

Anatomical, physiological and chemical differences between populations of *Pseudocyphellaria flavicans* (Hook. f. & Taylor) Vain. from Chile

Diferencias anatómicas, fisiológicas y químicas entre poblaciones de *Pseudocyphellaria flavicans* (Hook. f. & Taylor) Vain. de Chile

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ABSTRACT

Environmental conditions can affect lichen morphology, physiology and chemistry. Some functional traits; like thickness of thallus layers, photosystems condition, and presence of photoprotective and antioxidant compounds are key to colonizing new sites, and can change depending on light and moisture availability. In the present study, we compared the morphology, physiology and chemistry of the native Chilean lichen *Pseudocyphellaria flavicans* from two Coastal populations (Nahuelbuta and Contulmo, Araucanía district), and one Andean population (Ralco, Bío-Bío district). Ralco is a more closed secondary forest, and can show dryer periods compared to Nahuelbuta and Contulmo. We found differences in thalli anatomy between populations. Ralco individuals had thinner upper cortex than Nahuelbuta individuals. Contulmo individuals had similar upper cortex thickness compared to the other two populations. We also found differences in the response of the maximal efficiency of the PSII (Fv/Fm) to desiccation and in the *in situ* values measured. We did not find differences between populations in the chemical traits. The differences between populations could be attributed to differences in local environmental conditions, namely moisture and light.

KEYWORDS: *Pseudocyphellaria flavicans*, lichen ecophysiology, lichen anatomy, lichen chemistry, foliose lichens.

RESUMEN

Las condiciones ambientales afectan la morfología, fisiología y química de los líquenes. Ciertos rasgos funcionales, como el grosor de las distintas capas del talo, estado de los fotosistemas, y presencia de compuestos fotoprotectores y antioxidantes son claves para colonizar nuevos sitios y pueden variar dependiendo de la disponibilidad de luz y humedad. En este trabajo se comparó la morfología, fisiología y química de individuos del líquen nativo de Chile *Pseudocyphellaria flavicans* de dos poblaciones de la Cordillera de la Costa (Nahuelbuta y Contulmo, Región de la Araucanía) y una de la Cordillera de los Andes (Ralco, Región del Bío-Bío). Ralco es un bosque más cerrado y presenta periodos más secos, comparado con Nahuelbuta y Contulmo. Se encontraron diferencias significativas en la anatomía del talo entre poblaciones. Los individuos de Ralco presentaron una corteza superior más delgada en relación con los de Nahuelbuta. Los individuos de Contulmo presentaron una corteza similar a las otras dos poblaciones. También se encontraron diferencias en las curvas de respuesta de la eficiencia máxima del PSII (Fv/Fm) a la desecación y los valores medidos *in situ*. No se encontraron diferencias entre poblaciones en las variables químicas medidas. Las diferencias entre poblaciones podrían atribuirse a diferencias en las condiciones ambientales locales, principalmente humedad y luminosidad.

PALABRAS CLAVE: *Pseudocyphellaria flavicans*, ecofisiología de líquenes, anatomía de líquenes, química de líquenes, líquenes foliosos.

INTRODUCTION

Lichens result from the symbiotic association of at least one algal species (the photobiont) and one fungal species (the mycobiont). The stable association of both partners (holobiont) allows lichens to grow in different habitats and conditions, tolerating extremely stressful environments such as deserts and Polar Regions (Kappen & Breuer 1991, Kappen

2000, Kappen & Valladares 2007, Wirth *et al.* 2007). Foliose lichens (lichens with leaf-like habit) normally have clearly distinct layers: an upper cortex formed by glutinized hyphae where derivatives from the secondary metabolism precipitate; an algal layer where the photobiontal cells are distributed; a medullae formed by loosely attached hyphae that allows the exchange of gases necessary for photosynthesis; and a lower cortex with different structures for attachment (*i.e.* rhizines,

rhizinoze strands, hapteria, etc., Büdel & Scheidegger 2008).

Many anatomical, ecophysiological and chemical traits in foliose lichens depend on environmental conditions (Snelgar & Green 1981, Green & Lange 1991, Gauslaa *et al.* 2006). This is also true for photobiont availability, which may determine the presence of a given species in a particular environment (Beck *et al.* 2002, Yahr *et al.* 2006, Fernández-Mendoza *et al.* 2011, Vargas & Beck 2012, Nyati *et al.* 2013, 2014). Total thallus, upper cortex, algal layer and medulla thickness, for example, are increased in lichens highly exposed to sunlight, and even an epinecral layer is formed as protection to high sun exposition in some cases (Büdel 1990, Gaya 2009). Also, an increase in the amount of lichen metabolites that precipitate in the upper cortex have been observed, probably as an anti-UV screening system (Beckett *et al.* 2008, Green & Lange 1991). It has also been stated that high intensity light might induce the synthesis of melanic compounds and other colored metabolites with antioxidative properties, suggesting a functional response to high light (Solhaug *et al.* 2003). On the other hand, structural differences can have functional consequences, for example, in water storage capacity (Sancho & Kappen 1989, Valladares *et al.* 1993). Environmental conditions can also impact the reproductive output of the lichen, altering the formation of sexual structures (Ramstad & Hestmark 2001, Mikhailova 2007), and the vegetative reproduction of the whole lichen, altering the number of isidia and soredia per area (Mikhailova & Vorobeichik 1999, Mikhailova 2002, Sojo *et al.* 2008).

Lichens also alter their physiology in response to changes in abiotic conditions. Gas exchange, normally small in lichens, is highly dependent on water availability, as high amounts of water reduces the net carbon gain of the holobiont (Leisner *et al.* 1997, Beckett *et al.* 2008). The response of carbon gain to thallus water content, however, is not always simple. Most species reduce their photosynthesis at low water potentials, but some species also show a reduction at high water content (Green & Lange 1991), possibly due to increased diffusion resistance to CO₂ (Nash 2008). Small reductions in water potential can negatively impact photosynthesis and drop the maximal efficiency of PSII (Fv/Fm) below optimal values (Beckett *et al.* 2005, Kappen & Valladares 2007).

Members of the genus *Pseudocyphellaria* are very conspicuous, with some species among the largest known foliose lichens, often reaching up to 50 cm in diameter (Galloway 1992). They have heteromerous thalli with cyanobacterial or algal photobionts (Galloway 1992). The genus is characterized by the presence of scattered (sometimes very sparse) pseudocyphellae on the lower surface, and sometimes also in the upper surface. *Pseudocyphellaria flavicans* (Hook. f. & Taylor) Vain. is a corticolous lichen that can be found in Chile and Argentina, the Juan Fernandez Archipelago and from 37°50' S southwards to Tierra del Fuego and Staten Island in Argentina (Galloway 1992). The species can be found in a variety of habitats and it is

an epiphyte of shrubs and trees in areas with high humidity, in moderate to high light intensity areas, usually inside undisturbed temperate forests, from sea level up to 1500 m (Vainio 1913, Galloway 1992).

The present study addresses if three populations of *P. flavicans* from Southern Central-Chile differ in their anatomical, physiological and chemical traits, in relation to local environmental conditions; namely light and water availability. We selected thalli from one Andean population and from two populations from Nahuelbuta Mountain Range, close to the coast. The Andean population is located in a closed secondary forest, close to the town of Ralco, inside a protected area. Both Coastal populations are located also in secondary forests inside protected natural areas but receive higher precipitation throughout the year compared to the Andean population (Di Castri & Hajek 1976), mainly due to the oceanic influence.

MATERIALS AND METHODS

SAMPLES

Thalli of *Pseudocyphellaria flavicans* were collected from the field and stored in the laboratory. Samples were obtained from three populations: Nahuelbuta National Park (Malleco, Nahuelbuta Mountain Range, 37°47' S - 72°59' W, ~800-1000 masl), Contulmo Natural Monument (Malleco, Nahuelbuta Mountain Range, 38°01' S - 73°10' W, ~300-400 masl), and Ralco National Reserve (Bío-Bío, Andes, 37°51' S - 71°19' W, ~1200 masl). The first two sites correspond to protected natural areas close to the coast. Ralco correspond to a protected area in the Andes, with almost undisturbed, natural vegetation. Thalli were collected from September 2012 (Austral spring) to January 2013 (Austral summer). We took 10 hemispherical photos per site in the exact locations of the studied thalli. These photos were then analyzed with GLA software (Frazer *et al.* 1999) to calculate canopy openness, a measure of the light environment of the lichens.

ANATOMICAL TRAITS

We compared the number of isidia and apothecia per area in 5 randomly selected thalli per site. The reproductive structures were counted in 1cm² area, under an optical magnifier. Measures were made at 3 cm from the thallus margin.

On the same thalli we performed transversal section at 3 cm from the edge. We took digital photographs using an optical microscope (Primo Star, Carl Zeiss, Germany) with an attached digital camera (Canon EOS Rebel XS). We then measured the thickness of the upper cortex, algal layer, medullae, and lower cortex using image software (Axiovision Rel. 4.8).

ECOPHYSIOLOGICAL TRAITS

Sections of thalli from the field were put in hermetic plastic bags and transported to the laboratory where they were

kept for 1 week at room temperature in the dark. At the beginning of the desiccation experiment, 30 thalli segment per population were weighted with a digital scale (Baxtran PS1000). Fragments were then fully hydrated with tap water until constant weight was reached. Lastly, thalli were dried for 630 min at room temperature and weight was measured periodically every 35 min (see below). Relative water content was calculated as:

$$\text{RWC} = \frac{(\text{fresh weight} - \text{dry weight})}{(\text{fully hydrated weight} - \text{dry weight})}$$

RWC was used instead of water content (WC) because is commonly used in ecophysiological studies of desiccation-tolerant organisms and allows inter-species comparisons (see Atala *et al.* 2014). Maximal WC (fully hydrated weight) is difficult, but not impossible, to obtain in lichens, usually taking many hours to reach constant weight.

To determine the maximum efficiency of the PSII (Fv/Fm) we used a portable fluorimeter (Pocket PEA, Hansatech Ltd, King's Lynn, UK). Samples were collected and transported to the laboratory as described above. Following the same procedure described for the RWC, we measured Fv/Fm every 35 min up to 630 min. This experiment was conducted in the dark and samples were dark-adapted for 45 min previous to the first measurement of Fv/Fm. Temperature and relative humidity (RH) were constant at the time measurements were conducted. All populations were measured simultaneously, separating individual measurements by 2-5 min intervals. Fv/Fm was not expressed on an area basis because the used instrument determinate fluorescence parameters on a fixed leaf area.

CHEMICAL ANALYSIS

For the antioxidant analysis, the material was dried at room temperature under shade after washing with distilled water. After drying, the material was cleaned under a magnifying

glass and impurities were removed. A methanol extract was prepared by soaking 0,770 g of thalli each populations from in 210 mL of solvent. The maceration process of the extracts was performed during one hour at room temperature. The extracts were filtered and solvent was evaporated using a rotatory evaporator (IKA, RV10 digital) at 50 °C. Extracts were then filtered and concentrated. The crude extract was kept at 4 °C. The free radical scavenging activity of *Pseudocyphellaria flavicans* extract was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) (SIGMA). The ability of lichen extract to scavenge DPPH free radicals (%) was calculated according to the method described by Takao *et al.* (1994) with some modifications.

An aliquot of 2 ml of the extracted fraction (5, 40, 200, 400, 500, 1000 and 2000 µg/ml) was mixed with 2 ml (freshly) prepared DPPH solution in a concentration of 80 µg/ml. After 30 min incubation in dark at ambient temperature, the resultant absorbance was measured at 517 nm against a blank on a spectrophotometer (Genesys 10 UV, Thermoscientific). The IC50 values (concentration of extract in the reaction mixture needed to decrease by 50% the initial DPPH concentration) were determined by polynomial regression analysis of the obtained DPPH radical scavenging (% inhibition). The inhibition concentration (IC50) was the parameter used to compare the radical scavenging activity between the three *Pseudocyphellaria flavicans* populations. Analyses were done in triplicate (3 per population). All measurements were done within a month.

STATISTICAL ANALYSES

To analyze differences in traits between populations we conducted a one-way ANOVA and used the Tukey test as an *a posteriori* test. Fv/Fm curves were compare using a repeated-measures ANOVA. Standard errors were not included in Figure 1 to avoid confusion and crowding in the figure. Additionally, errors were small and usually fell within the dot in the graph (2-3%).

TABLE I. Ecological, anatomical and chemical traits of *P. flavicans* individuals from three populations. Contulmo and Nahuelbuta are Coastal populations and Ralco is an Andean population. Different letters show statistical differences (Tukey test, $p < 0.05$).

TABLA I. Rasgos ecológicos, anatómicos y químicos en individuos de *P. flavicans* de tres poblaciones. Contulmo y Nahuelbuta son poblaciones costeras y Ralco es una población de Los Andes. Letras diferentes indican diferencias estadísticas (test de Tukey, $p < 0.05$).

POPULATION	GAP OPENNESS (%)	UPPER CORTEX THICKNESS (µm)	ISIDIA/cm ²	ANTIOXIDANT ACTIVITY (IC50, µg/mL)
Contulmo	27.5 ± 8.0 ^a	35.8 ± 3.9 ^{ab}	34.4 ± 5.8 ^a	474.1 ± 94.7 ^a
Nahuelbuta	31.8 ± 5.5 ^a	57.0 ± 5.4 ^a	72.6 ± 3.1 ^b	520.1 ± 106.5 ^a
Ralco	19.1 ± 5.8 ^b	27.6 ± 3.1 ^b	77.2 ± 6.9 ^b	432.1 ± 59.4 ^a

RESULTS

We found differences in the light environment experienced by the thalli of the three populations (Table I, ANOVA $p < 0.001$). Ralco thalli were exposed to a more closed canopy compared to both Coastal populations (Table I, Tukey test $p < 0.05$). There were no differences in canopy openness between Nahuelbuta and Contulmo thalli (Table I, Tukey test $p > 0.05$).

ANATOMICAL TRAITS

The three populations of *Pseudocypbellaria flavicans* differed in their anatomy. Individuals from the Andean population (Ralco) had thinner upper cortex compared to Nahuelbuta (Table I, Tukey test $p < 0.05$). There were no differences in upper cortex thickness between both coastal populations and between Ralco and Contulmo (Table I, Tukey test $p > 0.05$). All three populations did not show statistical difference in algal layer, medullae, or lower cortex thickness (ANOVA $p > 0.05$, data not shown). Total thalli thickness followed the same pattern as upper cortex thickness (Tukey test $p < 0.05$, data not shown).

We found differences in the number of isidia/cm² between populations (Table I, ANOVA $p < 0.05$). Contulmo individuals had lower isidia density compared to Nahuelbuta and Ralco (Table I, Tukey test, $p < 0.05$). There were no differences in isidia/cm² between Nahuelbuta and Ralco (Table I, Tukey test, $p < 0.05$). Apothecia were scarce in all populations. We only found three individuals with few evident apothecia in the Contulmo population and two in the Nahuelbuta population. They averaged 1-2 apothecia/cm² (data not shown).

PHYSIOLOGICAL AND CHEMICAL TRAITS

Thalli from all populations loosed water similarly when desiccated (Fig. 1). However, the Fv/Fm changed differently when desiccating in the three populations (Fig. 1, Repeated Measures ANOVA, $p < 0.001$). All populations maintained a relatively constant Fv/Fm up to 50-60% RWC. After that, the Fv/Fm dropped. Ralco and Nahuelbuta individuals had a similar Fv/Fm kinetic when desiccating, but they had different initial values (at 100% RWC, data not shown). Contulmo thalli dropped their Fv/Fm more rapidly compared to Ralco and Nahuelbuta populations (Fig. 1). After 250 min of desiccation for Contulmo and ~400 min for Nahuelbuta and Ralco, corresponding to almost 0 % RWC, Fv/Fm dropped to 20-30 % of the initial values, and stabilized up to the end of the experiment (Fig. 1).

The extracts from the different populations showed free radical scavenging activity, but there were no statistically significant differences in the IC50 values between the studied *Pseudocypbellaria flavicans* populations (Table I, ANOVA, $p > 0.05$).

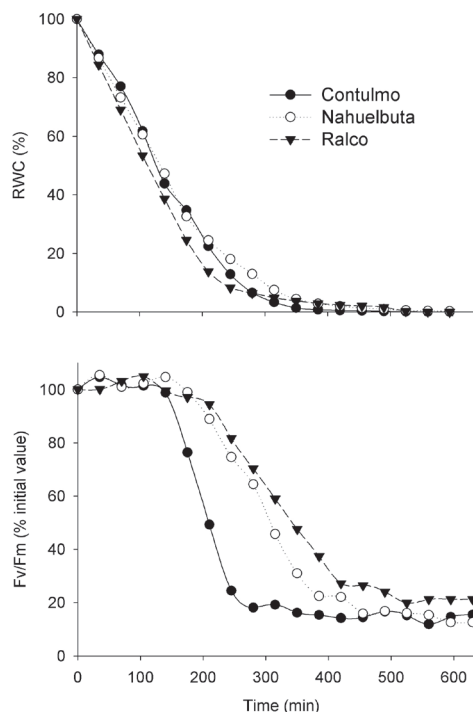


FIGURE 1. Response of the Fv/Fm (maximal efficiency of the PSII) and relative water content (RWC) to desiccation in *P. flavicans* thalli from three populations. Contulmo and Nahuelbuta are Coastal populations and Ralco is an Andean population. $n = 30$ per population. Populations statistically differed in their Fv/Fm (Repeated Measures ANOVA, $p < 0.001$). Fv/Fm values are expressed as % of initial measurement.

FIGURA 1. Respuesta del Fv/Fm (eficiencia máxima del fotosistema II) y contenido relativo de agua (RWC) a la desecación en talos de *P. flavicans* de tres poblaciones. Contulmo y Nahuelbuta son poblaciones costeras y Ralco corresponde a una población Andina. $n = 30$ por población. Se encontraron diferencias estadísticas en el Fv/Fm entre las poblaciones (ANDEVA de medidas repetidas, $p < 0.001$). Los valores de Fv/Fm corresponden al % del valor inicial medido.

DISCUSSION

The studied populations differed in anatomical and physiological traits. However, there were no differences between populations in the chemical traits included in our study. Both coastal populations tended to be similar in several traits, possibly reflecting the similar climatic conditions of both sites (Di Castri & Hajek 1976, Luebert & Plissock 2006). On the other hand, Nahuelbuta and Ralco populations were physiologically similar, possibly reflecting their similar altitudes and the resulting climatic convergences (Di Castri & Hajek 1976, Luebert & Plissock 2006). The

Andean population was located in a more closed (older) forest compared to coastal populations. Additionally, coastal populations receive higher precipitation compared to the Andean population, particularly the Contulmo population (Di Castri & Hajek 1976, Luebert & Plissock 2006).

The different light environments resulted in differences in anatomical traits such as upper cortex thickness. Both coastal populations were located in more open sites and Nahuelbuta individuals had thalli with thicker upper cortex compared to the other two populations. This higher upper cortex thickness might reflect part of the incident radiation (Büdel 1990) and usually contains antioxidants and anti-UV compound (Solhaug *et al.* 2003, Manojlovic *et al.* 2012). A thicker upper cortex has also been found in other light-exposed lichens, and it has been related to a direct result from direct sunlight exposition (Beckett *et al.* 2008, Green & Lange 1991), protecting algae from excess light, heat and photoinhibition. Other strategies to cope with high light intensity have been discussed for other groups of lichens. For example, stacking of the algae and fungal mycelia allow lichens to disperse the light and to lower the effect of direct sunlight (Vondrák & Kubásek 2013). Antioxidant capacity, however, was not statistically different in the three populations. This could be due to the low sample size or maybe the structural differences between populations were sufficient to cope with the differences in light and moisture between sites. Snelgar & Green (1981) considered the length of the rhizines as a functional trait related to water retention capability in more exposed sites. Their study was based on *Pseudocyphellaria dissimilis*, a species with a constant tomentum in the lower surface. This is in strong opposition with *P. flavicans* that has an unevenly distributed tomentum in the lower surface that is almost absent in the margins. Thus measurements of the rhizines would not have been functionally equivalent to the mentioned study on *P. dissimilis*.

Environmental conditions also seemed to affect the reproductive output of the lichens. Individuals from Contulmo had lower vegetative reproductive output compared to the other two populations. In Contulmo, there seems to be a higher lichen density per tree, resulting in higher competition (Atala & Vargas, unpublished data). In such conditions, it has been suggested that sexual reproduction is favored over asexual reproduction (Lücking 1999). Nevertheless, few apothecia were found in Contulmo thalli. Ralco individuals showed no apothecia at all, location where competition seemed limited due to the reduced lichen diversity and abundance (Atala *et al.*, unpublished data).

All populations reduced their RWC similarly when left to dehydrate. Individuals from Contulmo (moister site), however, reduced their Fv/Fm faster than the other two populations. When the RWC dropped to 50-40%, all thalli dropped their Fv/Fm, reaching values close to 0.1 (absolute value) in less than an hour. Individuals from Nahuelbuta had a lower initial Fv/Fm compared to the other two populations (0.4 vs. 0.6, data not shown), but the relative Fv/Fm curve behaved similarly to

Ralco in terms of slope. These differences in the Fv/Fm when dehydrating could be due to ecotypic differentiation between populations, although plasticity cannot be ruled out. After 400 min lichen from all populations dropped their Fv/Fm to values close to 0.1 (~20% of initial Fv/Fm) and remained constant until the end of the experiment (630 min).

Lichens, in general, are understudied organism, especially in Chile (but see Quilhot *et al.* 2012, Rubio *et al.* 2013, Vargas *et al.* 2013). This study is one of the first ecophysiological studies on Chilean *Pseudocyphellaria*. We found differences in functional traits between populations of *P. flavicans* but we currently do not know the underlying mechanisms explaining such differences. Future studies should address if *P. flavicans* populations can show plasticity in functional traits and/or local adaptation to light and water availability.

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