and (Rb) indicates 1h and 16h of rehydration, respectively. The amount of the indicated metabolites was determined by HPAEC/PAD. Values represent mean of three biological replicates. Different letters indicate significant differences ($p < 0.05$) between each desiccation–rehydration stage following Fisher's least significant difference (LSD) test ($P < 0.05$). Capital letters compare the sugar and sugar alcohol content in each treatment. Lower letter compares the sugar and sugar alcohol content among desiccation-treatments. b.d.l below detection limit.

	Relative percentage (%)										
	Control	Cycle 2					Cycle 4				
	T0	**T0'**	**Da**	**Db**	**Ra**	**Rb**	**T0''**	**Da**	**Db**	**Ra**	**Rb**
Coccomyxa simplex											
Myo-inositol	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	0.12 Bb	5.44 Aa	b.d.l.	b.d.l.	b.d.l.	b.d.l.
Glycerol	0.10 Ba	b.d.l.	1.55 Ba	1.42 Ba	5.13 Aa	0.41 Ba	1.75 Aa	3.76 Ba	0.78 Ba	43.50 Aa	35.30 Aba
Iso-erytritol	1.85 Ba	b.d.l.	b.d.l.	b.d.l.	0.04 Aa	2.36 Ba	8.69 Aa	0.25 Ba	1.23 Ba	37.72 Aba	0.14 Ba
Xylitol	0.33 Bb	0.27 Bb	1.30 Bab	1.99 Ba	19.49 Aab	0.01 Bb	0.87 Aa	0.15 Bb	0.05 Bb	b.d.l.	0.01 Bb
Arabitol	27.78 Aba	4.23 Ba	23.72 Ba	23.95 Aba	33.88 Aa	0.19 Ba	12.15 Aa	b.d.l.	0.16 Ba	b.d.l.	b.d.l.
Sorbitol	0.02 Ba	b.d.l.	0.04 Ba	0.14 Ba	0.04 Aa	7.45 Ba	8.60 Aa	b.d.l.	9.33 Ba	0.56 Ba	b.d.l.
Mannitol	63.56 Aa	94.09 Aa	65.28 Aa	49.37 Aa	31.93 Aa	63.07 Aa	26.96 Aa	92.96 Aa	68.20 Aa	18.21 Aba	64.55 Aa
Maltose	b.d.l.	b.d.l.	b.d.l.	0.20 Ba	b.d.l.	18.36 Ba	20.97 Aa	b.d.l.	10.88 Ba	b.d.l.	b.d.l.
Mannose	0.19 Ba	b.d.l.	0.88 Ba	2.12 Ba	1.13 Aa	5.48 Ba	13.52 Aa	b.d.l.	8.90 Ba	b.d.l.	b.d.l.
Glucose	6.17 Ba	1.41 Ba	7.24 Ba	20.82 Aba	8.35 Aa	2.54 Ba	1.05 Aa	2.88 Ba	0.46 Ba	b.d.l.	b.d.l.
Trebouxia TR9											
Myo-inositol	1.69 Bb	0.24 Bb	2.26 Bab	0.88 Bab	0.79 Aab	1.70 Bab	1.89 Bcab	5.84 Ba	10.31 Bb	1.19 Bab	0.18 Bb
Glycerol	4.52 Bab	0.38 Bab	0.86 Bab	0.47 Bab	0.35 Aab	1.03 Bab	1.06 Cb	1.27 Ba	9.14 Bab	1.79 Bab	b.d.l.
Iso-erytritol	26.27 Abab	1.09 Bab	0.93 Bab	0.92 Bab	11.05 Aa	13.56 Bab	1.85 Bcab	2.51 Bab	1.10 Bb	2.23 Bab	0.24 Bb
Xylitol	b.d.l.	0.25 Bab	0.85 Bab	0.46 Bab	b.d.l.	0.44 Bab	0.03 Cb	1.23 Bab	4.03 Ba	0.45 Bab	4.30 Bab
Arabitol	61.74 Aa	63.38 Aab	34.06 Bb	1.05 Bb	1.51 Ab	2.41 Bb	3.30 BCb	4.50 Bb	4.0 Bb	3.47 Bb	0.00 Bb
Sorbitol	2.46 Bb	26.02 Abb	59.62 Abab	85.67 Aa	55.00 Aab	78.45 Aab	80.83 Aab	73.46 Aab	71.4 Ab	82.1 Aab	0.89 Bb
Mannitol	2.00 Bb	1.30 Bb	0.81 Bb	4.05 Bb	25.63 Ab	1.65 Bb	4.26 BCb	2.94 Bb	b.d.l.	3.53 Bb	94.38 Aa
Maltose	b.d.l.	0.98 Bb	0.08 Bb	5.11 Ba	0.34 Ab	b.d.l.	b.d.l.	0.96 Bab	b.d.l.	b.d.l.	b.d.l.
Mannose	b.d.l.	4.57 Bb	0.54 Bb	2.02 Bab	5.13 Aab	b.d.l.	6.66 Bab	7.22 Ba	b.d.l.	3.15 Bab	b.d.l.
Glucose	1.32 Ba	1.78 Ba	b.d.l.	0.07 Ba	0.20 Aa	0.76 Ba	0.13 Ca	0.06 Ba	b.d.l.	2.11 Ba	b.d.l.

5. GENERAL CONCLUSIONS

The present PhD Thesis was aimed at studying the composition and metabolic changes and the defense systems in two lichen microalgae with different ecology and putatively distinct hydric responses, *Trebouxia sp.* TR9 and *Coccomyxa simplex*, subjected to D/R, and relate those physiological traits with the DT of these species. Two types of experiments were performed: (i) one D/R cycle – *Research paper I*, (ii) subsequent D/R cycles, allowing us to observe acclimation processes that often occur under natural conditions - *Research papers II and III*. The following general conclusions can be derived from these studies:

1. The dynamics of the hydric parameters are consistent with the hyphotesis that the DT strategy of each microalga is fitted according to the water regime of their respective habitat.

2. When subjected to daily slow desiccation cycles in air-dried conditions similar to those of their natural habitat (~ 25% RH - TR9 and ~ 56% RH - Csol), both species were able to fully recover their photosynthetic rates after the four D/R cycles.

3. Regarding to their metabolim, TR9 induced the synthesis of protective RFOs and antioxidant phenolics while Csol invested in the accumulation of ascorbic acid.

4. Polyols play an important role as osmoprotactants in both microalgae. However, TR9 maintained constitutively higher amounts of polyols that are unchanged during one cycle of D/R, contrasting with the lower but inducible levels of these metabolites observed in Csol.

5. After several D/R cycles, a species-specific modification of the expression pattern of *myo*-inositol and raffinose synthase related genes occurs in each microalga, possibly through the NO modulation of polyol biosynthetic pathway.

6. Some antioxidant encoding genes, such as TR9 MnSOD-1, MnSOD-3, MnSOD-4, FeSOD, GR-2 and Csol MnSOD-1, CAT, GR- 2 and GCL are likely induced by the increase in ROS formation provoked by cyclic D/R conditions.

7. The induction of antioxidant genes and *myo*-inositol and raffinose synthase related genes could be included as primed responses, suggesting a strategy of metabolic economy to cope with D/R stress. In addition, NO may also be related to a primed memory state, by assisting in the rapidly and more precise metabolic response to subsequent D/R cycles through influencing on the transcriptional expression of sugar alcohols-related genes.

8. The results presented in this thesis demonstrate that the desiccation tolerance in *Trebouxia sp.* TR9 and *Coccomyxa simplex* is species-specific and consistent with the ecological contexts in which these microalgae naturally occur. Moreover, the induction of antioxidant enzymes and polyol synthesis during cyclic D/R, suggest a primed memory response to oxidative stress.

APPENDIX

Additional productions published during the PhD time course.

Metabolic Changes on the Acquisition of Desiccation Tolerance in Seeds of the Brazilian Native Tree *Erythrina speciosa*

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Metabolic Changes on the Acquisition of Desiccation Tolerance in Seeds of the Brazilian Native Tree *Erythrina speciosa*

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Erythrina speciosa Andrews (Fabaceae) is a native tree of Atlantic forest from Southern and Southeastern Brazil. Although this species is found in flooded areas, it produces highly desiccation tolerant seeds. Here, we investigated the physiological and metabolic events occurring during seed maturation of *E. speciosa* aiming to better understand of its desiccation tolerance acquisition. Seeds were separated into six stages of maturation by the pigmentation of the seed coat. Water potential (WP) and water content (WC) decreased gradually from the first stage to the last stage of maturation (VI), in which seeds reached the highest accumulation of dry mass and seed coat acquired water impermeability. At stage III (71% WC), although seeds were intolerant to desiccation, they were able to germinate (about 15%). Desiccation tolerance was first observed at stage IV (67% WC), in which 40% of seeds were tolerant. At stage V (24% WC), all seeds were tolerant to desiccation and at stage VI all seeds germinated. Increased deposition of the arabinose-containing polysaccharides, which are known as cell wall plasticizers polymers, was observed up to stage IV of seed maturation. Raffinose and stachyose gradually increased in axes and cotyledons with greater increment in the fourth stage. Metabolic profile analysis showed that levels of sugars, organic, and amino acids decrease drastically in embryonic axes, in agreement with lower respiratory rates during maturation. Moreover, a non-aqueous fractionation revealed a change on the proportions of sugar accumulation among cytosol, plastid, and vacuoles between the active metabolism (stage I) and the dormant seeds (stage VI). The results indicate that the physiological maturity of the seeds of *E. speciosa* is reached at stage V and that the accumulation of raffinose can be a result of the change in the use of carbon, reducing metabolic activity during maturation. This work confirms that raffinose is involved in desiccation tolerance in seeds of *E. speciosa*, especially considering the different subcellular compartments and suggests even that the acquisition of desiccation tolerance in this species occurs in stages prior to the major changes in WC.

Keywords: carbohydrates, cell wall, legume, metabolic profiling, non-aqueous fractionation, seed maturation

INTRODUCTION

Physiological deterioration of seeds stored in gene banks is a huge problem that remains to be solved for large-scale, long-term seed preservation (Chmielarz, 2009). Seeds and fruits that do not mature and the difficulty to determine maturity are included among the biological constraints of reforestation, because of the requirement of a constant supply of high-quality seeds (Owens, 1995). The degree of seed orthodoxy or recalcitrance depends on the maturation degree at shedding and the characteristics of each species (Barbedo, 2018). Therefore, the understanding physiological behavior of seeds regarding desiccation tolerance is crucial to obtain seeds of high quality.

Desiccation is a natural process that occurs at the end of seed development preparing it to enter in a quiescent stage (Dasgupta et al., 1982; Beardmore and Whittle, 2005). Developing orthodox seeds acquire the ability to tolerate desiccation during the mid to late maturation stages, and their longevity increases logarithmically with the decrease in water content, allowing these seeds to retain vigor and viability throughout a storage period under defined conditions (Berjak and Pammenter, 1999; Berjak and Pammenter, 2008). However, acquisition of desiccation tolerance seems to be strongly influenced by environmental conditions and variable among different stages of development (Huang et al., 2012; Leduc et al., 2012; Barbedo et al., 2013; Caccere et al., 2013; Lamarca et al., 2013; Newton et al., 2013).

Seed desiccation is a very active event concerning gene expression and metabolism (Angelovici et al., 2010). The overlap of the metabolic processes associated with the desiccation tolerance and the events related to seed development represents the main hindrance in the study of the factors implicated in desiccation tolerance acquisition (Farrant et al., 2012). Metabolic changes occur during seed maturation, prior to dehydration, in a process dedicated to reserve synthesis and coupled to nutrient absorption (Borisjuk et al., 2004). This step of the development is accompanied by sucrose accumulation, cell expansion, and increase in the energetic status and metabolic fluxes in direction to storage products (Weber et al., 2005). At the metabolic levels, the reserve accumulation stage is characterized by a low oxygen state that causes a transitory stimulus for fermentative metabolism (Rolletschek et al., 2004). In *Arabidopsis* seeds, accumulation of storage compounds and decrease in sugars, organic acids, and amino acids were observed during the maturation stage, although alterations in these compounds were also found during seed desiccation (Fait et al., 2006). In pea (Rogerson and Matthews, 1977) and beans (Farrant et al., 1997), a decrease in the levels of sugars, followed by a decrease in respiratory rates, was observed during seed maturation. In *Arabidopsis*, a decrease in sugars and in respiratory rates, indicated by a decrease in intermediates of the Krebs cycle, was also observed (Fait et al., 2006).

Desiccation tolerance is acquired while reserve deposition occurs prior to the onset of maturation drying. Several cellular and metabolic processes have been connected with the acquisition of desiccation tolerance. They include changes in intracellular characteristics, such as reduction of the vacuolation degree, intracellular de-differentiation, decay of metabolic activity, presence of oleosine, and free-radical scavengers, as well as repair mechanisms during rehydration (Berjak and Pammenter, 1999; Oliver et al., 2000). Other important compounds, such as late embryogenesis abundant (LEA) proteins (Kermode, 1997; Wolkers et al., 2001), amphipathic molecules (Hoekstra et al., 1997), and soluble sugars and cyclitols (Koster and Leopold, 1988; Crowe et al., 1992; Centeno et al., 2016), are also considered important protective substances, mainly at the dehydrated state. Recently, a number of transcriptomic and proteomic studies have confirmed the abundance of such protective compounds associated with acquisition of desiccation tolerance in orthodox seeds (reviewed by Giarola et al., 2017).

The accumulation of sucrose and oligosaccharides during seed development, for instance, has been associated with desiccation tolerance in various species, i.e. *Brassica campestris* (Leprince et al., 1990), maize (Chen and Burris, 1990; Brenac et al., 1997), wheat (Black et al., 1999), lupin (Górecki et al., 1997), and pea (Corbineau et al., 2000). During seed drying, the formation of a glassy matrix might reduce the molecular mobility as an aspect on the protection of lipid membranes and macromolecules (Williams and Leopold, 1989; Buitink et al., 1999). Soluble sugars, such as sucrose and oligosaccharides, may form a glass during desiccation, resulting in molecular stabilization during desiccation (Williams and Leopold, 1989; Wolkers et al., 1998). This could even determine the longevity of the seed since this would vary according to the newly formed spatial interactions among molecules during desiccation (Walters, 2015). Moreover, vitrification of the intermembrane sugar solution could improve the resistance of the lipid phase transition in membranes during dehydration process (Bryant et al., 2001). In leaves of *Arabidopsis*, a non-aqueous fractionation (NAF) showed that raffinose is accumulated in the plastids after freeze–thaw cycles, and its role might be related to the stabilization of the photosystem II, located at plastid membranes (Knaupp et al., 2011). Cold acclimation also resulted in the accumulation of raffinose and galactinol in plastids fractions (Hoermiller et al., 2017). The accumulation of both compounds has been also related to work against deleterious effects of reactive oxygen species (ROS) in *Arabidopsis* (Nishizawa et al., 2008). In contrast, monosaccharide levels have a negative correlation with desiccation tolerance (Hoekstra et al., 2001). Fructose and glucose could be involved in Maillard reactions and would be a source of hydroxyl radicals in dehydrated tissues, thus favoring the appearance of browning (Koster and Leopold, 1988; Van der Toorn and McKersie, 1995). In *Medicago truncatula*, Vandecasteele et al. (2011) have suggested that the increase in sucrose/raffinose family oligosaccharides (RFO) ratio can negatively affect seed vigor.

Cell wall folding has been considered as another strategy to overcome cell desiccation, preventing possible rupture of the continuum cell wall-plasma membrane during water loss and after rehydration (Webb and Arnott, 1982). The extent and way of folding in related to their conformation and chemical composition. High proportions of arabinans, which are highly mobile polysaccharides and are able to absorb water, allow for higher wall flexibility, thus contributing to diminish mechanical stress during water loss (Moore et al., 2006; Farrant et al., 2012). Increased deposition of arabinose-containing polymers has been reported during development of orthodox seeds, e.g., of

Arabidopsis (Gomez et al., 2009) and *Tyslosema esculentum* (Mosele et al., 2011), suggesting that they might play a role in seed tissues that undergo desiccation.

Although many studies with seeds, carried out during the last several years, have contributed to a better understanding of some events connected to desiccation tolerance acquisition, most information has been gathered from experiments performed with crops. Seeds of tropical species show differences in the degree of desiccation tolerance at shedding. Frequently, these differences occur among seeds of the same species from different regions and even from different years from the same specimen (Daws et al., 2004; Lamarca et al., 2013). Tropical forests constitute a genetic diversity reservoir, which plays a crucial role in the environment stability. Most seeds are disseminated with high water content, and germination often occurs directly after dissemination, although seeds with hard coats, such as many legume and wind dispersed seeds from dry fruits, show orthodox storage behavior (Rodríguez et al., 2000).

Erythrina speciosa is a legume tree native of the Brazilian Atlantic Forest with ornamental potential and rich in bioactive secondary metabolites, which have been used in traditional medicine due to their antimicrobial, antimalarial, and antiparasitic properties (Amaral et al., 2019). Although this species is found in flooded areas, its seeds are remarkably tolerant to desiccation and can be stored for many years under different conditions without losing vigor. These orthodox seeds have dormancy by seed coat water impermeability, which varies according to the climate conditions during maturation (Molizane et al., 2018). The mature seeds of *E. speciosa* are composed of ca. 40% soluble carbohydrates, which are mainly represented by raffinose family oligosaccharides (45% of raffinose, 40% of sucrose 12% of stachyose) and small amounts of reducing sugars, such as glucose and fructose. Starch represents less than 2% of the storage material and lipids account for ca. 12% (Mello et al., 2010; Mello et al., 2011). A number of studies performed by our group with *E. speciosa* allowed us to understand the physiological characteristics and the biochemical composition of its seeds during maturation and after germination (Mello et al., 2010; Mello et al., 2011; Molizane et al., 2018). Thus, *E. speciosa* has become a potential interesting model for studies of classical orthodox seeds from tropical environments that accumulate mainly soluble sugars. The lack of reports of the metabolic changes associated to water loss in seeds of this species prompted us to investigate the physiological and metabolic events occurring during seed maturation in *E. speciosa* to establish a better understanding of the fundamental changes related to its acquisition of desiccation tolerance.

MATERIALS AND METHODS

Plant Material and Sampling

Fruits of *E. speciosa* Andrews were collected from fully grown plants growing in a homogeneous plantation located at the Parque Central de Santo André (23°40′ 20–50′′ S and 46°31′35–55′′W, 784 m alt.), state of São Paulo, Brazil, during the whole fruiting season, which lasts from August to October, the end of winter and beginning of spring in the Southern hemisphere. In the first year experiment, flowers were tagged on the day of their anthesis (DAA), and the development of fruits and seeds was analyzed by collecting fruits directly from the branches and classified as 20, 38, 42, 44, 57, and 60 DAA. Seeds were removed from the pods by hand, and their length, width, thickness, and color were also registered (four replicates of 10 seeds) before the evaluation of their morphological characterization (**Supplementary Figure S1**). Each sampling period was considered as a different stage of the seed development. In the second year experiment, based on the results obtained in the first experiment, six different stages (I to VI) of seed development were obtained from a single harvest. These six stages cover the end of the embryogenesis and the entire maturation process (**Figure 1**).

Analysis of Seed Desiccation Tolerance

Seeds from each stage of development were submitted to drying up to about 15% and 10% water content by closing them in desiccators with dry silica gel, at 25°C. After reaching the target water content, germination testes were carried out as described previously.

Seed Maturation and Germination

Seeds were weighed, and the water content (%, on a fresh weight basis) and the dry matter (mg seed^{-1}) were determined for each stage of maturity (four replicates of four seeds), after oven drying at 103°C ± 3°C for 24 h (ISTA, 2015). Before drying, the water potential of seeds was measured with a Decagon WP4 potentiometer (Pullman, USA) based on the dew point (Decagon Devices 2001), according to the manufacturer's instructions. For this procedure, lengthwise sectioned seeds were measured, using four replicates of five seeds each (Mello et al., 2011). Seeds from six different stages of development were submitted to germination tests in paper rolls. Four replicates of 15 seeds were placed between two layers of germination paper previously moistened with distilled water. After rolling the papers, the rolls were maintained in Marconi MA400 germination chambers (Piracicaba, Brazil) at 25°C ± 1°C, under continuous light. Germination was evaluated every 2 days from the sowing day, by registering the protrusion of the primary root (≥5 mm). After 30 days, the normal seedlings development was registered considering seeds with both normal radicular (single straight primary root) and shoots system (normal leaf morphology for the species). To estimate vigor, the Germination Speed Index (GSI) was calculated according Maguire's equation (Maguire, 1962), GSI = n1 /t1 + n2 / t2 + … n7 / t7; where n1, n2, … n7 are the number of germinated seeds at times t1, t2, … t7 (in days).

Sugar Extraction and Analysis

Seeds from the six different stages of development had their seed coat removed before the embryos were separated in embryonic axis and cotyledon and weighed for subsequent analyses. Embryonic axes and cotyledons from 30 fresh embryos (in triplicates) were boiled separately in 80% ethanol (3 ml·g^{-1}·fresh mass^{-1}) for 5 min for enzyme inactivation. The supernatants were recovered by centrifugation (1000*g* for 20 min), and the

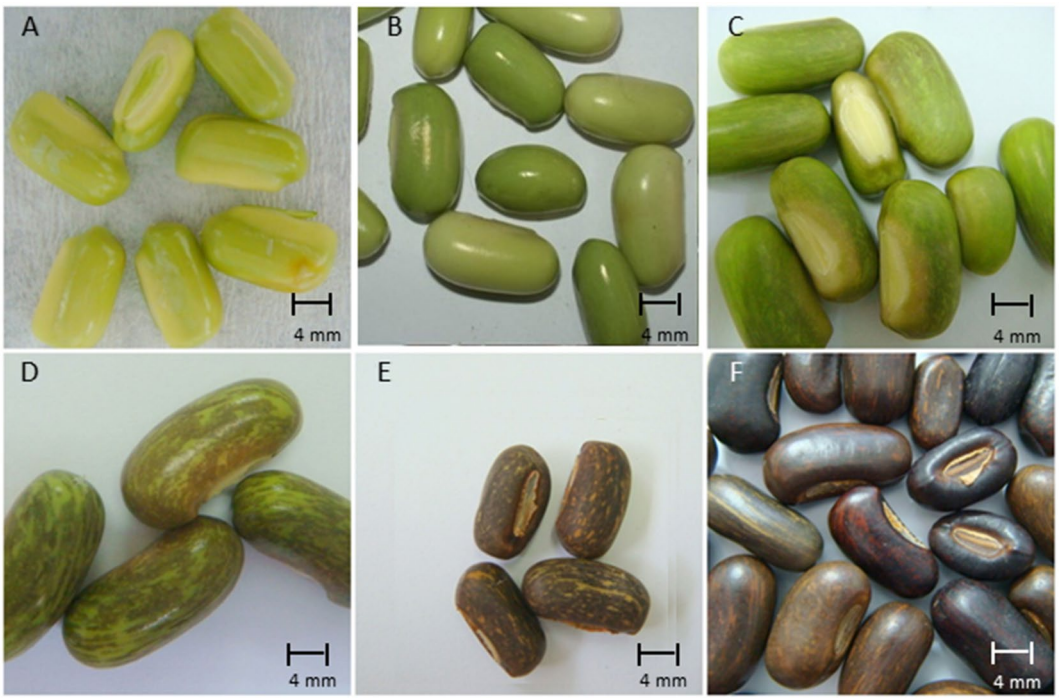

FIGURE 1 | Morphological aspects of seeds of *Erythrina speciosa* at six maturation stages (**A**: 20 DAA, stage I; **B**: 38 DAA, stage II; **C**: 42 DAA, stage III; **D**: 44 DAA, stage IV; **E**: 57 DAA, stage V; **F**: 60 DAA, stage VI) obtained in a single harvest.

residues were manually homogenized and re-extracted twice in boiling 80% ethanol. After centrifugation, the residues were washed with distilled water and recovered by centrifugation. The resulting ethanolic and aqueous supernatants were combined, concentrated in rotoevaporator, re-dissolved in 10 ml of distilled water and considered as the soluble sugar extracts. Residues were freeze-dried and used for quantification of starch and cell wall fractionation as described below. The amounts of total carbohydrates and reducing sugars in the ethanolic extracts were determined colorimetrically by the phenol-sulfuric acid method (Dubois et al., 1956) and Somogyi-Nelson procedure (Somogyi, 1945), respectively, using glucose as standard.

Cell Wall Extraction and Analysis

Aliquots of the ethanol-insoluble residue were extracted three times with chloroform: methanol (1:1, v/v) (10 ml·g^{-1}) at room temperature for 20 min, followed by extraction with acetone and ethyl ether for 10 min to remove lipids.

The residue after lipid removal was extracted with 90% dimethyl sulfoxide (DMSO) (10 ml·g^{-1}·dry residue^{-1}) for 16 h at room temperature. After centrifugation at 13,000*g* for 15 min, the residue was submitted to four additional extractions with DMSO, twice at room temperature for 4 h and twice overnight at 5°C. The residue was then exhaustively washed with distilled water, centrifuged, and subsequently treated with amyloglucosidase from *Aspergillus niger* (Megazyme, Ireland) in sodium acetate buffer 100 mM, pH 4.5 (0.1 unit enzyme mg^{-1}) for 24 h at 30°C to remove the remaining starch after the DMSO extraction. The residue was washed thoroughly with distilled water (10 ml·g^{-1}), chloroform: methanol (1:1, v/v), and acetone followed by ethyl ether (twice), acetone, and was recovered by filtration through a glass fiber filter (Whatman GF/A). Finally, the residue was freeze-dried and considered as the yield in crude cell walls (modified from Gorshkova et al., 1996).

One milligram of crude cell walls from embryonic axis and cotyledons was hydrolyzed in 500 µl 2 N trifluoroacetic acid (TFA) in an autoclave at 121°C (1.5 atm, 1 h). The acid was eliminated by evaporation, and the residue was dissolved in deionized water for further anion exchange chromatography analysis. The amounts of uronic acids were determined colorimetrically by *m*-hydroxydiphenyl assay (Filisetti-Cozzi and Carpita, 1991) using galacturonic acid (Sigma Co., USA) as standard.

Neutral sugars were analyzed by anion exchange chromatography in an ICS 3000 system (Dionex, USA), coupled with a pulsed amperometric detector (HPAEC/PAD) (Dionex, USA). The neutral monosaccharides were eluted isocratically in a Carbo-Pac PA1 column in 20 mM NaOH at a flow rate of 0.2 ml·min^{-1}, for 35 min and identified by comparing their elution times with commercial standards of fucose, arabinose, rhamnose, galactose, glucose, xylose, and mannose (Sigma Co., USA).

Metabolic Profile

The metabolic profiling was performed by GC-MS as described by Roessner et al. (2001), modified with the peak identification optimized for *E. speciosa* seeds. Due to the very low mass of embryonic axes from stage I, the metabolic profile was obtained

for stage II to VI. Cotyledons and embryonic axes separated from seeds were ground in liquid nitrogen, 100 and 50 mg were weighed, respectively, and extracted in 500 µl of chloroform/methanol/water mix (12:5:1) and 50 µl of adonitol (0.2 mg·ml^{-1} pyridin) as internal standard. The mixture was agitated in a vortex, warmed at 60°C for 30 min under agitation and centrifuged at 13,000 rpm for 2 min. From the upper phase (hydroalcoholic), 350 µl were then transferred to a new tube, and 350 µl of water were added. The mixture was then agitated and centrifuged at 13,000 rpm for 5 min. Three hundred microliters were taken and dried under vacuum for further derivatization. The dried samples were derivatized with 150 µl of pyridin, 50 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), and 50 µl of methoxiamine hydrochloride (0.2 mg·ml^{-1} pyridin) and injected in a gas chromatography–mass spectrometry (GC-MS) system composed of an Agilent GC 6890 series (Agilent, USA). GC was performed on a 30-m HP5 column with 0.25 µm film thickness (Supelco, Bellfonte, CA). The injection temperature was set at 230°C, the interface at 250°C, and the ion source adjusted to 150°C. Helium was used as the carrier gas at a flow rate of 1 ml·min^{-1}. The analysis was performed under the following temperature program: 5 min of isothermal heating at 70°C, followed by a 5°C·min^{-1} oven temperature ramp to 310°C, and a final 1 min of heating at 310°C. Mass spectra were recorded at 2 scan s^{-1} with a scanning range of 50 to 600 m/z. Both chromatograms and mass spectra were evaluated using the ChemStation program (Agilent, USA). The peaks were identified and quantified in comparison with authentic standards and the NIST Mass Spectral Library.

Estimation of Respiratory Rates Analysis

Respiratory rates were estimated using approximately 90 seeds per developmental stage, separated in three replicates. The O_2 consumption and CO_2 release were determined by an analyser model 6600 (Illinois Instruments, Inc., Johnsburg, EUA) as previously described by Lamarca and Barbedo (2012).

NAF

Subcellular fractionation of vacuolar, plastidial, and cytosolic compartments was done according to Knaupp et al. (2011). Briefly, 80 mg of freeze-dried tissue homogenate was suspended in 10 ml of heptane-tetrachlorethylene (ρ = 1.3 g cm^3), cooled on ice, and repeatedly sonified for 5 s with pauses of 15 s over a time course of 12 min (Branson Sonifier 250, output control 4; Branson). Subsequently, the sonified suspension was passed through nylon gauze, pore size 30 µm, and centrifuged. The sediment was re-suspended in heptane-tetrachlorethylene (ρ = 1.3 g cm^3) and loaded on a linear gradient of heptane-tetrachlorethylene (ρ = 1.3 g cm^3) to tetrachlorethylene (ρ = 1.6 g cm^3). After ultracentrifugation at 121,000*g* for 3 h, the gradient was fractionated into nine 1-ml fractions. These were divided into three subfractions of 0.3 ml and dried under vacuum. One subfraction was used for marker enzyme determination and another one for metabolite analysis. Alkaline pyrophosphatase as plastidial marker, UGPase as a cytosolic marker, and acid phosphatase as marker for the vacuolar compartment were measured essentially as described (Knaupp et al., 2011).

Statistical Analysis

The results were analyzed by applying *F* test ($p < 0.05$ as significance threshold) in an entirely randomized design, with four replicates, and a Tukey test (5%) was applied among treatments. For NAF of seed subcellular compartments, a correlation analysis of marker enzyme activities and metabolite distributions using Kendall's rank correlation (Kendall, 1938) was performed, applying a significance threshold of $p < 0.01$.

RESULTS

Although some increase in length and diameter was observed in fruits of *E. speciosa* from 20 to 30 DAA and from 20 to 45 DAA, respectively, fruits had almost achieved the maximum size already at the 20 DAA (**Supplementary Figure S1**). Conversely, seeds increased size substantially till 45 DAA, mainly because of the gain in dry matter (**Figure 2A**). From 45 DAA on, the seed size declined until 55 DAA (**Supplementary Figure S1**), mainly due to reduction in water content (**Figure 2A**). Indeed, water content is stable until 38 DAA, when an almost linear drop until the last stage (60 DAA) was observed (**Figure 2A**). Water potential remained constant until 44 DAA (**Figure 2A**) and decreased after this time point, reaching the lowest value at the end of seed development. The changes in water potential were not high as those observed for water content from 44 to 57 DAA. Neither the water content changed as much as water potential from 57 to 60 DAA. Thus, it seems that water status was not only dependent on water content but other active processes seemed to occur to maintain water flux from mother plant to the seeds. The results also showed that an important period to be considered in *E. speciosa* seed maturation is the interval from 40 to 55 DAA, when most changes occur.

The physiological behavior of *E. speciosa* seeds during maturation followed the classical pattern described for orthodox seeds, i.e., decrease of water content from more than 80% to *ca.* 10% at the end of maturation (**Figure 2A**). The dry mass accumulation was the same as expected for orthodox seeds, increasing from 20 to around 44 DAA (**Figure 2A**).

Seeds became germinable and produced normal seedlings around 42 DAA (**Figure 2B**), with increasing germination rates until 57 DAA. However, maximum vigor was reached only at the latest stage, 60 DAA (**Figure 2B**). Also, the results of germination after seed drying showed that desiccation tolerance started about 44 DAA, where 40% of the seeds were tolerant (**Figure 2B**). At 57 DAA, all seeds were able to germinate after drying. Despite showing about 15% of germination, 42 DAA seeds did not tolerate drying.

In the embryonic axis, there was a remarkable increase in the cell wall yield during seed maturation from stage II to stage IV (**Table 1**). Cell wall yield represents the amount of dry mass that is present as cell wall, and the proportion on the overall seed dry mass was 3.4 times lower in stage II compared with stage VI. In contrast, a decrease in the cell wall yield was observed in the cotyledons during seed maturation. The proportion of cell wall was ca. 1.4 lower in stage VI in comparison with stage I. The content of uronic acids decreased in the cell walls of both, embryonic axis and cotyledons, subsequent to stages II and I, respectively.

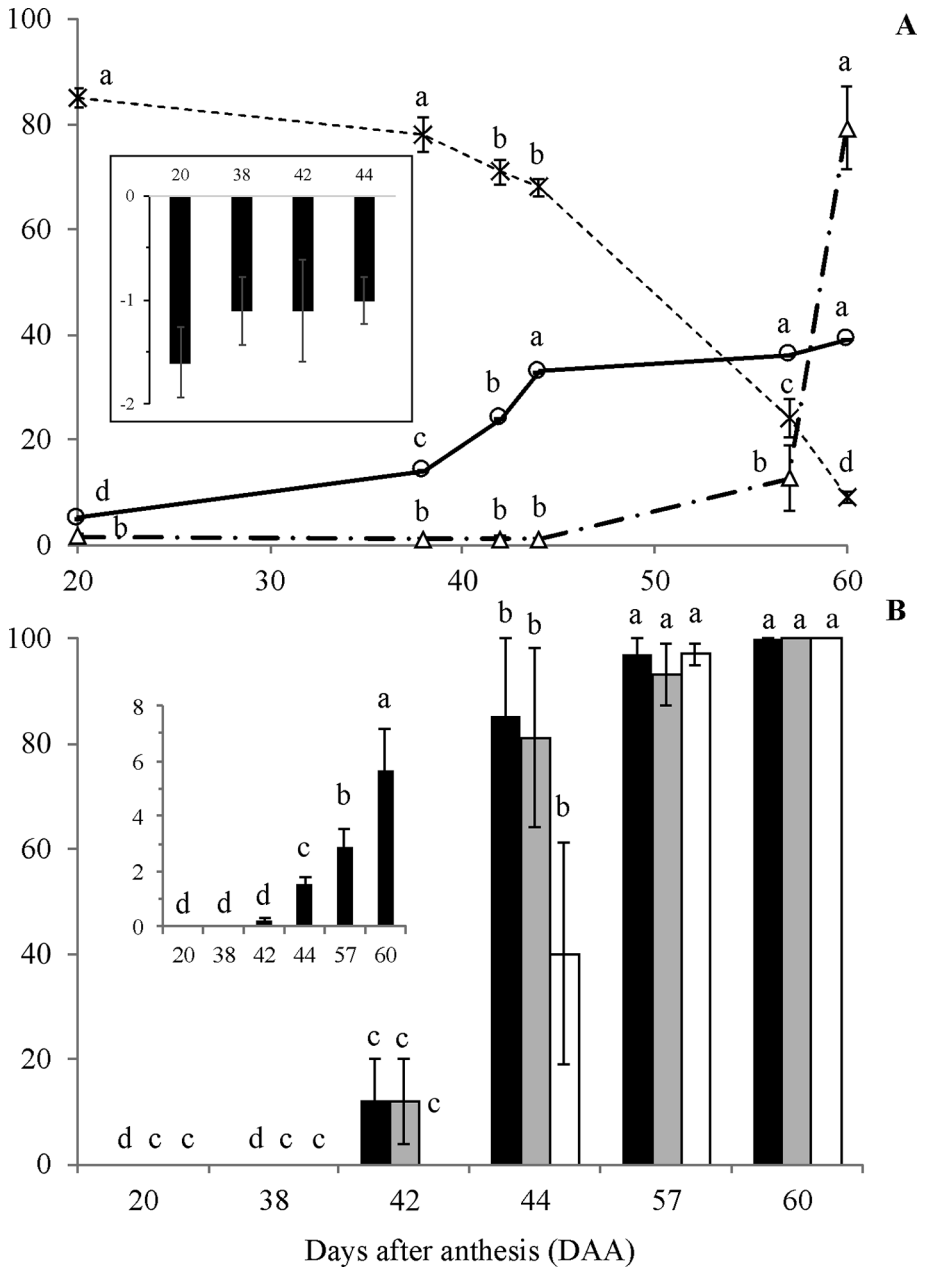

FIGURE 2 | Water content (**A**, –x–, %), dry mass (**A**, –o–, g/100 seeds), water potential (**A**, Δ, -MPa; A, small chart), germination (**B**, black columns, %), normal seedling development (**B**, grey columns, %), germination of dried seeds (**B**, white columns, %) and Germination Speed Index (**B**, small chart) of *Erythrina speciosa* obtained from stages I to VI. Four biological replicates (containing at least four seeds each) were used for water content, dry mass and water potential, and four biological replicates (containing 15 seeds each) were used for germination and normal seedling development analysis. Different letters indicate statistical difference among the developmental stages by Tukey test (5%).

Glycosyl composition analysis revealed that although xylose/mannose and galactose were the main monomers in cell walls of embryonic axis at stages II and III, arabinose increased significantly from the stage III on, predominating at stage IV (**Table 1**). The relative proportion of galactose was higher in stages V and VI compared with the beginning of seed maturation and coinciding with a significant reduction in glucose and xylose/mannose. Increased proportion of arabinose was also observed in cotyledons during seed maturation, peaking at stage IV and presenting almost the same proportion of the predominant sugar galactose. As observed in the embryonic axis, glucose was also reduced in the cotyledon cell walls at the last stages of maturation.

The content of total soluble sugars presented a remarkable increase at stages V and VI, but their levels were 3.9 times higher

TABLE 1 | Cell wall yield, uronic acid content, and relative percentage of neutral monosaccharides in embryonic axis and cotyledons of *Erythrina speciosa* seeds during different stages (I-VI) days after anthesis (DAA).

DAA	Embryonic axis						Cotyledons					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Cell wall yield (mg g seed DM^{-1})												
	–	144.7a	355.8b	473.9c	473.9c	488.1c	388.1ab	436.6a	345.8bc	374.4ab	377.9ab	280.1c
Content of uronic acids (mg g cell wall DM^{-1})												
	–	29.7a	22.2b	20.9b	21.6b	22.9b	35.7a	19.0b	22.2ab	22.5ab	21.7b	25.4ab
Neutral sugars (relative %)												
Fucose	–	1.7a	1.1a	0.9a	1.0a	1.0a	1.0a	1.2a	1.0a	0.9a	1.3a	1.3a
Rhamnose	–	4.1a	2.6b	2.3b	2.4b	2.4b	2.3ab	2.5b	1.6a	1.7a	1.8ab	1.6ab
Arabinose	–	18.8a	25.1b	29.1c	28.5c	27.9bc	26.1a	26.7ab	25.3a	32.4bc	30.7abc	35.8c
Galactose	–	28.5a	27.9a	26.8a	34.2b	33.9b	40.4ab	31.8c	31.0c	33.2ac	43.4b	35.8ac
Glucose	–	16.6a	14.4ab	12.2b	9.8c	9.7c	13.5a	22.4b	21.4b	10.9a	3.7c	3.8c
Xylose/ mannose	–	30.3a	28.9a	28.7a	24.1b	25.1b	16.7ac	15.3c	19.6ab	20.9b	19.2b	21.1ab

Letters compare each value (line) within each organ among different maturation stages by Tukey 5%.
– not analyzed due to low yield.

in embryonic axis than cotyledons (**Table 2**). At stage I, soluble sugars were represented by equal amounts of the monosaccharides fructose and glucose and the disaccharide sucrose in embryonic axis. The relative proportion of sucrose reached ca. 60% at stage II, decreasing later, probably due to its consumption for synthesis of the raffinose family oligosaccharides (RFO), raffinose, and stachyose, as indicated by values observed at the late stages of development. These RFO together summed up to 45% of the soluble sugars at stage VI. In the cotyledons, a similar tendency was observed, as sucrose decrease from stage III to VI, and accumulation of RFO was observed from stage III (**Table 2**, **Supplementary Figure S2**).

The metabolic profile showed a decrease in the levels of organic acids in embryonic axis of *E. speciosa* during seed development, the major changes occurred during the first stages of development (**Figure 3**). The decrease in the level of succinic, citric, and malic acids suggests a reduction in mitochondrial metabolism. Those changes were more pronounced between the stages II and III (**Figure 3**). Metabolic profiles in *E. speciosa* axis also showed a decrease in amino acids between stages II and III, from whereon these compounds were below the detection limit, except for tryptophan and tyrosine (**Figure 3**). A Principal Component Analysis (PCA), based on the relative abundance of compounds found at the metabolic profile, brings a holistic view concerning the deactivation of the metabolism (**Figure 4**). There is a great switch on the metabolism, especially on embryonic axis, from stage II to stage IV. This switch corresponds to a metabolic shutdown marked by decreased levels of TCA cycle and other primary metabolites (**Figure 3**) and is in agreement with recordings of respiratory rates (**Figure 5**).

The disaccharide trehalose was also detected only in *E. speciosa* axes, and an increase in its level was found especially between maturation stages IV and VI (**Figure 3**). Sucrose was found during all stages of *E. speciosa* seed development in both axes and cotyledons (**Figure 3**). The metabolic profiles show an antagonistic behavior of sucrose and raffinose between maturation stages II and III (**Figure 3**).

NAF of seed subcellular compartments based on density was performed for stages I and VI. Marker enzyme activities for plastids (alkaline pyrophosphatase), cytosol (UGPase), and vacuole (acid phosphatase) identified the cytosol as lightest compartment, while plastids showed a broad distribution. The vacuole peaked at an intermediate density of 1.42 $g{\cdot}ml^{-1}$ in stage I, while it was much lighter in stage VI, showing a distribution similar to the cytosol (**Figures 6A**, **B**). To prevent metabolite diffusion during compartment separation, NAF uses density gradient centrifugation of subcellular particles generated by ultra-sonication of lyophilized tissue (see Materials & Methods). This does not yield purified organelles, but a distribution over the gradient fractions, which, based on marker enzyme activities, is used for mathematical assignment of metabolites to the different compartments. Only sugars and sugar alcohols could be significantly correlated with marker enzyme activities (**Supplementary Table 1**). During stage I, hexoses, raffinose, stachyose, and *myo*-inositol showed strongest correlation with the vacuole, while raffinose was significantly associated also with the cytosol. At stage VI, however, sucrose, raffinose, and stachyose were significantly associated with cytosol and vacuole, while the sugar alcohol xylitol was found only in the vacuole. The weaker association of sugars with plastids during stage I was completely lost in stage VI (**Figures 6A**, **B** and **Supplementary Table 1**).

The concentration of glucose, fructose, sucrose, and raffinose were evaluated by HPLC in the different fractions obtained from cotyledons from the two selected developmental stages. In general, we could observe that concentrations of glucose and fructose tend to be higher in stage I (**Figures 7A, B**). This pattern can also be observed for sucrose in the fractions enriched with cytosol. On the other hand, a greater concentration of raffinose is present in the later stages of development (**Figures 7A–D**).

TABLE 2 | Relative percentage of total soluble sugars, monosaccharides, and oligosaccharides in embryonic axis and cotyledons of *Erythrina speciosa* seeds during different stages (I to VI, DAA).

	Embryonic axis						Cotyledons					
Stages	**I**	**II**	**III**	**IV**	**V**	**VI**	**I**	**II**	**III**	**IV**	**V**	**VI**
(DAF)	20	38	42	44	57	60	20	38	42	44	57	60
Content soluble sugar (mg g DM^{-1})	57.7a	72.1a	39.4a	65.6a	196.7b	294.8c	21.2a	14.4a	17.3a	20.0a	58.3b	75.4c
Sugars (relative %)												
Glucose	21.0Aab	17.2Ac	20.1Ab	12.4Aa	11.4Aa	14.4Aa	24.5Ab	30.0Aab	26.3Ab	19.5Ad	24.6Ab	26.5Acd
Fructose	29.3Ab	16.4ABc	28.0ABb	42.4Ba	22.6ABbc	20.5ABa	37.9Ac	26.9Aab	28.4Ab	55.2Aab	49.6ABc	34.6ABd
Sucrose	49.7BCb	61.1Cd	26.9ABb	3.1Aa	11.9Aab	20.9Aa	37.6Bc	41.2Bb	29.6Bb	0.3Ac	0.0Aa	2.4Aa
Raffinose	0.0Aa	5.3Ab	25.0Ab	35.6Aa	32.2Ac	28.6Aa	0.0Aa	1.8Ba	15.7ABab	21.2ABa	12.9ABab	20.9ABbc
Stachyose	0.0Aa	0.0Aa	0.0Aa	6.4ABa	21.9Cabc	15.7BCa	0.0Aa	0.0Aa	0.0Aa	3.8Aa	12.9Aab	15.6Aab

Letters compare means of triplicates in each organ by Tukey 5%. Capital letters compare the sugar content among stages. Lower letter compares the relative sugar content in each stage.

DISCUSSION

As expected for seeds with orthodox behavior, the water content in *E. speciosa* seeds decreased during maturation until it reached ca. 10%, when germination reached 100%. It is important to emphasize that seed germination at stage VI without scarification was *ca.* 10% (data not shown) and that the highest values shown in Fig. 2B correspond to scarified seeds. Seed coat impermeability in *E speciosa* seeds is a frequent, but variable, occurrence. Both its occurrence and degree are dependent on climatic conditions during seed maturation (Molizane et al., 2018). Thus, in this work, the dormancy was near the maximum and was probably established in a very short period (stages V and VI), coinciding with the highest reduction in the water potential (**Figure 2A**), which corresponds to hydration level 1, according to Vertucci (1990).

The low content of starch, as previously reported by Mello et al. (2010), together with the high abundance of soluble sugars and the increased accumulation of RFO during seed maturation (**Table 2**) are also in agreement with the orthodox behavior of *E. speciosa*. Interestingly, the highest increase in raffinose accumulation in the embryonic axis and cotyledons of these seeds was observed from stage II to IV, before the sharp drop observed in the seed water content (**Figure 2**). In contrast, in *Inga vera*, a legume that has recalcitrant seeds, sucrose, and starch were the major reserves in mature seeds, and RFO were not detected all over the seed development and maturation (Caccere et al., 2013).

Investigation of seed cell walls of *E. speciosa* revealed a composition similar to that of other leguminous seeds, but with high levels of arabinose, as reported for marama beans (Mosele et al., 2011). Accumulation of arabinose in the cell walls was observed in the embryonic axis at the beginning of seed development of *E. speciosa,* before the highest decrease in water content. Conversely, a reduction in the proportion of this sugar from 40% to 35% was observed in the cotyledons from stage I to VI, respectively (**Table 1**). High proportion of arabinose, ranging from 38% to 60% of the cell walls, has been found in some orthodox seeds (Shiga and Lajolo, 2006; Gomez et al., 2009) contrasting with recalcitrant seeds as *I. vera*, in which the low proportion of arabinose in the cell walls was found in the embryonic axis (Caccere et al., 2013).

The changes observed in the seed cell wall composition of *E. speciosa* are probably due to cell wall adaptation to the maturation. High levels of arabinose, supposedly from long side chains of rhamnogalacturonan I, has been related to the increase of cell wall flexibility, by diminishing strong interactions among acidic pectic polysaccharides during tissue water loss (Moore et al., 2008). In leaf tissues of resurrection plants, the abundance of arabinose-containing polymers has been associated with the ability to survive the desiccation and rehydration process (Moore et al., 2009). Therefore, it is reasonable to suppose that the increased arabinose content in embryonic axis of *E. speciosa* has a role in the ability of cell walls to remain flexible during dehydration, avoiding cell wall damage due to water loss during seed maturation and contributing to its orthodox behavior. In *Arabidopsis* seeds, which do not contain starch, it was demonstrated that the arabinans accumulated in developing, and mature embryos are mobilized

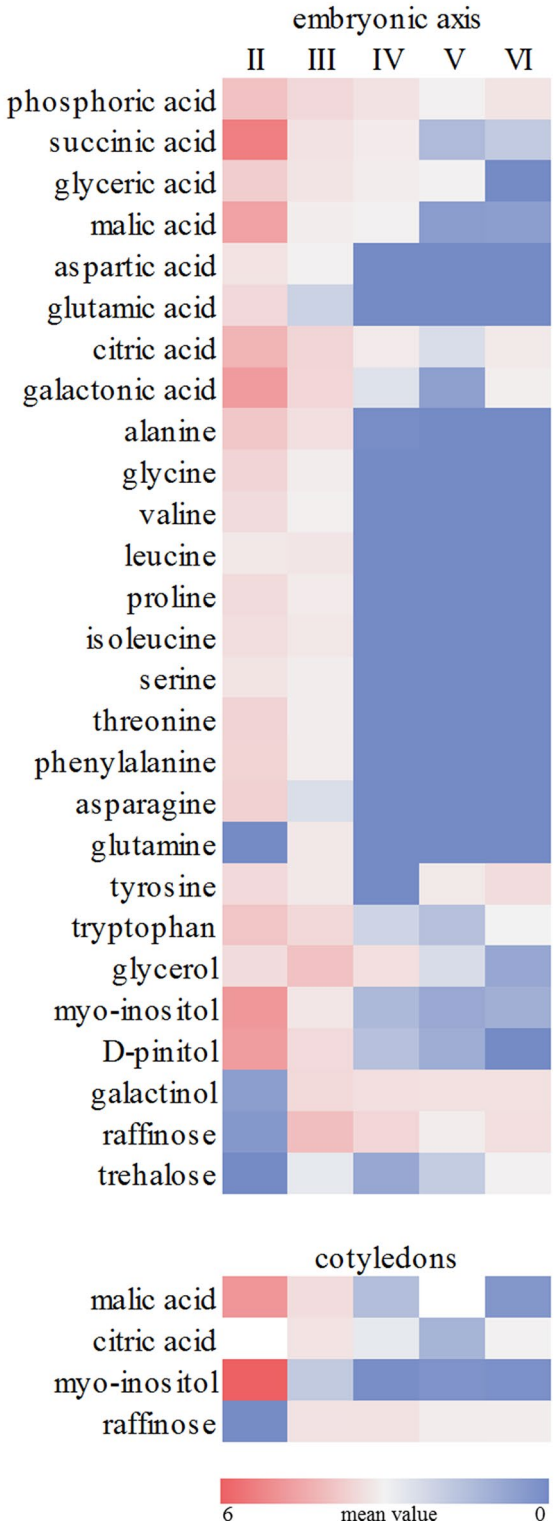

FIGURE 3 | Relative abundance of metabolites. Colours represent the relative metabolite abundance among the different developmental stages (II to VI). Values are the mean of at least five (embryonic axes) or three (cotyledons) biological replicates. Red and blue colors represent values higher or lower, respectively. The scale bar indicates the magnitude of variation among the developmental stages (0- to 6-fold the mean value).

during seedling establishment (Gomez et al., 2009). Similar to *Arabidopsis*, *E. speciosa* has very little starch and accumulates arabinose-containing polymers during seed development. This suggests that arabinans, besides playing a role associated with desiccation tolerance during maturation of *E. speciosa* seeds, could also serve as a storage reserve, providing carbon to the embryo during early seedling growth.

Accompanying the water loss, there is a decay of metabolic activity in the seeds, which reduces the risk of ROS formation. Moreover, during maturation, seeds accumulate compounds, such as raffinose, which are supposed to protect the seeds against desiccation and ROS (Nishizawa et al., 2008). However, in *E. speciosa*, these biochemical changes (**Figure 3**) occurred early in seed maturation, when the water content was still high (**Figure 2A**), indicating a preparation of the metabolism for further water loss, which occurred primarily between stage IV and stage V (**Figure 2A**). This is even more evident when considering the water potential, which decreased from stage V on. According to Vertucci (1990), seeds from stage I to stage IV would be classified as hydration level 5, which means high level of hydration. Stages V and VI correspond to hydration levels 2 and 1, respectively.

Comparing different strategies of species propagation, *I. vera* seeds are dispersed with high content of water (hydration level), which allows storage only for a period shorter than 20 days at room temperature (Barbedo and Cicero, 2000). Caccere et al. (2013) studied these seeds observed high metabolic activity from the early stages of development until the end of the maturation, and Bonjovani and Barbedo (2019) showed that oxidative processes are involved in this activity. Our results indicate an opposite strategy in *E. speciosa* seeds, characterized by a dramatic decline in metabolism, which could classify them as orthodox. However, based on the hypothesis of Barbedo et al. (2013), seeds of *E. speciosa* simply complete their development, while this does not occur with *I. vera* seeds.

The changes observed in the embryonic axis can be explained by the need of reserve material from cotyledons. The decline of the metabolic level in axis of *E. speciosa* can also be explained by the decrease of sucrose, suggesting a reduction of substrate for glycolysis (**Figure 3**). The decay of metabolic activity can be inferred from the decrease in succinic, malic, and citric acids, which are intermediates of the Krebs cycle. Moreover, a general decrease in amino acids is also observed. Plants rarely oxidize amino acids for energy provision; however, amino acids can be used by other metabolic pathways at the central carbon metabolism and also provide carbon skeletons which may enter into the tricarboxylic acid cycle (TCA). Inside TCA cycle those can continue the respiration process or be used to produce other metabolites for different biosynthetic pathways (Nelson and Cox, 2001), such as synthesis of protective molecules involved in desiccation tolerance. Supporting this hypothesis, respiratory rates (**Figure 5**) decreased during maturation.

The accumulation of trehalose in the axes suggests an important role in desiccation tolerance, as previously observed in yeast and other organisms tolerant to desiccation (Crowe et al., 1992). This disaccharide, together with sucrose, may develop an important role in glass state formation (hydration level 1; Crowe et al., 1998)

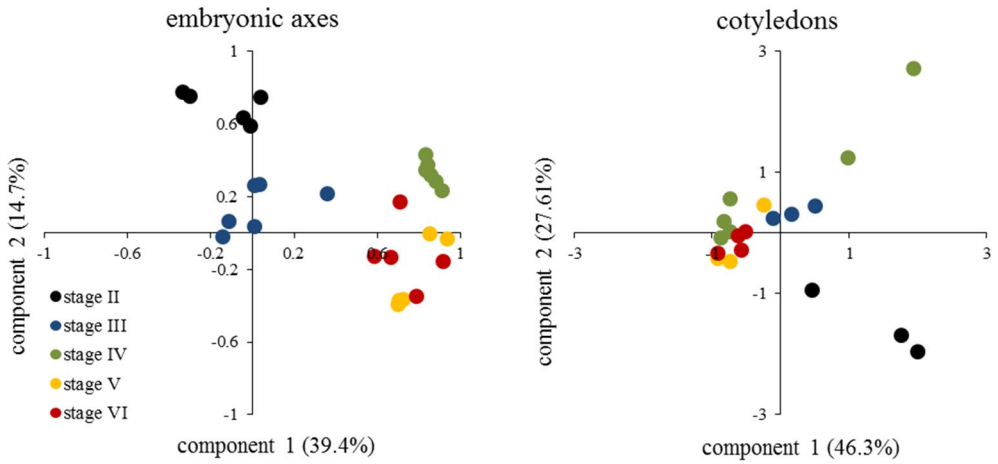

FIGURE 4 | Principal Component Analysis from metabolic profile of embryonic axes and cotyledons of *Erythrina speciosa* seeds at different developmental stages (stage II to stage VI). At least five (embryonic axis) or three (cotyledons) biological replicates were used for the analysis.

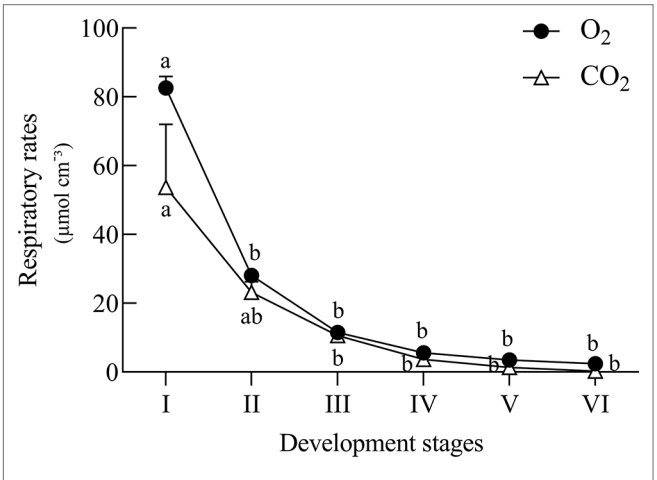

FIGURE 5 | Estimated respiratory rates of *Erythrina speciosa* seeds at six developmental stages. Values are the mean of three biological replicates. Different letters indicate statistical difference among the developmental stages according to Tukey test (5%).

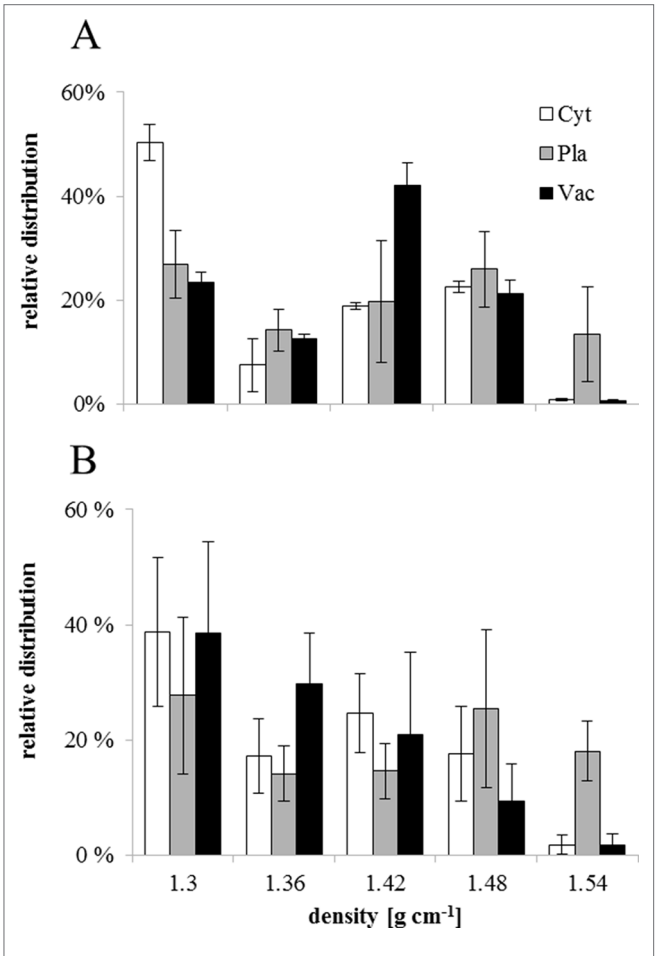

FIGURE 6 | Relative distribution of sub-cellular compartments on different densities obtained by non-aqueous fractionation of *Erythrina speciosa* seeds on stage I **(A)** and stage VI **(B)**. Cyt, cytosol (white); Pla, plastids (gray); Vac, vacuole (black). Values are given as mean of the percentage ratio of four biological replicates ± standard deviation.

and might be involved as a protective molecule (de Klerk and Pumisutapon, 2008; Mollo et al., 2011), besides its function as a signal for sink activity (Smeekens et al., 2010).

According to Castillo et al. (1990), sucrose is the major soluble carbohydrate found in mature seeds of many species and acts as a substrate for metabolic reactions that occur at low temperatures. In a recent overview, Fàbregas and Fernie (2019 and references therein), reported experiments reveal that other sugars, such as RFOs, glucose, and fructose, accumulate earlier than other metabolites in response to stress. The authors emphasize a fine-tuned sequence of metabolic responses to desiccation with RFO accumulation followed by hexoses, sugar alcohols, and sucrose in leaf tissue of various species. The rapid accumulation of RFO, galactinol, and *myo*-inositol points to an important role during

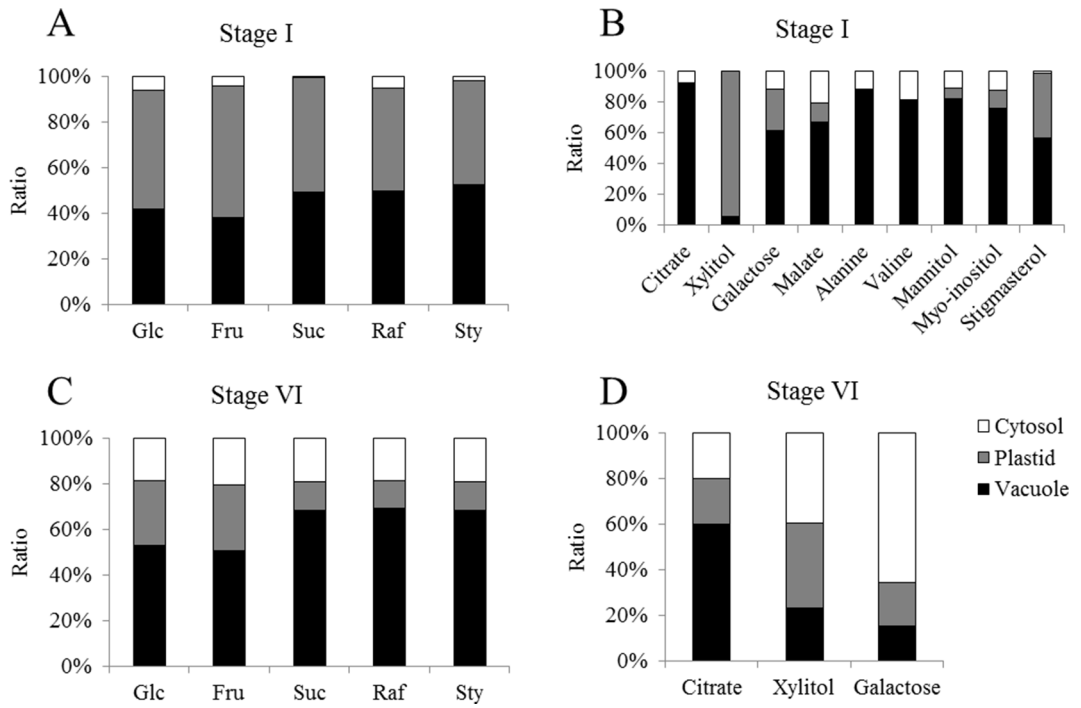

FIGURE 7 | Relative distribution of metabolites over the cellular compartments cytosol (white), plastids (gray) and vacuole (black) from cotyledons of *Erythrina speciosa* seeds at two developmental stages (stage I, active metabolism; stage VI, dormant seeds). Values are given as mean of four biological replicates. Glc, glucose; Fru, fructose; Suc, sucrose; Raf, raffinose; Sty, stachyose. Relative abundance of sugars **(A)** and other metabolites **(B)** on stage I and sugars **(C)** and other metabolites **(D)** on stage VI.

metabolic activity, which might include scavenging of ROS (Nishizawa et al., 2008). Rapid accumulation of raffinose was also obtained in *E. speciosa* seeds, where peaks for precursors *myo*-inositol, galactinol, and sucrose were observed already in stage II or III. Although raffinose likewise peaked in stage II, its decline in later stages was shallow as compared with sucrose, thus pointing to an important role also in mature seeds.

In axes, we observed a reduction in sucrose levels during maturation, suggesting that, in *E. speciosa*, this sugar seems not essential for the protection of cell membranes. Alternatively, sucrose may be used to synthesize RFOs (Castillo et al., 1990), which include raffinose, stachyose, and verbascose. Raffinose accumulation following stage II supports this hypothesis (**Table 1**). Thus, in *E. speciosa* seeds, sugars, such as trehalose and raffinose, appeared more directly involved in seed vitrification, which slows down enzymatic reactions. This event contributes to avoid degradation of cellular components of seeds and prevent membrane injuries and consequently the breakdown of cell compartments (Hoekstra et al., 2001), thus augmenting longevity of dry seeds (Leopold et al., 1994; Buitink et al., 2000). Also, as hypothesized by Walters (2015), the more stable the glasses, the longer the seed storability.

In studies carried out by Mello et al. (2010), raffinose and stachyose were the predominant sugars in embryonic axes and cotyledons of *E. speciosa* in addition to sucrose found in these seeds. According to Leprince et al. (1992) and Berjak and Pammenter (1999), the accumulation of RFO could be the result of a monosaccharide conversion, thus reducing the availability of respiration substrate, and consequently decreasing metabolic activity during desiccation and storage. This hypothesis may explain the high respiratory rates found at stage II, which decreased gradually until stage V (**Figure 5**), when physiological maturity was reached. In this stage of development, levels of amino acids and some organic acids were low (**Figure 3**), indicating a shift in carbon use (**Figure 4**) toward RFOs production as a means of decreasing the availability of respiration substrate. According to Mello et al. (2011), the precursor of raffinose synthesis, galactinol, could also be related to acquisition of desiccation tolerance in *Erythrina* seeds. Moreover, in a recent study, Hoermiller et al. (2017) inspected all metabolites quantitatively relevant in primary metabolism, i.e., amino-acids, carboxylic acids, amines, and sugar alcohols, and showed that cold acclimation causes substantial re-arrangements of metabolites within the cell. Sugars, especially those involved in raffinose metabolism, were shifted from the vacuole to the plastids (Hoermiller et al., 2017) to protect membranes involving the photosynthetic apparatus. The increased levels of galactinol during maturation observed in axes, together with decreased contents of pinitol and myo-inositol, which are substrates for the synthesis of galactinol, supports the idea that polyols may also be involved in acquisition of stress tolerance, i.e., desiccation, in seeds of *E. speciosa*, similar to the leguminous *Caesalpinia echinata* (Borges et al., 2006).

The analysis of subcellular distribution of metabolites revealed differences in the concentration of glucose, fructose, sucrose, and

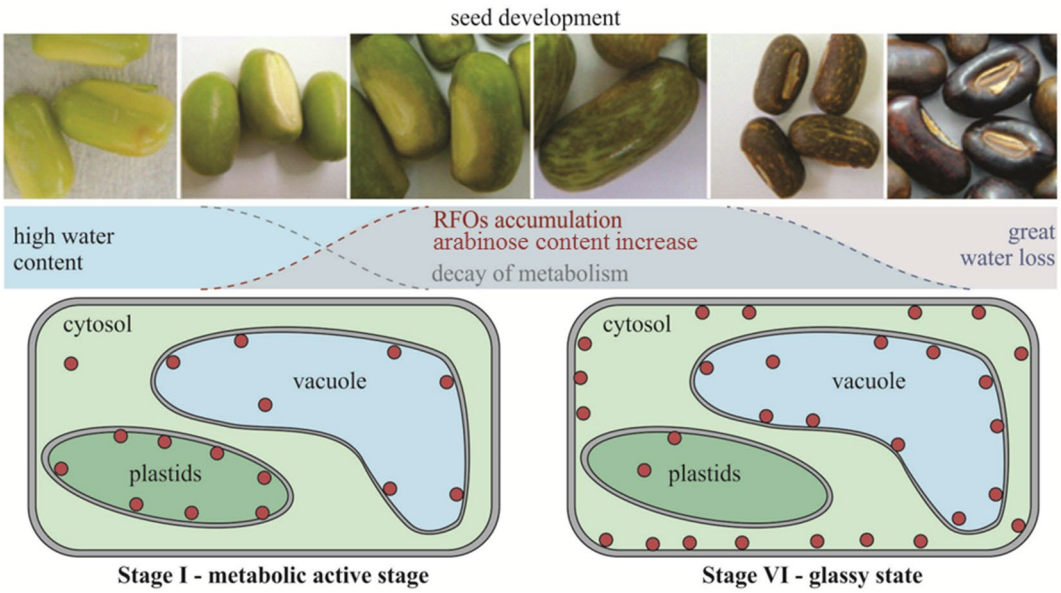

FIGURE 8 | Schematic timeline of events of *Erythrina speciosa* seeds development. Red circles represent raffinose family oligosaccharides and their sub-cellular localization on cells of stage I and stage VI.

raffinose among subcellular compartments. The results showed an apparent increase of all sugars in the cytosol of mature cotyledons, when comparing stages I and VI (**Figure 7**). The concentrations of sugars in the vacuole also tended to increase. Similar results were previously observed by Knaupp et al. (2011), who compared non-acclimated and cold acclimated leaves of *Arabidopsis thaliana* and noted that sugars increased after acclimation in the cytosol. On the other hand, these authors found higher ratio of sucrose and raffinose in the plastids in the acclimated plants.

In *E. speciosa*, these sugars decreased during seed development and were not associated with plastids, especially in stage VI (**Figure 7**). Although RFO and related genes might be involved in cold (Bachmann et al., 1994; Egert et al., 2013) and freezing tolerance (Knaupp et al., 2011), as well as in desiccation tolerance (Steadman et al., 1996; Mello et al., 2011), their role as cellular protectants appears to be tissue and organism-dependent. While Knaupp et al. (2011) found a protective function in plastids, stabilizing the photosystem II, but not in the cytosol in *Arabidopsis* leaves, sucrose, raffinose, and stachyose became associated with the cytosol in seeds of *E. speciosa* during maturation. An additional role of oligosaccharides may reside in their ability to preserve the liquid crystalline state of cellular membranes in the dry state (Sun et al., 1994). Thus, a re-allocation of sucrose and RFO from vacuolar stores during seed maturation in *E. speciosa* might be an important prerequisite of seed longevity. Mobilization of these vacuolar stores might be responsible for the reduction of density that was observed when comparing distribution of subcellular compartments in density gradient for stage I and stage VI seeds (**Figure 6**).

The combined results suggest, therefore, that in *E. speciosa* reduction of metabolic activity in seeds occurs mainly between the stages II and III before reaching the physiological maturity in the stage V of development. In addition, the accumulation of raffinose may also result from changing the use of carbon, decreasing the availability of substrate for respiration and therefore, the metabolic activity during the drying and storage, as illustrated in the diagram in **Figure 8**. This work confirms the hypothesis of the involvement of the RFO in the desiccation tolerance in seeds of *E. speciosa* and that its accumulation occurs in stages prior to major changes in water content. It highlights that the sub-cellular localization of RFO is crucial to protect cell membranes during seed development in the different cell compartments.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

AFH and FK obtained most of the experimental data. KS performed cell wall analysis. AGH and DC performed the non-aqueous fractionation analysis. AFH wrote the first draft of the manuscript. CB, MB, and DC designed the study. AFH, AGH, CB, MB, and DC contributed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01356/full#supplementary-material

SUPPLEMENTAL FIGURE 1 | Morphological parameters of *Erythrina speciosa* seeds during development. Fruit length (A), Fruit diameter (B), Seed length (C), Seed width (D), Seed thickness (E) and Seed dry mass (F). Values are given as mean of six biological replicates considering fruit analysis (three fruits each replicate) and four biological replicates for seed analysis (ten seeds each replicate) ± Standard Deviation.

SUPPLEMENTAL FIGURE 2 | Soluble carbohydrates profile by HPAEC/PAD from embryonic axes (left) and cotyledons (right) of Erythrina speciosa seeds during development. (a, d) 38, (b, e) 42 e (c, f) 44 days after anthesis. C - cyclitols; G - glucose; F - fructose; S - sucrose; R - raffinose; St - stachyose.

SUPPLEMENTAL TABLE 1 | Correlation values between metabolites and marker enzymes (for sub-cellular compartments) and their respective *p* values at stage I and stage VI.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.