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The influence of growth form and substrate on lichen ecophysiological responses along an aridity gradient

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Abstract In this paper, we investigated whether growth form and substrate in lichens influence their physiological responses along an aridity gradient. Thalli of the foliose lichen *Parmotrema perlatum* and the fruticose lichen *Ramalina canariensis* were transplanted in selected rural/forested sites of Southern Portugal characterized by a different aridity index. Physiological parameters including photosynthetic performances, assimilation pigments, ergosterol content and sample viability were measured prior to exposure (winter) and after 6-month exposure (summer). Photosynthetic performances were also investigated in common native foliose and fruticose epiphytic lichens and in fruticose terricolous species. Both transplanted and native lichens showed a decrease in photosynthetic performances in summer and lower performances in sites classified as drier and higher performances in humid forested sites. No relevant differences occurred between epiphytic foliose and fruticose growth forms. However, terricolous fruticose samples showed a significant difference in humid and drier sites and between winter and summer, probably due to microclimatic conditions similarly to other biological crusts.

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Introduction

Mediterranean drylands are among the most highly threatened ecosystems by present and predicted climate changes (Christensen et al. 2007) due to the scarce knowledge existing about these ecosystems, the high impact of anthropogenic activities and the rich biodiversity of Mediterranean environments (Bakkenes et al. 2002; Lloret et al. 2004).

Characteristics of Mediterranean drylands are strong seasonality, with hot, long and dry summers alternating with wet and even cold winters (Rambal et al. 2003) and limited water availability during summer that, consequently, is the main driver of vegetation composition, cover and dynamics (Ramos et al. 2015; Matos et al. 2015).

Lichens are sensitive both to climatic conditions and pollution, hence their use as bioindicators of ecosystem functioning in Mediterranean environments (Matos et al. 2015 and references therein). As we move to drier environments, lichen species composition changes in response to increasing water stress (Matos et al. 2015) and eutrophication from dust (Pinho et al. 2014). This change in species composition is related to species functional traits, lichen characteristics that influence their response to the environment.

Grouping species with a common value of a trait will result in a functional group (e.g. fruticose or nitrophytic), which in lichens can be seen as groups of species with a common response to an environmental driver (Diaz and Cabido 2001). For example, in several studies, authors observed that crustose and foliose functional groups were associated with higher aridity than fruticose (Matos et al. 2015 and references

therein), most likely due to their different capacity to maintain thallus hydration (Kershaw 1985). Similarly, the existence of molecular and biochemical mechanisms for coping with environmental stresses defines the tolerance to nitrogen (Munzi et al. 2017; Pirintsos et al. 2009), oxidative stress (Kranner et al. 2005), extreme conditions (de Vera et al. 2004) and other abiotic factors.

Thus, lichen functional diversity (i.e. lichen functional groups identity and abundance) has been frequently used as an ecological indicator of environmental changes in the Mediterranean area (Matos et al. 2015; Pinho et al. 2014). Compared to functional diversity, physiological and metabolic responses to environmental changes are faster, providing a useful tool to detect early stress symptoms (Munzi et al. 2012; Pirintsos et al. 2011). Moreover, physiological parameters also allow us to investigate how lichens respond at cellular and molecular level to changing biotic and abiotic factors (e.g. Munzi et al. 2017; Nicolardi et al. 2012).

In order to provide an insight into the relationship between ecosystem functioning and regional climatic trends, we have combined physiological and functional approaches. We tested the physiological responses of transplanted and native lichens with a different functional trait (growth form) and growing on different substrates, in rural/forested sites of Southern Portugal characterized by different aridity, where the aridity index was calculated based on site potential evapotranspiration and total precipitation. This widely used method (UNEP 1992) provides a reliable indication of the water available considering climatic conditions and the vegetation contribution. Our working hypothesis was that the physiological parameters of the lichens vary according to the aridity of the site and the characteristics of the species. In particular, we aimed at answering the following questions: (1) does the aridity gradient affect physiological responses of transplanted foliose and fruticose lichen samples? (2) do growth form and substrate influence their physiological responses along the aridity gradient?

To answer the first question, thalli of the lichens *Parmotrema perlatum* (Huds.) M.Choisy and *Ramalina canariensis* J.Steiner (respectively foliose and fruticose growth forms) were transplanted along an aridity gradient and their responses recorded. Physiological parameters were investigated prior (winter) and after 6 months of exposure (summer). To answer the second question, the photosynthetic performances were investigated in the commonest epiphytic and terricolous native lichens at the experimental sites.

The results not only confirmed the suitability of lichen physiological parameters as biomonitors of stresses induced under dry conditions in Mediterranean ecosystems, but also showed that drought stress may have a different effect on lichens with different traits (e.g. substrate requirements), contributing to the knowledge required for a correct ecosystem protection and management.

Materials and methods

Study area and sampling design

The transplant experiment was carried out in a Mediterranean environment in Southern Portugal (Fig. 1). The National Forest Inventory 2005/06 (AFN 2010) was used as the basis to select the sampling sites. Out of the 336,000 available, the sampling sites were chosen by several criteria, in order to reduce the effect of variables excluded from this study: (1) "holm-oak woodland" land-use type; (2) within drylands, altitude between 150 and 300 m and slope less than 5°; (3) with a luvisol soil type and dominantly acidic (pH < 6.5); (4) dominated by sedimentary and metamorphic lithology; (5) without record of fire. This means that these factors (land-use type, altitude, inclination, type and soil pH, type of soil and fire) did not influence our results. This resulted in 2730 points from which a random sampling was made: nine sampling sites were selected and distributed into three groups stratified by the aridity index (Trabucco and Zomer 2009) (see Table 1 for identification and characteristics of the groups). The vegetation type in the selected sites was a typical Mediterranean evergreen woodland, dominated by Holm-oak (*Quercus ilex* L.). The trees occur scattered in the landscape, in a matrix dominated by annual herbaceous plant species. These annual plants occur mainly in extensive pastures, grazed with low intensity by cattle or sheep. The aridity index was calculated using a broadly used approach, dividing the site potential evapotranspiration by the total precipitation (UNEP 1992). Because it considers the amount of water reaching the site as rain and the amount of water that leaves the ecosystem by evaporation, it is a universal and robust measure of the amount of water available to vegetation in ecosystems. All areas with an aridity index (AI) below 0.65 are indicated as drylands and our sampling sites ranged between the semi-arid and the lower limit of the humid zone (UNEP 1992). For practical reasons, within the manuscript, the sites were divided into three categories referred to as drier, intermediate and humid.

Lichen material and transplant experiment

Branches of *Pinus* sp. carrying the fruticose lichen *Ramalina canariensis* were collected in Tróia peninsula (Baía de Setúbal) and bark pieces of *Quercus suber* carrying the foliose lichen *Parmotrema perlatum* were collected in "Companhia das Lezírias", a large agriculture, cattle and forest farmstead in Portugal, in an area where grazing, farming and agricultural practices ended a few decades ago. The two species were chosen to compare lichens with similar, quite wide ecological requirements (Nimis and Martellos 2017), but belonging to different functional groups (foliose and fruticose growth forms). Water loss in fruticose lichens occurs more rapidly than in foliose lichens (Larson and Kershaw 1976), which in

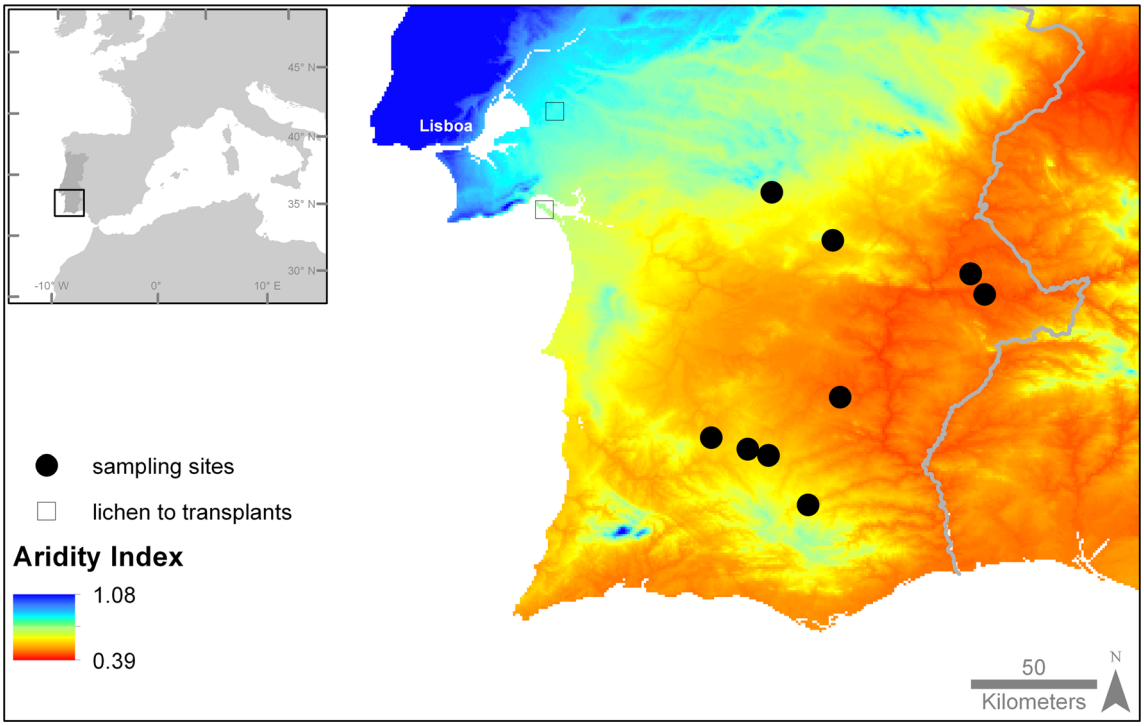

Fig. 1 The transplant experiment was carried out in a Mediterranean environment in Southern Portugal

dry conditions spend more time inactive. The transplant experiment lasted from January to July 2014. Samples (branches and bark pieces with lichen thalli) were exposed chiefly on local *Q. suber* or alternatively *Q. ilex* at about 2.5 m above the ground, simulating the conditions of their native environment. About 15 thalli per species per site were transplanted. In parallel, the commonest epiphytic and terricolous native lichens available at each site were collected and analysed (the same species between humid, intermediate and drier sites). Concerning native samples, our attention focused on photosynthetic performance, in order to test by means of a simple indicator, whether functional traits, i.e. growth form (foliose, fruticose) and substrate (epiphytic, terricolous) influence physiological response along the aridity gradient. For this purpose, epiphytic foliose species (broad lobed parmelioid lichens, namely *Parmelina tiliacea*, *Flavoparmelia caperata*, *P. perlatum*, *P. hypoleucinum*), epiphytic fruticose species (*R. canariensis*) and the terricolous fruticose species *Cladonia rangiformis* were selected. In addition to photosynthetic performances, photosynthetic pigments, ergosterol content and sample viability (assessed as dehydrogenase activity) have been investigated in the transplants.

Photosynthetic performance

The "vitality" of the lichen photobiont was firstly checked by the maximum quantum yield of primary photochemistry as inferred from chlorophyll *a* fluorescence emission: $F_V/F_M = (F_M - F_0)/F_M$, where F_0 and F_M are minimum and maximum chlorophyll *a* fluorescence and $F_V = (F_M - F_0)$ is the variable fluorescence. In addition, the performance index (PI_{ABS}), a global indicator of the photosynthetic performance was calculated to express the overall vitality of the samples. The parameter PI_{ABS} combines in a single expression the three functional steps of the photosynthetic activity (light absorption, excitation energy trapping and conversion of excitation energy to electron transport), resulting in a very sensitive indicator of stress suitable for application to physiological and environmental screenings (Strasser et al. 2000). Prior to the measurements, samples were sprayed with mineral water until

Table 1 Main characteristics of the sampling points ($N = 9$). The climate variables refer to long-term averages of 1950–2000 (Hijmans et al. 2005). For practical reasons, within the manuscript, the sites were divided into three categories referred to as drier, intermediate and humid

Identification	Aridity index	Precipitation (mm)	Average annual temperature (°C)	Altitude (m)
Drier	0.45–0.48	479–548	>17.5°	165–218
Intermediate	0.54–0.57	612–633	15–16 °C	199–252
Humid	0.57–0.69	664–769	15–16 °C	262–284

completely moisten; excess water was removed by hand-shaking; then, the samples were kept hydrated in humid paper for 24 h at 18–20 °C and protected from excess light (50 μmol s^{-1} m^{-2} photons PAR). Measurements were carried out with a Plant Efficiency Analyser (Handy PEA, Hansatech Ltd., Norfolk, UK). Prior to recording fluorescence emission samples were dark-adapted for 10 min, exposed for 1 s to a saturating excitation pulse (3000 μmol s^{-1} m^{-2}) of red light (650 nm) from a LED into the fluorimeter sensor. Up to ten replicates were measured for each species at each site, both for transplanted and native samples.

Content of photosynthetic pigments

About 20 mg of lichen material were dissolved in 1 mL of dimethylformamide and homogenized with Ultraturrax for 1 min. The homogenate was centrifuged at 10,000 rcf for 10 min and 50 μL of the resulting supernatant were analysed by HPLC (Waters LC I Plus) using an Ascentis Supelco C18 column (250 × 4.6 mm, porosity 5 μm). The concentrations of photosynthetic pigments were determined according to Suzuki et al. (1993) using water/methanol/acetone as mobile phase with a flow rate of 1 mL/min. Runs were monitored at 440 nm. Quantification of chlorophyll *a* and chlorophyll *b* was performed using calibration curves of standards from Sigma-Aldrich (USA). Three replicates were measured for each species at each site.

Sample viability

Triphenyltetrazolium chloride (TTC) reduction to triphenylformazan (TPF) is a good indicator of dehydrogenase activity (dark respiration) and was used to assess sample viability according to Bačkor and Fahselt (2008). About 20 mg of lichen material were incubated in the dark for 20 h in 2 mL of 0.6% TTC and 50 mM phosphate buffer solution. Solutions were then removed and samples washed in distilled water. Water-insoluble formazan was extracted with 6 mL of ethanol at 65 °C for 1 h. Tubes were then centrifuged at 4000*g* for 10 min and absorbance read at 492 nm. Results were expressed as absorbance units/g (dw). Three replicates were measured for each species at each site.

Ergosterol content

Samples of 100 mg of lichen material were homogenized for 10 min in 99% ethanol. Extracts were transferred to 1.5-mL Eppendorf tubes and shaken in the dark at 25 °C for 30 min, then vortexed and centrifuged at 10,000*g* for 20 min. The resulting supernatant was immediately analysed by HPLC in a Kromasil 100 C18 column (150 × 4.6 mm, particle size 7 μm) as separator, with flow rate 0.8 mL min^{-1} and isocratic elution with methanol as mobile phase (Dahlman et al. 2002). Total analysis time was 15 min. Ergosterol absorption at 280 nm was measured with a UV detector (Ecom LCD 2084). A standard curve was prepared ranging 1–200 μg ergosterol (Sigma-Aldrich, USA) dissolved in 1 mL of ethanol. As ergosterol is sensitive to light, all steps were conducted almost in the dark. Three replicates were measured for each species at each site.

Statistics

The Kolmogorov-Smirnov test was used to check the normality of data distribution ($p < 0.05$). Data not matching a normal distribution were treated with Box-Cox transformation. One-way ANOVA was run to check the significance of differences for each physiological parameter according to the aridity gradient; the Tukey test was applied for post hoc comparisons (significance level at $p < 0.05$). At each site, differences between winter and summer values were checked by the *t* test ($p < 0.05$).

Results

Both transplanted and native lichens responded with lower performances in sites classified as drier and higher performances in more humid sites. Transplanted thalli of *P. perlatum* showed lower photosynthetic performances (F_V/F_M, PI_{ABS}) and an altered ergosterol content in drier sites (Table 2). Higher values of fluorescence parameters were recorded in transplanted thalli of *R. canariensis* in those sites classified as humid, with PI_{ABS} showing twice the value found in drier ones (Table 2). Ergosterol content was not affected by the aridity gradient.

The content of photosynthetic pigments increased in humid sites with respect to pre-exposure values in *P. perlatum*, while in *R. canariensis*, it decreased overall; although in both cases, no significant differences emerged between the sites. Viability of *R. canariensis*, as reflected by dehydrogenase activity (dark respiration), increased in humid and intermediate sites with respect to drier sites and with respect to pre-exposure samples, while in *P. perlatum*, there was a weak tendency for higher sample viability in humid sites with respect to drier sites.

Climatic conditions are the most important factor affecting physiological performance. As far as native lichens were concerned, spatial differences between humid, intermediate and drier sites were not detected during winter. Seasonal variations (summer vs winter) consisted in a decrease of the photosynthetic performances in drier sites during summer (Table 3). In particular, lower values of F_V/F_M and PI_{ABS} were measured in epiphytic lichens (foliose and fruticose thalli) growing in drier sites. No differences between winter and summer were detected in humid and intermediate sites (Table 3). Furthermore, under the experimental conditions, no relevant differences

Table 2 Transplanted samples. Physiological parameters in *Parmotrema perlatum* and *Ramalina canariensis*. *F* and *P* of ANOVA. n.s. not significant. Values followed by a different letter are statistically different according to the exposure conditions (Tukey test, $P < 0.05$)

	Parameters	Pre-exposure	Humid	Intermediate	Drier	ANOVA
Parmotrema perlatum	F_V/F_M	0.689 ± 0.033a	0.687 ± 0.073a	0.677 ± 0.039a	0.547 ± 0.159b	$F = 7.654$ $P = 0.000$
	$PI_{(ABS)}$	1.330 ± 0.742a	1.030 ± 0.791a	0.930 ± 0.544a	0.534 ± 0.240b	$F = 5.014$ $P = 0.004$
	Chlorophylls ($a + b$) (μg/mg)	0.65 ± 0.07b	1.19 ± 0.13a	0.87 ± 0.36ab	0.94 ± 0.18ab	$F = 4.065$ $P = 0.024$
	Sample viability A_{492}/g dw	8.16 ± 2.02	10.72 ± 1.77	10.72 ± 4.30	7.43 ± 3.70	n.s.
	Ergosterol (μg/mg)	0.66 ± 0.06a	0.70 ± 0.13a	0.66 ± 0.11a	0.47 ± 0.05b	$F = 7.263$ $P = 0.002$
Ramalina canariensis	F_V/F_M	0.651 ± 0.055a	0.621 ± 0.138a	0.640 ± 0.145a	0.522 ± 0.151b	$F = 6.118$ $P = 0.001$
	$PI_{(ABS)}$	1.552 ± 0.680ab	1.597 ± 0.694a	2.101 ± 1.120a	1.004 ± 0.507b	$F = 6.794$ $P = 0.001$
	Chlorophylls ($a + b$) (μg/mg)	0.36 ± 0.06a	0.14 ± 0.06b	0.16 ± 0.06b	0.19 ± 0.06b	$F = 10.27$ $P = 0.001$
	Sample viability A_{492}/g dw	2.57 ± 0.76b	3.96 ± 0.82a	4.64 ± 1.13a	3.08 ± 0.64b	$F = 8.245$ $P = 0.001$
	Ergosterol (μg/mg)	0.30 ± 0.03	0.33 ± 0.05	0.31 ± 0.05	0.31 ± 0.06	n.s.

were found between epiphytic foliose and fruticose growth forms. Overall, comparing humid with drier sites in summer and accounting only for epiphytic lichens (both foliose and fruticose growth forms), lower F_V/F_M and PI_{ABS} were detected in those sites classified as drier. In terricolous lichens, F_V/F_M and PI_{ABS} significantly decreased in summer at all sites. Terricolous lichens (i.e. the fruticose species *C. rangiformis*) appeared to be more affected than epiphytic ones and the lowest performances (as reflected by PI_{ABS}) corresponded to the drier sites (Table 3).

Discussion

Photosynthetic performance of foliose and fruticose lichens varied along the aridity gradient in a dry environment

Table 3 Photosynthetic parameters (F_V/F_M, PI_{ABS}) in native epiphytic lichens (foliose and fruticose thalli) and native terricolous lichens. *F* and *P* of ANOVA. n.s. not significant. Values followed by a different letter are statistically different according to the site (Tukey test, $P < 0.05$). Values in italic indicate differences between winter and summer

	Parameters	Season	Humid	Intermediate	Drier	ANOVA
Native epiphytic foliose lichens	F_V/F_M	Winter	0.698 ± 0.077	0.695 ± 0.028	*0.708 ± 0.027*	n.s.
		Summer	0.669 ± 0.085a	0.652 ± 0.081a	*0.498 ± 0.144b*	$F = . P = 0.$
	$PI_{(ABS)}$	Winter	2.237 ± 1.162	2.228 ± 0.976	*3.249 ± 1.381*	n.s.
		Summer	2.323 ± 1.662a	2.173 ± 1.795a	*0.892 ± 0.788b*	$F = . P = 0.$
Native epiphytic fruticose lichens	F_V/F_M	Winter	0.729 ± 0.025	0.701 ± 0.056	*0.710 ± 0.020*	n.s.
		Summer	0.697 ± 0.038a	0.664 ± 0.075a	*0.605 ± 0.056b*	$F = 9.271$ $P = 0.000$
	$PI_{(ABS)}$	Winter	3.253 ± 1.409	2.551 ± 0.911	*2.388 ± 0.687*	n.s.
		Summer	2.969 ± 1.423a	2.639 ± 1.188a	*1.367 ± 0.485b*	$F = 6.323$ $P = 0.004$
Native terricolous fruticose lichens	F_V/F_M	Winter	*0.667 ± 0.040*	*0.692 ± 0.050*	*0.646 ± 0.076*	n.s.
		Summer	*0.345 ± 0.173*	*0.327 ± 0.118*	*0.239 ± 0.120*	n.s.
	$PI_{(ABS)}$	Winter	*1.509 ± 0.356*	*1.940 ± 0.376*	*1.998 ± 1.050*	n.s.
		Summer	*0.451 ± 0.284a*	*0.368 ± 0.347ab*	*0.124 ± 0.135b*	$F = 3.425$ $P = 0.048$

confirming that lichen physiological parameters depend on water availability (Pirintsos et al. 2011). In particular, transplanted samples of *P. perlatum* and *R. canariensis* showed lower values of photosynthetic parameters (F_V/F_M, PI_{ABS}) in the driest sites and higher values in those sites classified as humid.

Previous studies carried out under stressed conditions in Mediterranean landscapes, showed that sensitive lichens undergo an alteration of photosynthetic performances and a decrease of photosynthetic pigments in parallel with chlorophyll degradation (Paoli et al. 2010; Pirintsos et al. 2011). These effects are induced by a combination of factors, such as high light irradiance and temperature and low water availability, which influence the water status of the thalli (hence metabolic activity) and are particularly harmful for fruticose lichens (Paoli et al. 2010; Pirintsos et al. 2011).

Being poikilohydric organisms, the photosynthetic activity and occurrence of lichens in extreme environments such as deserts reflect the presence of a regular water supply (Lange et al. 2001). Therefore, driven by aridity, transplanted lichens may undergo faster changes than native species. However, in the desiccated state, photosynthesis in desiccation-tolerant lichens shuts down and excess absorbed energy is dissipated without damaging the photosynthetic apparatus (Kranner et al. 2008). Photosynthesis re-activation occurs in a very short time, within "time-lags" ranging from a few minutes to a few hours when lichens are rehydrated with water or high atmospheric humidity (Lidén et al. 2010). Consequently, although our findings suggest that the photobiont is a reliable target of drought stress, further work is needed to investigate how to use algal photosynthetic parameters as early biomonitors in the case of aridity.

When considering functional traits, the lichen growth form in transplanted and native species was not relevant to the response to aridity. Thallus morphology drives the rate of evaporation (water loss): evaporation being mainly a physical process, the lower surface/volume ratio of foliose lichens corresponding to a higher ability to prolong thallus hydration and hence active metabolism (Kershaw 1985), while fruticose lichens are more dependent on external water supply. However, the use of species already adapted to drought stress can explain the absence of differences between foliose and fruticose species under the experimental conditions.

Interestingly, physiological response of native lichens along the aridity gradient was influenced by substrate; terricolous species showing significant differences between summer and winter and the lowest performances in summer, both in humid and drier sites. Biological soil crusts (BSCs), including terricolous lichens, must endure frequent desiccation–hydration cycles and steep temperature gradients, since temperature may decrease from 40 to 25 °C within 5 cm from the soil surface (Kershaw 1985). The need to cope with more severe conditions can be reflected in a higher responsiveness of terricolous lichens than epiphytic ones.

This higher sensitivity is particularly relevant because terricolous lichens are an important component of BSCs in arid and semi-arid ecosystems (Maestre et al. 2011). In water-limited ecosystems such as Mediterranean evergreen woodlands, BSCs provide a wide variety of ecosystem services influencing for example the cycle of soil nutrient, carbon and nitrogen, local hydrology, soil structure and stability and water infiltration (Concostrina-Zubiri et al. 2016; Morillas et al. 2016 and references therein).

Our findings showed that the physiological response of foliose and fruticose lichens changed along an aridity gradient and that the response was influenced by substrate requirements while growth form was not relevant under the experimental conditions. This not only provides information on the potential use of lichen physiological parameters as biomonitors of stresses induced under dry conditions, but contributes to integrate the existing knowledge about structure and functioning of Mediterranean drylands and the expected effects of changes in climatic conditions on these ecosystems.

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